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Protein Side-Chain Translocation Mutagenesis *via* Incorporation of Peptoid Residues

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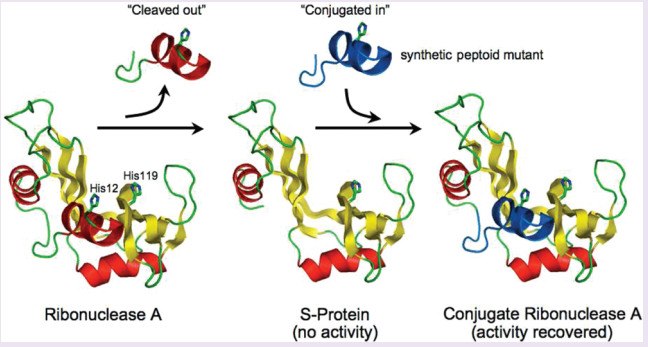
Supporting Information

ABSTRACT: For the last few decades, chemistry has played an important role in protein engineering by providing a variety of synthetic tools such as chemoselective side-chain modifications, chemical conjugation, incorporation of non-natural amino acids, and the development of protein-mimetic heteropolymers. Here we study protein backbone engineering in order to better understand the molecular mechanism of protein function and to introduce protease stable, non-natural residues into a protein structure. Using a combination of genetic engineering and chemical synthesis, we were able to introduce peptoid residues (N-substituted glycine residues) at defined positions into bovine pancreatic ribonuclease A. This results in a side-chain translocation from the Cα carbon to the neighboring backbone nitrogen atom. To generate these peptoid substitutions, we removed the N-terminal S-peptide of the protein by proteolysis and chemically conjugated synthetic peptide-peptoid hybrids to the new N-terminus. A triple peptoid mutant containing a catalytic His12 peptoid mutation was active with a k_{cat}/K_m value of 1.0×10^4 M^{-1} s^{-1}. This k_{cat}/K_m value is only 10-fold lower than the control wild-type conjugate and comparable in magnitude to many other natural enzymes. The peptoid mutations increased the chain flexibility at the site of peptoid substitution and at its C-terminal neighboring residue. Our ability to translocate side chains by one atom along the proten backbone advances a synthetic mutagenesis tool and opens up a new level of protein engineering.



Precision engineering of protein structure is essential to evolve new function, control specificity, improve stability, and elucidate mechanisms of action and chain folding.[1] Site-directed mutagenesis with natural amino acids, and a growing number of non-natural amino acids[2,3] are foundational tools that use the biosynthetic machinery to produce defined protein mutants or variants. On the other hand, advances in organic synthesis have made the total chemical synthesis of proteins feasible.[4−6] Peptides made by solid-phase synthesis can be linked together using native chemical ligation technology to generate full-length proteins. The chemical approach greatly expands the diversity of chemical modification that can be introduced into protein structures. In between biosynthesis and total synthesis lies protein semi-synthesis, where synthetic organic chemistry is used to produce peptidic variants, which can be chemically conjugated to biosynthetically expressed polypeptide domains.[7,8] Semi-synthesis has emerged as a powerful tool to produce defined protein variants, as it combines the synthetic flexibility of organic chemistry with the scalability and efficiency of biosynthesis.

Improvements in these techniques are enabling the construction of proteins that contain increasing departures from natural side chains and backbone linkages. Backbone mutation is of particular interest, as it allows the exploration of main chain hydrogen bonding and electronic effects, main chain stability and modulation of residue spacing. Expansion of the genetic code has enabled the incorporation of α-hydroxy acids in place of amino acids at particular residues, resulting in a backbone amide to ester mutation.[9−12] This mutation perturbs backbone hydrogen bonds, and has provided a useful chemical tool to dissect the effect of backbone hydrogen bonds on protein folding and stability since a single backbone hydrogen bond in hydrophobic surroundings can contribute up to 1.2 kcal/mol in protein thermodynamic free energy.[9] Several groups have introduced peptidomimetic residues into structured polypeptides by solid-phase synthesis,[13−16] expanding the diversity of protein backbone variants. β-Amino acids, which include an additional backbone methylene unit,[17−19] have been selectively introduced into protein coiled-coil tertiary structures in order to understand the side-chain packing preference at the interfaces between helices.[14,15] Using backbone thioester exchange, Gellman's group identified suitable α/β peptide segments that pair up with α-peptide segments in the context of helical coiled-coil.[20]

In our effort to design protein-mimetic polymers using polypeptoids (N-substituted glycines),[21−23] we seek to understand

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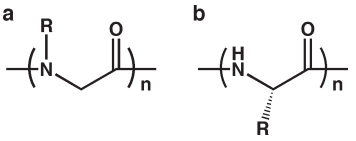

Figure 1. Peptoid and peptide. (a) Peptoid or N-substituted glycine oligomer and (b) peptide for comparison.

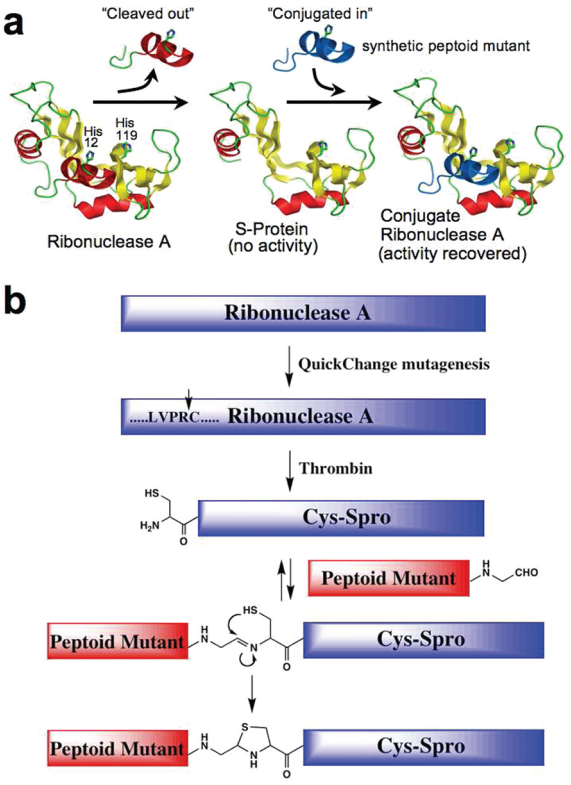

Figure 2. Strategy for protein peptoid mutagenesis. (a) The S-peptide segment of ribonuclease A was replaced with a peptide-peptoid hybrid using N-terminal proteolysis and conjugation. (b) Schematic diagram of the thiazolidine ligation between the C-terminal aldehyde of the synthetic peptide-peptoid hybrid and the N-terminal cysteine of the recombinant S-protein. A thrombin cleavage site and cysteine were introduced by site-directed mutagenesis. After the thrombin cleavage, a new N-terminal cysteine (Cys-Spro) was generated and conjugated to the aldehyde group of the peptide-peptoid hybrid.

the structure, dynamics, and functionality of peptoid residues in biomolecular recognition and catalysis. To explore this in the context of a folded protein structure, we chose to introduce single peptoid residues into a well-studied enzyme using semi-synthesis. Peptoids are of particular interest for protein engineering because of their ease of synthesis,[22] diverse alphabet of side chains,[24] and their protease stability.[25,26] Peptoids are sequence-specific N-substituted glycine polymers, where the side chains linked to the backbone nitrogens (Figure 1).[21] In comparison to a peptide, the position of the side chain in peptoids is shifted one atom, from the α carbon (Cα) to the backbone nitrogen, resulting in altered conformational properties,[27,28] protease stability,[25,26] achirality, and an absence of backbond hydrogen bond donors. Despite these differences, peptoid polymers are capable of potent biological activities[29] and of folding into protein-like structures based on the hierarchical assembly of helix[30,31] and sheet[32] secondary structures.

We chose bovine pancreatic ribonuclease A (RNase A) because its structure and mechanism are well understood[33] and because it is particularly well-suited to semi-synthesis. Ribonuclease A is a small single-domain protein (124 amino acid residues) and efficiently cleaves RNA by phosphodiester hydrolysis.[33] The N-terminal region (residues 1−20), called the S-peptide, can be readily removed by proteolysis, and the resulting protein (called S-protein) is completely devoid of enzymatic activity.[33] These two fragments can be reconstituted to create an active noncovalent complex RNase S. Furthermore, the S-peptide contains one of the critical active site catalytic residues (His12). Because the S-peptide is relatively short, it provides an ideal target to introduce synthetic backbone mutations that directly impact enzymatic activity.

In this study, we explore how peptoid point mutations affect ribonuclease A structure and function. We utilized the thiazolidine peptide ligation strategy[34,35] to conjugate a series of synthetic peptide-peptoid hybrids to a recombinant ribonuclease S-protein. Using this strategy, synthetic peptoid residues could be incorporated into ribonuclease A. We targeted multiple positions, including catalytic and noncatalytic residues, in the S-peptide segment for peptoid mutation and tested how the enzyme activity is changed by the single-atom translocation of individual side chains along the backbone. This semi-synthetic side chain translocation approach opens up a new strategy for protein backbone engineering and offers the potential for improving the protease resistance of proteins.

RESULTS AND DISCUSSION

Protein Peptoid Mutant Construction. In light of the challenges to introduce peptoid mutations into proteins genetically,[36] we preferred instead to replace a peptide segment in protein with a synthetic peptide-peptoid hybrid *via* a ligation strategy (Figure 2). We targeted the N-terminal S-peptide of ribonuclease A for the protein peptoid mutagenesis. Our strategy was to proteolytically cleave off the N-terminal S-peptide and then chemically conjugate the peptide-peptoid hybrid to the cleavage site as shown in Figure 2a. It is known that without the N-terminal S-peptide, the S-protein has no enzymatic activity.[33] Thus, the impact of the peptoid mutations on the enzymatic activity can be readily measured.

We attempted to utilize the native chemical ligation strategy[4] to conjugate the synthetic peptide-peptoid hybrids to the N-terminal cysteine of the ribonuclease S-protein. However, this method requires the preparation of a peptide-peptoid C-terminal thioester, which turned out to be difficult and inefficient due to incompatibilies with the peptoid synthesis conditions (*e.g.*, prolonged exposure to primary amines or bromoacetic acid). Using either the HS-CH_2CH_2COLeu-MBHA resin or 4-Fmoc-hydrazinobenzoyl resin,[37,38] only poor yields were obtained. We instead utilized the thiazolidine ligation strategy, which requires a peptide-peptoid C-terminal aldehyde.[35,39] This thiazolidine chemistry was first reported in 1937[35] and has been utilized in the total synthesis of proteins such as HIV-1 protease.[39]

To set up the thiazolidine ligation, an N-terminal cysteine is required. We thus introduced a thrombin cleavage site and a cysteine at position 20 and 21 of ribonuclease A, respectively, by two rounds of site-directed mutagenesis (see Methods). The amino acids TSAAS from position 17 to 21 were changed to

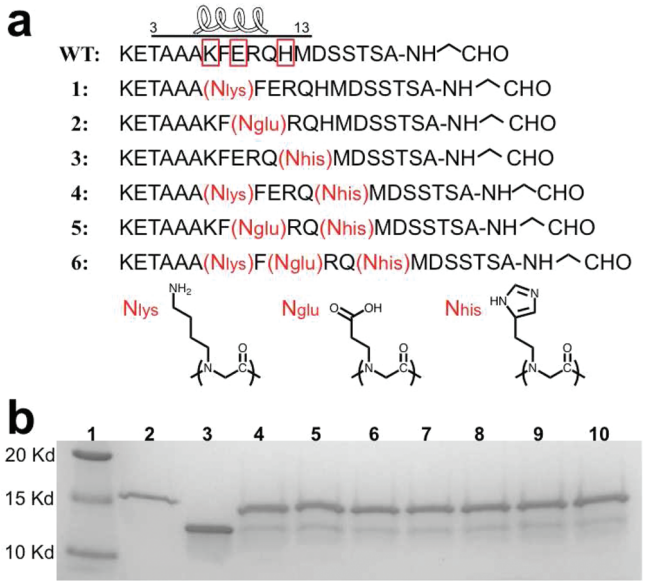

Figure 3. Sequences of peptide-peptoid hybrids and their conjugation to Cys-Spro. (a) Sequences of peptide-peptoid hybrids in this study. We used the peptoid analogues Nlys, Nglu, and Nhis to replace Lys, Glu, and His, respectively. The chemical structures of these analogues are shown at the bottom of panel a. Red boxes in the WT sequence indicates peptoid mutation sites. Residues 3−13 form a helical structure in the context of wild-type ribonuclease A. (b) SDS-PAGE of the protein conjugates using the peptide-peptoids listed in panel a. Lane 1: size marker; Lane 2: the 6× his-tagged ribonuclease A variant containing a thrombin cleavage site and cysteine at the position 20 and 21; Lane 3: S-protein with N-terminal cysteine after the thrombin cleavage; Lane 4: **WT**-Spro conjugate; and Lanes 5−10: **1**-Spro to **6**-Spro conjugate.

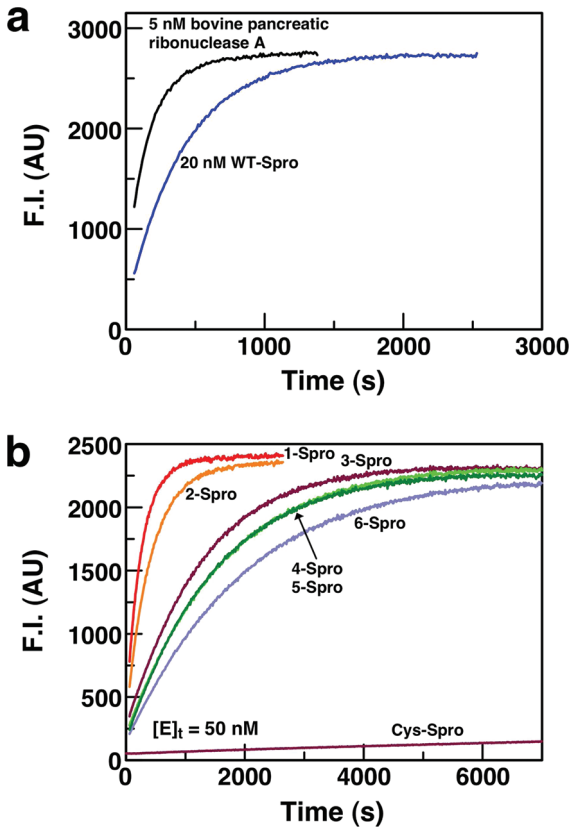

Figure 4. Enzyme kinetics of the ribonuclease A peptoid mutants. The time-dependent fluorescence recovery of the substrate by (a) bovine pancreatic ribonuclease A (5 nM) and **WT**-Spro (20 nM) and (b) **1**-Spro to **6**-Spro, and Cys-Spro, all at 50 nM. The total concentration of the substrate was 1 μM.

LVPRC that is a substrate for thrombin, which cleaves the amide between arginine and cysteine (Figure 2b). A new N-terminal cysteine S-protein is generated after the thrombin cleavage. A C-terminal aldehyde from the synthetic peptide-peptoid hybrid can react with this terminal cysteine to form the thiazolidine ring. This ligation is specific to the N-terminal cysteine and not to any other cysteines in the protein. The aldehyde group is first captured by the free amine at the N-terminus of the protein, and subsequently the free thiol of the N-terminal cysteine attacks the imine to form a stable thiazolidine five-membered ring (Figure 2b). This ligation strategy provides a useful method to introduce synthetic compounds to proteins in a site-specific manner.

Synthesis and Conjugation of Peptide-Peptoid Hybrids. We synthesized several C-terminal aldehyde S-peptide analogues containing various peptoid substitutions. To generate the C-terminal aldehyde, we utilized an aminomethyldioxolane-modified Sasrin solid-phase resin (Methods).[31] After the cleavage of compounds from the resin and treatment of sodium periodate, we were able to efficiently and directly generate a series of peptide-peptoid hybrid aldehydes in good yield for the thiazolidine ligation (Figure 3a; Figure S1 and Table S1 in Supporting Information).

We chose three positions for peptoid mutagenesis: Lys7, Glu9, and His12. These three residues belong to the α-helical region of the S-peptide (Figure 2a).[40] Lys7 is involved in RNA binding *via* electrostatic interaction with the phosphate backbone of RNA. His12 is critical for RNA cleavage, acting as a catalytic base to deprotonate the 2′-OH of RNA.[33] Glu9 forms a salt-bridge to His12 and stabilizes the helical structure in this S-peptide.[41] We changed these peptide residues to the chemically analogous peptoid residues Nlys, Nglu, and Nhis, whose chemical structures are shown at the bottom of Figure 3a. We synthesized single (compounds **1**, **2**, and **3**), double (compounds **4** and **5**), and triple (compound **6**) peptoid mutants to explore the various combinations of these three peptoid substitutions (Figure 3a). This set allows us to see how the peptoid substitutions affect both the helical structure and the enzyme kinetics.

Image analysis of the SDS-PAGE for the thiazolidine ligation (Figure 3b) showed 85% yield on average for the conjugation between the recombinant S-protein and the peptide-peptoid hybrids. To confirm that the conjugation occurs at the N-terminal cysteine of the protein, we tested the ligation using ribonuclease S-protein that contains an N-terminal serine. In this case, we did not observe any conjugation. Thus, the conjugation occurs at the N-terminal cysteine of the S-protein.

Effect of Peptoid Mutations on the Enzyme Kinetics. For the quantitation of enzymatic activity, we utilized a commercially available fluorogenic ribonuclease substrate, RNaseAlert (Integrated DNA Technologies, Inc.). This substrate has one cleavable RNA residue in the middle of a short DNA oligomer with a fluorescence donor and quencher located at each end of the sequence. As the substrate is cleaved, the fluorescence is recovered. The enzyme kinetics was monitored by fluorescence recovery. We measured the residual activity of the purified recombinant S-protein with an

Table 1. Parameters for Ribonuclease Enzyme Activity

	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})
bovine pancreatic ribonuclease A	51 ± 9	(1.1 ± 0.4) × 10^6	56.1
WT-Spro	81 ± 24	(1.1 ± 0.5) × 10^5	8.9
1-Spro	64 ± 9	(7.4 ± 0.2) × 10^4	4.7
2-Spro	62 ± 7	(4.7 ± 0.1) × 10^4	2.9
3-Spro	55 ± 6	(1.5 ± 0.2) × 10^4	0.8
4-Spro	51 ± 5	(1.4 ± 0.1) × 10^4	0.7
5-Spro	58 ± 7	(1.3 ± 0.1) × 10^4	0.8
6-Spro	50 ± 4	(1.0 ± 0.1) × 10^4	0.5

N-terminal cysteine (Cys-Spro) as shown in Figure 4b to see if there was any significant contamination of intact uncleaved ribonuclease A. The Cys-Spro construct had 0.06% of the residual activity relative to bovine pancreatic ribonuclease A, based on the ratio of the initial kinetic slopes of substrate cleavage (Figure 4b). This residual activity was not significant enough to affect the kinetic analysis of all of our ribonuclease variants.

From the kinetics shown in Figure 4, we were able to obtain k_{cat}/K_m values for each peptoid mutant (Table 1). Compared to wild-type bovine pancreatic ribonuclease A, introduction of the artificial thiazolidine ring at the conjugation site (**WT**-Spro) lowered the k_{cat}/K_m value by 1 order of magnitude. Compared to the **WT**-Spro, the single peptoid mutation at catalytic His12 (**3**-Spro) lowered the k_{cat}/K_m by 1 order of magnitude to 1.5 × 10^4 M^{-1} s^{-1}, which indicates that the precise location of the histidine in this position is critical for high efficiency in enzyme activity. The other single peptoid mutants **1**-Spro and **2**-Spro decreased the k_{cat}/K_m by 1.5- and 2.3-fold, respectively, as compared to **WT**-Spro.

The double and triple peptoid mutants containing the catalytic His12 peptoid mutation had no further significant impact on the enzyme kinetics (Figure 4 and Table 1), although the triple peptoid mutant **6**-Spro slightly lowered the k_{cat}/K_m compared to the double peptoid mutants **4**-Spro and **5**-Spro. Thus, the enzyme kinetics of these double and triple peptoid mutations are limited by the peptoid mutation at the catalytic His12. The triple peptoid mutant **6**-Spro is still active with the k_{cat}/K_m value of 1.0 × 10^4 M^{-1} s^{-1}, which is comparable in magnitude to many other natural enzymes.[42]

To understand further details of the enzyme kinetics, we carried out a competition assay with an unlabeled ribonuclease substrate, A(rU)AAA. This unlabeled substrate competitively inhibits the turnover of the labeled substrate. By monitoring the fluorescence recovery in the presence of different concentrations of the unlabeled substrate from 0 to 100 μM, we were able to obtain the profile of concentration-dependent inhibition of the labeled substrate cleavage (Figure S2 in Supporting Information). From this profile, the K_m value for the protein conjugate was determined.[43] As shown in Table 1, we found that there is no significant difference in the K_m values. Thus, it is the turnover rate (k_{cat} value) that is predominantly affected by the peptoid mutations. The binding of the substrate to the protein conjugates is not significantly changed by introduction of the peptoid mutations.

Effect of Peptoid Mutations on the Peptide Helical Structure. To understand the impact of the peptoid mutation on the peptide helicity, we utilized circular dichroism (CD) spectroscopy and molecular dynamics (MD) simulations. The S-peptide has been a model peptide for the study of helical structure and the helix-coil transition.[44−46] In aqueous solution, the S-peptide has no significant secondary structure. However, low-dielectric organic solvents such as 2,2,2-trifluoroethanol (TFE) stabilize the helical structure of the S-peptide with a pronounced CD signal in the far-UV region.[47] As shown in Figure 5, the peptide **WT** showed a typical helical pattern of CD with minima at 208 and 222 nm. This CD spectrum is in line with the previously reported CD of S-peptide.[47] This **WT** has only an extra aldehyde group at the C-terminus, and it has no effect on the peptide helicity.

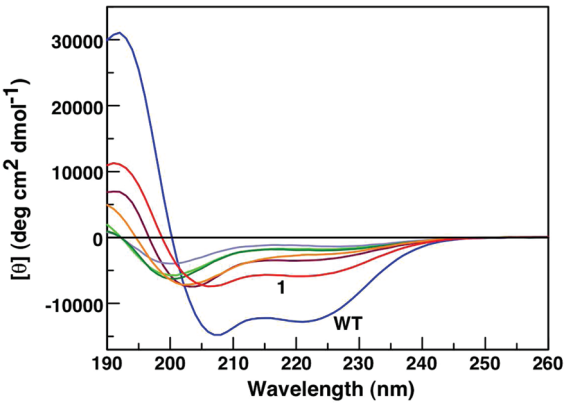

Figure 5. CD spectra of the peptide-peptoid hybrids. The order of CD signal at 222 nm is **WT** > **1** > **3** > **2** > **4** > **5** > **6**. The CD measurement was carried out in the presence of 90% TFE and 10% water.

All peptoid mutations destabilized the helical structure (Figure 5). The order of CD signal at 222 nm in Figure 5 is as follows: **WT** > **1** > **3** > **2** > **4** > **5** > **6**. The relative ratio of CD signal at 222 nm to the **WT** is 0.46, 0.27, 0.20, 0.15, 0.14, and 0.10, respectively. The helical structure was destabilized further with each additional peptoid mutation introduced into the same sequence. This propensity of structural destabilization by peptoid residues roughly correlates with the decrease in enzyme activity (Table 1). This correlation suggests that the helical stability at the N-terminus of ribonuclease A is one of the determining factors for enzyme activity.

The single peptoid mutant, compound **1**, decreased the CD signal at 222 nm by 2-fold as compared to the **WT**. Other single peptoid mutants (compounds **2** and **3**) decreased the CD signal further. These results indicate that the helix-destabilizing effect at position Glu9 and His12 is more pronounced than at position Lys7.

To understand why the peptoid mutation has a significant effect on the helical structure, we carried out MD simulations using the helical segment from residue 3 to 13 with a single peptoid mutation at Lys7. This mutation (compound **1**) decreased the CD signal by 2-fold from the **WT** (Figure 5). We performed 10 ns MD simulation using the GROMACS simulation package and ffG53a6 force field (Supporting Information).[48] From this MD simulation, we found that the effect of the peptoid mutation is local, mainly at the site of mutation and at the C-terminal neighboring residue. In the peptoid mutant, the backbone phi- and psi-angles were more flexible at Lys7 and Phe8 than for the wild-type peptide during the MD simulation (Figure 6). There is no significant difference of backbone angles between the wild-type peptide and peptoid mutant at other residues. This is correlated with the degree of backbone fluctuation as shown in the b-factor during the simulation (Figure S3 in

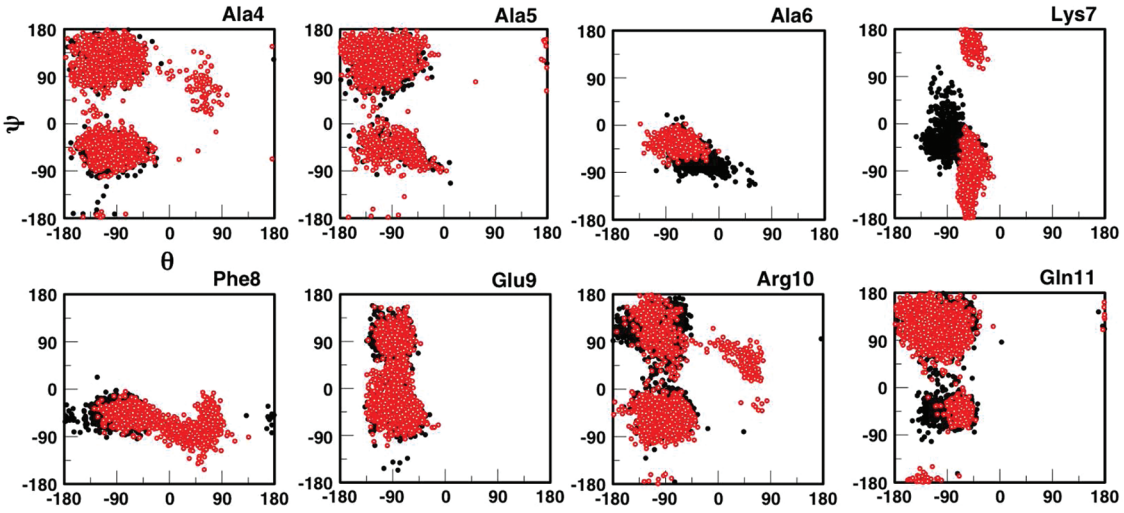

Figure 6. Ramachandran analysis of the MD simulation. Using the N-terminal helical segment (TAAAKFERQHM) and the single peptoid mutant (TAAA(Nlys)FERQHM) of ribonuclease A, we carried out a 10 ns MD simulation using the GROMACS simulation package with ffG53a6 force field. The phi- and psi-angles of the wild-type peptide sampled during the simulation are colored black at each residue, and those for the Lys7Nlys single peptoid mutant are colored red.

Supporting Information). Interestingly, the backbone phi-angle at the C-terminal neighboring residue (Phe8) was allowed to sample the angles from 0 to 90° during the simulation, which is energetically unfavorable in peptides (Figure 6; Figure S3 in Supporting Information). In peptides, the backbone phi-angle is generally limited to a range of angles from −180° to 0. This MD simulation suggests that the peptoid mutation increase the chain flexibility in the peptide helix, which results in the loss of helicity (Figure 5).

In this study, we developed a synthetic tool to introduce peptoid point mutations into a protein, effectively a side-chain translocation mutagenesis. A triple peptoid mutant containing a catalytic His12 peptoid mutation was active with a k_{cat}/K_m value of 1.0×10^4 M^{-1} s^{-1}. This k_{cat}/K_m value is only 10-fold lower than that of the control wild-type conjugate (**WT**-Spro) and comparable in magnitude to those of many other natural enzymes.[42] We expect that with further advancements in combinatorial chemistry synthesis and screening, coupled with computational design, it will be possible to identify peptoid helical sequences that can replace the entire N-terminal region of ribonuclease A. Peptoid helices are attractive candidates for domain substitution because of their excellent thermal,[24] chemical,[49] and protease stability.[25,26] Many proteins can be reconstituted by domain substitution and are potential candidates for this approach.[50] This peptoid substitution provides a unique opportunity to study the effect of side-chain dislocation on protein secondary structure and function and to potentially fortify protease-susceptible protein hotspots. This work is an important step toward engineering proteins with increased stability and potentially altered specificity.

METHODS

Plasmid Construction, Protein Expression, and Purification. Prof. Ron Raines at the University of Wisconsin kindly provided the protein expression plasmid pBXR for the wide-type bovine pancreatic ribonuclease A.[51] Using the QuickChange II site-directed mutagenesis kit (Agilent Technologies), we introduced a thrombin cleavage site and cysteine at positions 20 and 21 of ribonuclease A, respectively. By two rounds of this mutagenesis, we first changed the amino acids TSA to LVP at positions 17−19 and then AS to RC at position 20−21. We carried out additional mutagenesis to insert six histidines before position 1 for ease of protein purification. We utilized the QuickChange Primer Design Program (Agilent Technologies) to design the mutagenesis DNA primers. The change of DNA sequences was confirmed by sequencing.

We used the *E. coli* strain BL21(DE3) for protein expression. The ribonuclease A variant was expressed and purified as described previously with some modifications.[51] The bacterial cell pellet from a 1-L culture was suspended in 20 mM Tris-HCl (pH 7.5) buffer containing 6 M urea, 0.5 M NaCl, 1 mM EDTA, 20 mM DTT, and 30 mM imidazole for 30 min at 37 °C. This suspension was then centrifuged for 15 min at 30,000*g*. The supernatant was subjected to a 5 mL HisTrap FF column (GE Health Sciences) to purify the protein. The ribonuclease A variant was eluted by a buffer containing 20 mM Tris-HCl (pH 7.5), 6 M urea, 1 mM EDTA, 0.5 M NaCl, and 0.5 M imidazole and then dialyzed exhaustively (>48 h) in the presence of the refolding buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM reduced glutathione, and 0.2 mM oxidized glutathione. The amount of protein was quantified using the extinction coefficient of the ribonuclease A, $\varepsilon_{1cm}^{0.1\%} = 0.72$ at 277.5 nm.[51]

Synthesis of Peptide-Peptoid Hybrids with C-Terminal Aldehyde. The peptide-peptoid hybrids were synthesized on the Aapptec Apex 396 synthesizer. We used standard solid-phase Fmoc chemistry for the peptide synthesis and the submonomer cycle to introduce the peptoid mutations.[22] To generate the C-terminal aldehyde, we utilized the Sasrin resin (0.6 mmol/g) coupled with 2,2-dimethyl-1,3-dioxolane-4-methaneamine that we developed previously.[31] For peptide synthesis, 2 mL of 0.4 M Fmoc-protected amino acids in *N*-methylpyrrolidinone (NMP) was added to the resin-bound amine with 0.4 M hydroxybenzotriazole (HOBT) and 137 μL of DIC (0.92 mmol). The reaction mixture was incubated at RT for 2 h. The Fmoc group from the N-terminal amino acid was then deprotected with 20% 4-methlypiperidine in DMF. Between the amide coupling and the deprotection, the resins were washed with DMF (5 × 2 mL). To introduce peptoid residues, the submonomer cycle was employed by bromoacetylation and amine displacement as described previously.[22] We added at 1.5 M 1 mL of the free base of *N*-*t*-Boc-1,4-diaminobutane, *tert*-butyl β-alanine, and histamine for Nlys,

Nglu, and Nhis, respectively, during the amine displacement. All solvents and reagents were obtained from commercial sources and used without further purification.

The peptide-peptoid hybrids were purified by reverse-phase HPLC as described previously.[30,31] They were cleaved from the resin with 95:2.5:2.5 trifluoroacetic acid (TFA)/water/triisopropylsilane (TIS) (v/v/v) for 2 h at RT. The crude product was subjected to reverse-phase HPLC with a Varian Dynamax C18 column (10 μm, 21.4 mm × 250 mm) using a gradient of acetonitrile from 5% to 70% with 10 mL min^{-1} in water and 0.1% TFA. To generate the C-terminal aldehyde, the initially purified products were treated with 10 mL of 5 mM sodium periodate in 20 mM sodium phosphate buffer (pH 7.0) for 30 min and then injected on the reverse-phase HPLC to purify the aldehyde products. All final products were analyzed by analytical reverse-phase HPLC (5−95% gradient of acetonitrile at 1 mL min^{-1} over 30 min at 60 °C with a Grace Vydac C4 column) for purity and ESI-Q-FTICR mass spectrometry (Bruker) for identity. Final products were lyophilized, dissolved in water and stored at −70 °C. Approximate concentrations of products were determined by weight after lyophilization. All peptides were purified to more than 95% purity by analytical HPLC. The molecular weights determined by the ESI-Q-FTICR mass spectrometry are listed in Table S1 in the Supporting Information. The analytic HPLC profile and the mass spectrum for compound **6** are shown in Table S1 in the Supporting Information.

Protein Cleavage and Conjugation. The purified ribonuclease A variant was cleaved with the Thrombin CleaneCleave kit (Sigma) and then subjected to reverse-phase HPLC with a Varian Dynamax C4 column (10 μm, 21.4 mm × 250 mm) using a gradient of acetonitrile from 10 to 70% with 10 mL min^{-1} in water and 0.1% TFA to remove the N-terminal peptide segment. After lyophilization, the resulting ribonuclease S-protein with N-terminal cysteine was further purified as a flow-through of a 1-mL HisTrap FF column (GE Health Sciences) with the buffer of 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 30 mM imidazole, to remove any intact 6× his-tagged ribonuclease A. The flow-through was collected, dialyzed with 50 mM Tris-HCl (pH 7.5) and 0.2 M NaCl, and used for further thiazolidine modification. We measured the residual activity of this S-protein with N-terminal cysteine (Cys-Spro) as shown in Figure 4b. The Cys-Spro had only 0.06% of residual activity relative to bovine pancreatic ribonuclease A, based on the ratio of the initial kinetic slopes of substrate cleavage (Figure 4b).

For the thiazolidine conjugation, 100 μM S-protein with N-terminal cysteine was treated for 1 h with 100 mM DTT in 20 mM Tris-HCl (pH 7.5) and 0.2 M NaCl to reduce the thiols. Then, the peptide-peptoid hybrid (final 1 mM) was added with sodium acetate buffer (pH 5.4, final 150 mM). The reaction mixture was incubated for 2 days. The final protein conjugates were extensively dialyzed, first with 50 mM Tris-HCl, 0.2 M NaCl, pH 7.0; second with 50 mM Tris-HCl, 0.2 M NaCl, 1 mM reduced glutathione, and 0.2 mM oxidized gluthathione, pH 7.0, for the oxidative refolding; and finally with 50 mM Tris-HCl, 0.1 M NaCl pH 7.0. It has been previously shown that recombinant ribonuclease A recovered from this oxidative refolding procedure has an identical activity to natural ribonuclease A isolated from bovine pancreas.[51]

Ribonuclease Enzyme Assay and CD Spectroscopy. For the ribonuclease activity assay, we utilized a commercially available ribonuclease substrate, RNaseAlert (Integrated DNA Technologies, Inc.). The assay is based on fluorescence recovery. Fluorescence measurements were carried out on a fluorescence 96-well plate reader (SpectraMAX GEMINI EM, Molecular Devices Corp.) in a buffer containing 50 mM Tris-HCl (pH 7.0), 35 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.05% Triton X-100, and 1 μM substrate. The excitation and emission wavelength was 490 and 520 nm, respectively. One nM to 50 nM of the ribonuclease A variants were used for the enzyme assay.

The enzyme kinetic parameters were obtained as described previously.[43] One of the enzyme kinetic parameters, k_{cat}/K_m was determined using the eq 1.

$$I = I_f - (I_f - I_0)\, e^{-(k_{cat}/K_m)[E]t} \quad (1)$$

where I is the fluorescence intensity at a given time, and I_f and I_0 are the fluorescent intensity of the final product and initial substrate, respectively.

We carried out a competition assay with an unlabeled ribonuclease substrate, A(rU)AAA (IDT, Inc.) to obtain K_m values. By monitoring the fluorescence recovery in the presence of different concentrations of the unlabeled substrate from 0 to 100 μM, we were able to obtain the profile of concentration-dependent inhibition of the labeled substrate cleavage and fit this profile with the eq 2 to determine the K_m.[43] In this analysis, we used initial linear rates of enzyme kinetics (Figure S2 in the Supporting Information).

$$\text{relative activity} = K_m/(K_m + [S]) \quad (2)$$

where the relative activity is the ratio of initial rates to the absence of unlabeled substrate at a given concentration of the unlabeled substrate [S].

CD measurements were carried out with 100 μM of each compound in the presence of 90% TFE and 10% water using a Jasco 715 spectropolarimeter. A 0.1 cm path-length quartz cell was used for far-UV CD.

MD Simulation. Using the N-terminal helical segment (TAAAKFERQHM) and the single peptoid mutant (TAAA(Nlys)FERQHM) of the ribonuclease A, we carried out the MD simulation. We used the GROMACS simulation package with ffG53a6 force field (www.gromacs.org).[48] The atomic coordinates of the helix were obtained from the previous X-ray crystal structure (PDB code: 1FS3).[40] We virtually moved the side chain of Lys7 to the backbone nitrogen using PyMol (DeLano Scientific LLC). We generated a peptoid residue topology for Nlys at the position 7 combining the topology and force parameters from proline and lysine for the backbone and side chain, respectively. Both structures were first minimized by a steepest descent energy minimization. Then, we added a rhombic dodecahedron periodic box with a minimal distance of 1 nm between periodic images, solvent waters with SPC model, and ions to neutralize the system. After the energy minimization of the solvated system, 5 ps of position restrained MD, unrestrained NVT and NPT simulation were conducted to allow the solvent annealing and the temperature and pressure coupling prior to the production NPT simulation for 10 ns with the integration step size of 2 fs. The coordinates were stored every 10 ps.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http:// pubs.acs.org.

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