Synthetic Peptides

Dynamic Covalent Chemistry on Self-Templating Peptides: Formation of a Disulfide-linked β-Hairpin Mimic**

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The self-assembly of supramolecular structures is usually dependant on reversible noncovalent interactions.[1] Advantages of reversible self-assembly include mechanisms for error-correction through dynamic equilibration leading to the most stable structure and minimization of synthetic effort.[1b] However, thermodynamically stable assembled states often exhibit low kinetic stability. One approach to generate thermodynamic constructs that are kinetically stable is the concept of dynamic covalent chemistry where noncovalent interactions can be used to template covalent bond formation.[2] Dynamic covalent chemistry can generate an equilibrium mixture of interconverting molecules. The exchange mechanism may be stopped by changing the reaction conditions. The distribution of molecules shifts upon inclusion of a template to favor individual library members that bind to the template.[3] A specialized case of template-directed synthesis where the template is an integral part of the structure it helps to form has been termed "covalent capture".[4] Reversible covalent capture using dynamic chemistry to form stable peptide-based assemblies has been demonstrated for the oligomerization of helical peptide bundles.[5] Herein, we describe the first studies on the dimerization of β-sheet-forming peptides by covalent capture under both reversible and irreversible conditions.

The objective was to explore whether self-templating of β-sheet-forming peptides occurs during the process of dimer formation by dynamic covalent chemistry. We designed two peptides $\mathbf{A_{SH}}$ and $\mathbf{B_{SH}}$ of length 4 mer and 10 mer respectively (Figure 1) containing $(Leu–Lys)_n$ repeats[6] known to predispose the peptide to form β-sheets.[7] The cationic nature of the $(Leu–Lys)_n$ motif also disfavors aggregation.[8] As the length of the peptide would dictate the degree of noncovalent interactions[9] two different values of n were chosen; $n=1$ ($\mathbf{A_{SH}}$) and $n=4$ ($\mathbf{B_{SH}}$). Of the several possible covalent coupling motifs, we have chosen to exploit the coupling of the thiol–disulfide system since it is water compatible, relatively fast, and can be switched on or off by changing the pH value.[10] Thiols were incorporated into the peptides as N-terminal Cys residues with a Gly spacer to offer some conformational flexibility for disulfide bond formation.[5]

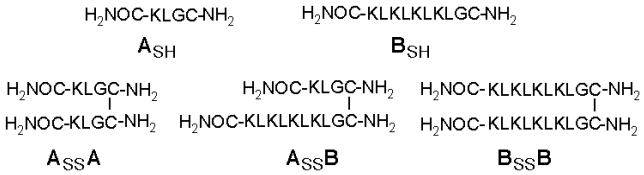

Figure 1. Sequences of peptides $\mathbf{A_{SH}}$ and $\mathbf{B_{SH}}$ and the various dimers potentially formed by covalent capture.

A mixture of $\mathbf{A_{SH}}$ and $\mathbf{B_{SH}}$ can dimerize to form two possible homodimers ($\mathbf{A_{SS}A}$ or $\mathbf{B_{SS}B}$) or a heterodimer ($\mathbf{A_{SS}B}$; Figure 2). We have investigated dimerization of equimolar solutions of $\mathbf{A_{SH}}$ and $\mathbf{B_{SH}}$ under irreversible and reversible conditions of covalent capture. Product distributions were analyzed from UV–HPLC traces of aliquots of the reaction mixtures (Figure 3) and quantified from a comparison of their areas under the traces with those of purified standards.[11] When a solution containing equimolar $\mathbf{A_{SH}}$ and $\mathbf{B_{SH}}$ in buffer

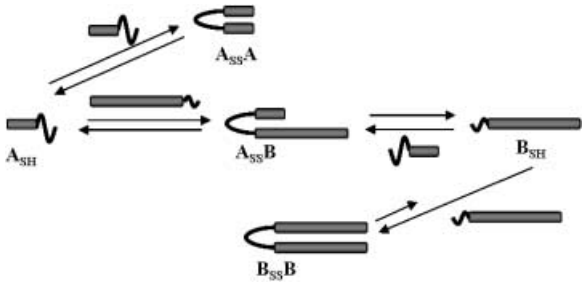

Figure 2. Schematic representation of reversible covalent capture in simple linear peptides leading to preferential formation of covalently linked peptide partners.

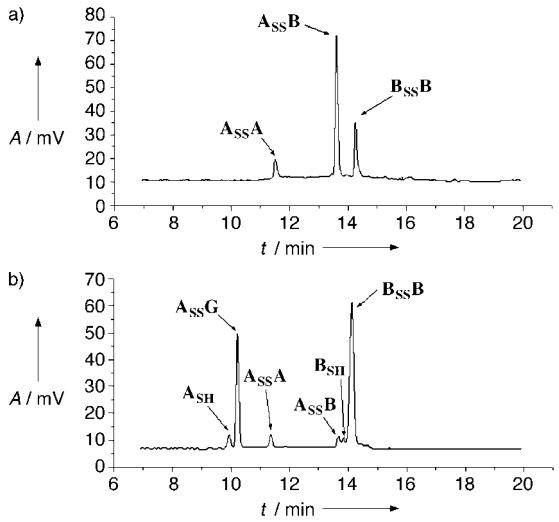

Figure 3. HPLC traces of aliquots of reaction mixtures where equimolar amounts of $\mathbf{A_{SH}}$ and $\mathbf{B_{SH}}$ were a) oxidized by air, b) equilibrated in buffer containing $\mathbf{G_{SH}}$ and $\mathbf{G_{SS}G}$ (where G = glutathione). Note that these raw data peaks have not been normalized for the difference in extinction coefficients at 220 nm for individual peptides.

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was subjected to air oxidation[12] dimers $\mathbf{A_{SS}A}$:$\mathbf{A_{SS}B}$:$\mathbf{B_{SS}B}$ were obtained in a 1.0:2.4:1.1 molar ratio. This essentially statistical distribution reflects that dimer formation is not influenced by noncovalent interactions. Even when the completely oxidized reaction mixture was left stirring for 72 h, the change in product distribution was negligible. The same results were obtained when we oxidized the mixture of thiols using $K_3[Fe(CN)_6]$ in buffer.

To explore dimerization under reversible conditions we employed a redox buffer comprising oxidized ($\mathbf{G_{SS}G}$) and reduced ($\mathbf{G_{SH}}$) forms of glutathione.[5] As this peptide-based buffer was used in large excess over $\mathbf{A_{SH}}$ and $\mathbf{B_{SH}}$, it also serves as a competitor to reduce the effect of nonspecific interactions between other peptide components. Equimolar solutions of $\mathbf{A_{SH}}$ and $\mathbf{B_{SH}}$ were equilibrated in $\mathbf{G_{SH}}$/$\mathbf{G_{SS}G}$ redox buffer for 12 h. HPLC analysis of the products revealed only trace amounts of the statistically favored disulfide $\mathbf{A_{SS}B}$ and a dramatic increase in the quantity of $\mathbf{B_{SS}B}$ (Figure 3b).[13] A new peak corresponding to $\mathbf{A_{SS}G}$ where $\mathbf{A_{SS}G}$:$\mathbf{A_{SS}A}$ was 13:1 was also observed. This feature is attributable to a concentration effect (initial $[\mathbf{G_{SH}}]$:$[\mathbf{A_{SH}}] = 12.5:1$) because of the relatively low stabilization resulting from self-recognition of $\mathbf{A_{SH}}$. All the oxidized $\mathbf{B_{SH}}$ is present in the form of $\mathbf{B_{SS}B}$ with no $\mathbf{B_{SS}G}$ detected despite the 13-fold excess of $\mathbf{G_{SH}}$. This result is indicative of $\mathbf{B_{SS}B}$ being a particularly stabilized structure. The molar ratio of $\mathbf{A_{SS}B}$:$\mathbf{B_{SS}B}$ under reversible conditions was 1:86 which reflects a significant contribution of noncovalent stabilization energy resulting from self-recognition of $\mathbf{B_{SH}}$. The product distribution was unchanged when the reaction was left to equilibrate under argon for up to 2 weeks (data not shown), which suggests that this is a true thermodynamic distribution. To confirm that the system had indeed reached a thermodynamic equilibrium, a 1.0:2.4:1.1 mixture of $\mathbf{A_{SS}A}$:$\mathbf{A_{SS}B}$:$\mathbf{B_{SS}B}$ generated by air oxidation in buffer was re-equilibrated in the presence of $\mathbf{G_{SH}}$/$\mathbf{G_{SS}G}$ (500 μM/125 μM; Supporting Information). The product distribution was evaluated at various time intervals by HPLC. After equilibration for 36 h a product distribution identical to that shown in Figure 3b was obtained, which confirms our model. The same results were obtained when the equilibration was effected by a neutral redox reagent employing oxidized and reduced forms of β-mercaptoethanol (Supporting Information). Thus, for this system, the introduction of reversible conditions allows an error-correction step in the covalent capture of a peptide partner.

To determine the origin of stabilization in $\mathbf{B_{SS}B}$, the structure of $\mathbf{B_{SS}B}$ in water was studied using NMR spectroscopy. The influence of dimerization on structure was gauged by comparison with the monomer $\mathbf{B_{SH}}$, used as the reference compound. ^{1}H NMR spectra were recorded in 10% (v/v) D_2O and 90% (v/v) H_2O and assigned using a combination of TOCSY and NOESY 2D data sets (See Supporting Information for details of experiments and spectra). A comparison of the 1D spectra of dimer $\mathbf{B_{SS}B}$ (80 μM) and monomer $\mathbf{B_{SH}}$ (80 μM) revealed a high degree of similarity. Moreover, TOCSY on $\mathbf{B_{SS}B}$ clearly identified only eight Leu–Lys spin systems not 16 which indicates that the peptide backbones in $\mathbf{B_{SS}B}$ have C_2 symmetry (Supporting Information). Perturbations to the H_α and N–H chemical shifts are characteristic of secondary structure formation by peptides.[14,15] Substantial H_α upfield shifts ($\Delta\delta = 0.3$ ppm) were observed at C^{10} and C^{11} residues at the center of $\mathbf{B_{SS}B}$ which is indicative of a turn conformation at these residues. Keeping in mind the C_2 symmetry of $\mathbf{B_{SS}B}$, this suggests that the disulfide linkage is at the center of a turn with both the peptide backbones propagating in the same direction. The $^3J_{N\alpha}$ coupling constants of $\mathbf{B_{SS}B}$ and $\mathbf{B_{SH}}$ at 300 K are shown in Table 1 (see Figure 5 for numbering scheme). The $^3J_{N\alpha}$ coupling constants for $\mathbf{B_{SH}}$ are close to the ideal random coil value of 6.0 Hz. In contrast, for $\mathbf{B_{SS}B}$ the values for the $(Lys–Leu)_4$ residues are generally over 7.0 Hz some reaching even 9.0 Hz—characteristic of them being present in β-sheet conformation.[16] G^9 and G^{12} showed ΔJ values of −0.7 Hz indicating that they form part of the turn in $\mathbf{B_{SS}B}$. More evidence for folded structure is provided by the observation of long-range NOEs between residues in the two strands of $\mathbf{B_{SS}B}$. A number of cross-strand NH–NH interactions between hydrogen-bonded residues (L^2–K^{18}, K^{16}–L^6, L^6–K^{14}, K^{14}–L^8, L^8–G^{12}) were also observed.[17] (Figure 4). Furthermore, the temperature coefficients ($\Delta\delta/\Delta T$) of N–H protons on Lys residues at various points on the sheet decreased progressively from the N-terminus to the C-terminus (Supporting Information). Similar results were obtained for the corresponding Leu residues. This result demonstrates that N–H protons nearer the turn are more strongly hydrogen bonded than those near the C-

Table 1: Coupling constants ($^3J_{N\alpha}$) of various residues on $\mathbf{B_{SS}B}$ and the monomer $\mathbf{B_{SH}}$ at 300 K.

$\mathbf{B_{SH}}$ Residue	$^3J_{N\alpha}$ [Hz]	$\mathbf{B_{SS}B}$ Residue	$^3J_{N\alpha}$ [Hz]
K^1	6.83	K^1, K^{20}	7.80
L^2	6.60	L^2, L^{19}	7.26
K^3	6.43	K^3, K^{18}	7.65
L^4	6.54	L^4, L^{17}	6.95
K^5	6.34	K^5, K^{16}	9.14
L^6	6.10	L^6, L^{15}	6.97
K^7	6.62	K^7, K^{14}	7.39
L^8	6.54	L^8, L^{13}	9.68
G^9	5.81	G^9, G^{12}	5.11
C^{10}	–	C^{10}, C^{11}	–

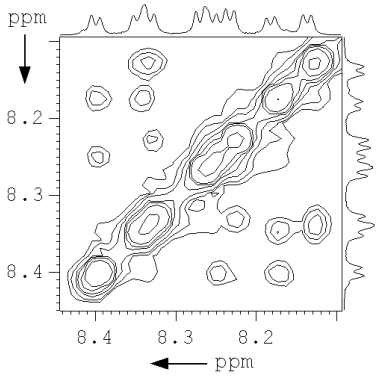

Figure 4. Portion of the NOESY spectrum of $\mathbf{B_{SS}B}$ at 300 K in 10% D_2O/90% H_2O illustrating cross-strand NH–NH NOEs that are consistent with the proposed folded structure.

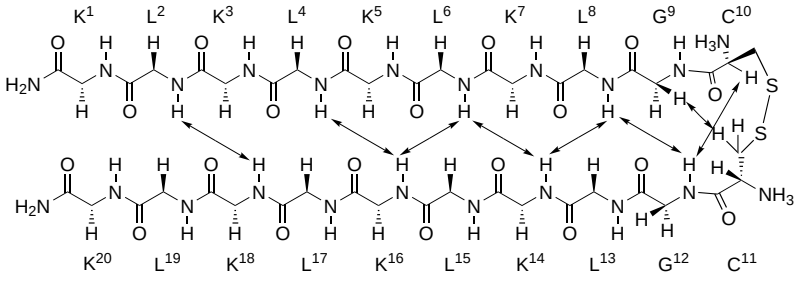

Figure 5. NOEs between non-adjacent residues observed in NOE NMR spectroscopy analysis of $\mathbf{B_{SS}B}$ at 300 K.

termini. This situation is completely consistent with $\mathbf{B_{SS}B}$ being present in a hairpin-type conformation as shown in Figure 5. Though disulfide linkages are known to stabilize β-hairpins[13] and have been proposed to act as turn scaffolds between helices,[18] this is the first example of a disulfide as a turn scaffold in a synthetic peptide yielding a β-hairpin-type conformation in water.

Biological systems frequently employ reversible covalent capture to stabilize inter- and intramolecular assemblies.[19] This is the first example of dynamic covalent chemistry applied to peptides to produce a thermodynamically stabilized β-sheet assembly.

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