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Protein Prosthesis: A Semisynthetic Enzyme with a β-Peptide Reverse Turn

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A long-term goal of biological chemists is to create new proteins with desirable properties.[1] One potentially fruitful approach is to remodel the frameworks designed by nature.[2] The genetic code limits the components of natural proteins to 20 or so α-amino acids. Methods that overcome this limitation but still rely on the ribosome are similarly limited to a subset of α-amino acids and α-hydroxy acids.[3] Total chemical synthesis of proteins is limited to relatively small polypeptides.[4] In contrast, the new method of expressed protein ligation (EPL) enables the semisynthetic incorporation of a limitless variety of nonproteinogenic modules into proteins of variable size (Figure 1).[5] Granted this freedom, chemists must now identify those synthetic components that are truly useful.

Here, we report on the first incorporation of a synthetic module composed of β-amino acids into an enzyme. We demonstrate that the resulting semisynthetic enzyme not only retains full catalytic activity, but also gains conformational stability. This effort links two active fields, the re-engineering of natural enzymes and the development of "foldamers",[6] nonnatural oligomers with well-defined folding preferences. Oligo-β-acids ("β-peptides") are among the most thoroughly characterized foldamers to date.[7] When the residues are preorganized properly, β-peptides have greater secondary structural stability, on a per-residue basis, than do conventional peptides. These attributes are encouraging us to explore polypeptides with heterogeneous backbones, that is, with backbones containing more than one type of building block. In particular, we are interested in creating analogues of natural proteins in which the α-amino acid residues required for biological activity are retained but structural components are replaced with more stable nonnatural segments. The prospective benefits of success include endowing chemotherapeutic proteins with the ability to survive longer in vivo or retain activity after oral administration.

As our target enzyme, we chose ribonuclease A (RNase A; EC 3.1.27.5), which has been the object of much seminal work in protein chemistry.[8,9] RNase A has eight cysteine residues that form four disulfide bonds in the native enzyme. In EPL, a modified intein is used to create a biosynthetic protein fragment containing a C-terminal thioester. The thiolate of an N-terminal cysteine residue in a synthetic peptide attacks the thioester to produce, ultimately, an amide bond within a semisynthetic protein.

The β-turn is an especially favorable way for a polypeptide chain to reverse its direction, as is necessary for its folding into a compact structure.[10] Reverse turns are critical to protein conformational stability[11] and many protein−protein interactions.[12] In addition, turns are often a preferred site for degradation by proteolytic enzymes.[13] Hence, biological chemists are seeking to create effective turn mimics.[14,15] In general, these mimics are designed to minimize the conformational entropy of the unfolded state by pre-organization of the turn. In native RNase A, residues Gly112-Asn113-Pro114-Tyr115 form a Type VI reverse turn within a hairpin (**1**). We suspected that Asn113-Pro114 could be replaced by a reverse-turn mimic composed of two β-amino acid residues, *R*-nipecotic acid-*S*-nipecotic acid (*R*-Nip-*S*-Nip, **2**). This di-β-peptide unit has been shown to form a N−H···O═C hydrogen bond within a 12-membered ring[16a] and to promote β-hairpin formation when flanked by α-amino acid residues[16b] or β-amino acid residues.[16c,d] β-Peptides such as *R*-Nip-*S*-Nip are resistant to degradation by proteolytic enzymes.[17]

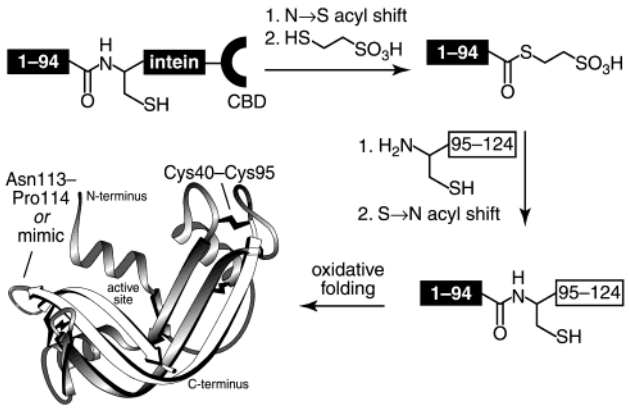

Figure 1. Semisynthesis of ribonuclease A by expressed protein ligation. N−Terminal segment (black box) is produced by biosynthesis; C-terminal segment (white box, including Cys95) is produced by chemical synthesis and can contain nonnatural modules.

We used EPL to replace Asn113-Pro114 with the nonnatural module **2**. Specifically, we produced RNase A fragment 1−94 as a fusion protein with the *mxe*-intein and a chitin-binding domain (CBD, Figure 1). Ligation to a peptide corresponding to residues 95−124 resulted in a protein that by mass spectrometry contained the expected amino acid residues.

Enzymatic catalysis provides an extremely sensitive measure of native protein structure.[18] RNase A(1−94) has no detectable ribonucleolytic activity. In contrast, the activity of the semisynthetic variant containing the *R*-Nip-*S*-Nip module is indistinguishable from that of the wild-type enzyme produced in three distinct ways.[19] Moreover, replacing Asn113-Pro114 with the *R*-Nip-*S*-Nip module does not diminish the conformational stability of the enzyme but slightly enhances it [$\Delta T_m = (1.2 \pm 0.3)$ °C, Table 1].

Thus, this β-peptide module is not only tolerated by the protein structure but actually increases its conformational stability. In marked contrast, replacing Pro114 with the natural α-amino acids glycine or L-alanine causes a large decrease in conformational stability ($\Delta T_m = -10$ °C).[21]

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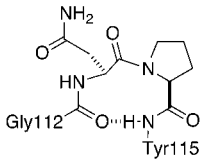
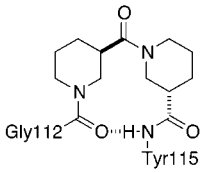
Table 1. Properties of Ribonuclease A Variants



1



2

residues 113-114	origin	T_m (°C)[a]	k_{cat}/K_M (10^7 $M^{-1}s^{-1}$)[b]
R-Nip-*S*-Nip (**2**)	semisynthesis	64.6 ± 0.2	1.6 ± 0.4
R-Nip-*R*-Nip	semisynthesis	ND[c]	0.03 ± 0.01[d]
Asn-Pro (**1**)	semisynthesis	63.4 ± 0.2	1.5 ± 0.2
Asn-Pro (**1**)	*E. coli*	63.5 ± 0.1	1.4 ± 0.3
Asn-Pro (**1**)	bovine pancreas	63.6 ± 0.2	1.5 ± 0.2

[a] T_m is the temperature at the midpoint of the thermal transition between the native and unfolded states. Values (±SE) were determined by UV spectroscopy in 50 mM sodium phosphate buffer (pH 8.0) containing NaCl (25 mM) and protein (0.2 mg/mL). [b] Values (±SE) were determined at 25 °C in 0.10 M MES−NaOH buffer (pH 6.0) containing NaCl (0.10 M) and 6-carboxyfluorescein-dArU(dA)$_2$-6-tetramethylrhodamine as substrate.[20] [c] ND, not determined. [d] Estimated with unpurified material.

The diastereomeric *R*-nipecotic acid-*R*-nipecotic acid (*R*-Nip-*R*-Nip) module, which cannot adopt the conformation of a reverse turn,[16a−c] should be an ineffective reverse-turn mimic. To test this hypothesis, we used EPL to replace Asn113-Pro114 with *R*-Nip-*R*-Nip (Table 1). Almost all of the resulting protein failed to fold into an active conformation. The low ribonucleolytic activity detected in this preparation most likely resulted from the presence of enantiomeric impurities (≤2%) in *R*- and *S*-Nip.[16a−c]

Dipeptide segments in other natural proteins have been replaced with nonnatural turn mimics.[14,15] In most cases, the natural polypeptide has been relatively small (≤55 residues) to facilitate total synthesis. Proteins in this size range typically do not display catalytic activity, and comparisons between natural proteins and their chimeric analogues have necessarily focused on structural criteria. When thermal stabilities have been compared, the natural polypeptide has always had a higher T_m than the chimeric analogue, in contrast to our observations. One chimeric enzyme containing a reverse turn mimic has been reported,[14b] but this molecule was impure and not compared with its natural biosynthetic counterpart. Our findings thus represent a unique contribution, as the observation of full enzymatic activity by chimeric RNase A is an exceedingly stringent indicator that native tertiary structure has been maintained.[18] In addition, comparison of the diastereomeric *R*-Nip-*S*-Nip and *R*-Nip-*R*-Nip turn segments demonstrates that the nonnatural segment does not serve merely as a passive linker, but rather that the specific conformational propensity of the prosthetic segment is critical for successful replacement of a natural reverse turn. These results suggest that it will be extremely fruitful to examine other chimeric proteins in which natural structural elements are replaced with foldameric equivalents of enhanced chemical and conformational stability.

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Supporting Information Available: Procedures and additional data for all syntheses and analyses reported herein (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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