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Transposition of Protein Sequences: Circular Permutation of Ribonuclease T1

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Abstract: A general procedure has been developed for the simple and rapid construction of circularly permuted proteins. The polymerase chain reaction (PCR) was utilized to amplify the gene for ribonuclease T1 (RNase T1) in two separate pieces, and these fragments were recombined in reverse order to create the circularly permuted gene. The circularly permuted analog of RNase T1 was assembled by first removing the disulfide bond between Cys-2 and Cys-10 through substitution with alanine residues to create the mutant (C2A, C10A). The original amino- and carboxyl-terminal ends of (C2A, C10A) were then covalently linked with the peptide Gly-Pro-Gly, and new termini were introduced between residues Gly-34 and Ser-35. The bacterially expressed and circularly permuted variant of RNase T1, cp35S1, was 28% as active as the native enzyme in the catalytic hydrolysis of RNA, and thus the mutant protein must fold to a conformational state quite similar to that of the native enzyme. Amino acid sequence analysis and mass spectrometry have confirmed the primary structure of the reconstructed protein. Thermodynamic stabilities at pH 5.0 of wild-type RNase T1, (C2A, C10A), and cp35S1 proteins were found to be 10.1, 6.4, and 4.2 kcal/mol, respectively.

Introduction

The relationship between protein structure and function is of significant interest to many biological chemists. However, the fundamental principles and rules that relate the primary sequence to the three-dimensional structure of folded proteins are largely unknown. A basic prerequisite for the prediction of protein tertiary structures will therefore involve a greater understanding for how small modifications to the precise amino acid sequence will influence the kinetics and thermodynamics of specific protein-folding pathways. These subtle alterations to the primary sequence of proteins at defined locations can be made via site-directed mutagenesis and circular permutations.

The creation of a circularly permuted variant of a protein involves the linear reorganization of the primary amino acid sequence such that the original amino and carboxy termini are covalently closed and new termini are created at an alternate site within the sequence. The concept of a circularly permuted protein was first developed by Goldenberg and Creighton with bovine pancreatic trypsin inhibitor (BPTI) by utilization of chemical coupling methods on the purified protein.[1] Kirshner's laboratory pioneered the bacterial expression of circularly permuted proteins with the characterization of variants of phosphoribosyl anthranilate isomerase (PRAI) and dihydrofolate reductase (DHFR).[2,3] More recently, a circularly permuted variant of T4 lysozyme has also been characterized.[4] The major drawbacks in the methods used to date to create the genetic sequences for these circularly permuted variants are that multiple and laborious recombinant DNA manipulations (approximately eight in each case) have been required. In contrast, the protocol outlined in this article

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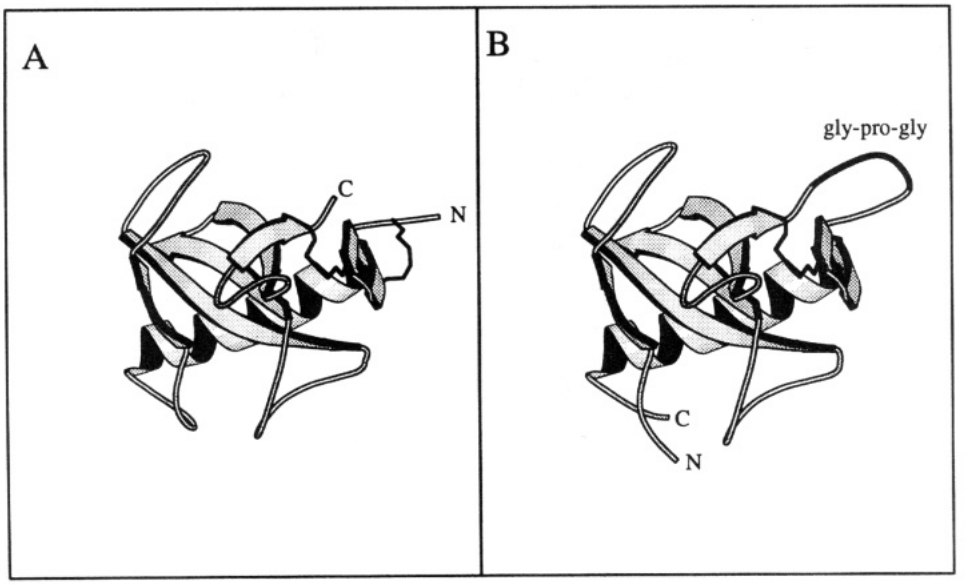

Figure 1. Ribbon diagram for the structure of native RNase T1 and the circularly permuted sequence: (A) three-dimensional structure of RNase T1; (B) three-dimensional structure of cp35S1 as modeled with Insight II from BIOSYM. The representations were made with MOLSCRIPT.[21] The original coordinates were from the crystal structure determined by Martinez-Oyanedel *et al.*[6]

is a general method requiring only four oligonucleotide primers and three polymerase chain reaction (PCR) amplification steps. We have used this general PCR methodology to construct a circular permutation of ribonuclease T1 (RNase T1).

RNase T1 is an excellent model for investigating the effects of circularly permuted sequences on protein folding and stability. It is a small α,β globular protein of 104 residues (Figure 1A),[5] and the native structure contains two disulfide bonds. The three-dimensional structure has been determined to 1.5-Å resolution by X-ray crystallography,[6] and the complete NMR spectrum has been assigned.[7] Recently, the folding pathway of the native oxidized protein was determined using the hydrogen–deuterium amide exchange 2D NMR technique.[8] RNase T1 has also been well characterized with regard to the thermodynamics[9] and kinetics of protein folding.[10]

General Circular Permutation PCR Methodology

The general method developed to circularly permute RNase T1 utilizes the polymerase chain reaction (PCR). This procedure can be effectively applied to other protein systems as well, as it is a relatively simple and straightforward technique for rearranging the primary amino acid sequence of any protein. The overall strategy for this approach is shown in Figure 2. Two sets of oligonucleotide primers are required. The first primer set, designated *linker B* and *cpB* (circularly permuted), is used in the first PCR step to create and amplify the DNA fragment (fragment B) that contains the sequence encoding the new N-terminal portion of the circularly permuted protein followed by the linker that connects the C-terminal and N-terminal ends of the original protein. The second primer set, designated *linker A* and *cpA*, is used in the second PCR step to create and amplify the DNA fragment (fragment A) that encodes the same linker as used above, followed by the new C-terminal portion of the circularly permuted protein. Fragments A and B, which share the same linker sequence, are joined together in a third PCR step to make the full-length gene encoding the circularly permuted protein. Multiple circularly permuted analogs of the same protein can easily be made by varying the oligonucleotide primers, *cpA* and *cpB*. Conversely, multiple peptide linkers can be sampled by a systematic variation of the primers, *linker A* and *linker B*. This general method of creating circularly permuted proteins through the use of PCR technology can be further extended to make even more complex rearrangements of protein sequences.

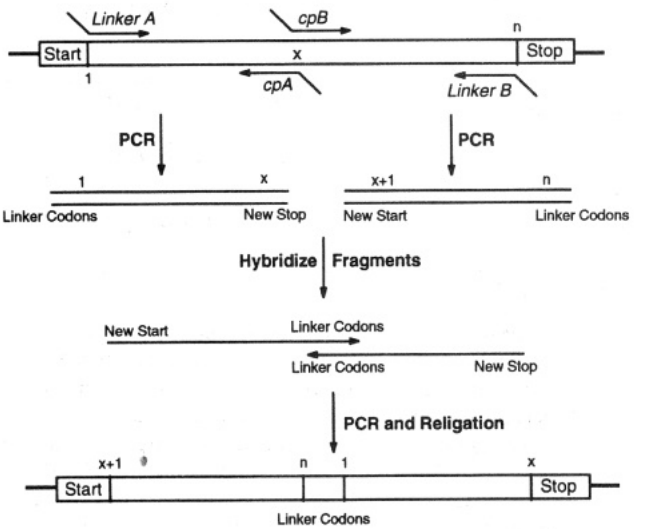

Figure 2. General PCR protocol for the creation of circularly permuted proteins.

Experimental Methods

Materials. Urea, hen egg lysozyme, and Type II-C ribonucleic acid core were purchased from Sigma. All other reagents and restriction enzymes were purchased from either Promega, Perkin-Elmer, Stragene, or United States Biochemical Corp. The oligonucleotides that were used for mutagenesis and sequencing were synthesized by the Gene Technology Laboratory of the Biology Department at Texas A & M University. The plasmid pMac5-8 and the *Escherichia coli* strain WK6 were generous gifts from Professor C. N. Pace (Texas A & M University).

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Table 1. PCR Primers Used in the Creation of Ribonuclease T1 Mutants[a]

primer	sequence
primer A	5'CAA CAG GGA CAC CAG GAT3'
StyI-B	5'CA AGC CGC CTT GGT CAC AGG3'
StyI-C	5'CCT GTG ACC AAG GCG GCT TG3'
primer D2	5'CGA GGT GAA TTA GCT TGG3'
C2,10A	5'C CCT GTG ACC AAG GCG GCT GCT GAC TAC ACT TGC GGT TCT AAG GCT TAC TCT3'
linker A	5'TGT ACA GGT CCG GGT GCT GCT GAC TAC ACT TGC GGT TCT3'
linker B	5'AGC AGC ACC CGG ACC TGT ACA TTC AAC GAA GTT GTT ACC3'
cpA	5'TGG GTA AGA AGC TTA TCC AAC AGT TTC ACC GTC3'
cpB	5'A GAC GGT GCC AAG GCG GCT TCC AAT TCT TAC CCA CAC3'

[a] Underlined nucleotides indicate mutagenic sites.

Site-Directed Mutagenesis. The plasmid, pMac5-8, encodes the gene for RNase T1 downstream from the signal peptide portion of the alkaline phosphatase gene, *phoA*.[11] The genes are under the transcriptional control of the *tac* promoter. The protein is expressed as a *phoA*-RNase T1 fusion product which undergoes posttranslational cleavage of the leader peptide, thus allowing high accumulations of RNase T1 in the periplasmic space.

The pMac5-8 plasmid contains a unique *Hin*dIII restriction site immediately following the stop codons for the RNase T1 gene and a unique *Eco*RI restriction site upstream from the *tac* promoter region. To facilitate the cloning of the circularly permuted variants, a unique *Sty*I restriction site was silently inserted into the portion of the gene encoding the *phoA* leader sequence using the overlap extension PCR method for site-directed mutagenesis.[12] The four primers used for this procedure are shown in Table 1 and have been designated *primer A*, *StyI-B*, *StyI-C*, and *primer D2*. *Primer A* has been designed to anneal upstream of the *Eco*RI site, while *primer D2* anneals downstream of the *Hin*dIII site. *StyI-B* and *StyI-C* are complementary to one another and contain the base changes necessary for the creation of the *Sty*I restriction site. The PCR mutagenic procedure was accomplished in three steps. In the first PCR step, *primer A* and *StyI-B* were used with the pMac5-8 plasmid as the template to generate the fragment AB. The second PCR step was performed with *StyI-C* and *primer D2* and the pMac5-8 plasmid to generate fragment CD. Fragments AB and CD were then combined in a third PCR step along with *primer A* and *primer D2* to generate and amplify the full-length AD fragment containing both the *Eco*RI and *Hin*dIII restriction sites along with the new *Sty*I restriction site. This fragment was restricted with *Eco*RI and *Hin*dIII and cloned into the similarly restricted pMac5-8 plasmid, creating the new plasmid, pKW01, which contains the silent *Sty*I restriction site within the RNase T1 gene.

The pKW01 plasmid was used as the template to engineer a RNase T1 mutant that eliminated one of the two disulfide bonds in the native protein by changing Cys-2 and Cys-10 to Ala-2 and Ala-10, respectively. The new protein was designated (C2A, C10A) RNase T1. This set of mutations was made in a single PCR step using only two primers, *C2, 10A* and *primer D2*, (see Table 1) since the two mutation sites were close enough to be encompassed by one mutagenic primer. The resulting PCR fragment and the pKW01 plasmid were separately restricted with *Sty*I and *Hin*dIII. The purified insert and vector were combined and ligated together to yield a new plasmid, pKW02, which contains the *Sty*I restriction site as well as the codon changes to replace the original cysteine residues at positions 2 and 10 with alanines.

The third mutant, encoding the circularly permuted RNase T1, was created with the general PCR procedure outlined in the previous section utilizing four oligonucleotide primers. The primers, designated *cpA*, *cpB*, *linker A*, and *linker B* are shown in Table 1. In the first PCR step, the fragment between the *Eco*RI and *Hin*dIII sites containing the RNase T1 gene was excised from the plasmid pKW02 and used as the template for the *cpB* and *linker B* primers. The *cpB* primer consisted of a portion of the *phoA* leader sequence containing the newly engineered *Sty*I site followed by the codon for an extra alanine and then the codons for Ser-35 through His-40. The extra alanine was inserted to allow for the proper processing of the mature protein from the *phoA* fusion product. The *linker B* primer consisted of the codons for Gly-97 through Thr-104, the codons for the Gly-Pro-Gly linker, and finally the codons for Ala-1 through Ala-2. In the second PCR step, the same pKW02 fragment was again used as the template with a new set of primers, *cpA* and *linker A*. The *linker A* primer consisted of the codons for Cys-103 through Thr-104, the codons for the Gly-Pro-Gly linker, and the codons for Ala-1 through Ser-8. Finally, the *cpA* primer consisted of the codons for Asp-29 through Gly-34, a stop codon, and the *Hin*dIII site. The amplified fragments from each PCR step were isolated and combined in a third PCR step with the *cpA* and *cpB* primers to create an amplified fragment containing the circularly permuted sequence. This circularly permuted gene was cloned into the pMac5-8 plasmid isolated from a *Sty*I and *Hin*dIII digestion of pKW02 to yield a new plasmid, pKW03. The newly constructed gene for the circular permuted variant of RNase T1 (cp35S1) thus encodes a 108 amino acid protein, beginning with an alanine, followed by Ser-35 through Thr-104 of the original protein, the Gly-Pro-Gly linker, and Ala-1 through Gly-34 of the original protein. The ribonuclease T1 genes contained within the plasmids pKW01, pKW02, and pKW03 were completely sequenced to ensure that no unwanted mutations were incorporated during the PCR amplification steps. The construction of these plasmids from pMac5-8 is summarized in Figure 3.

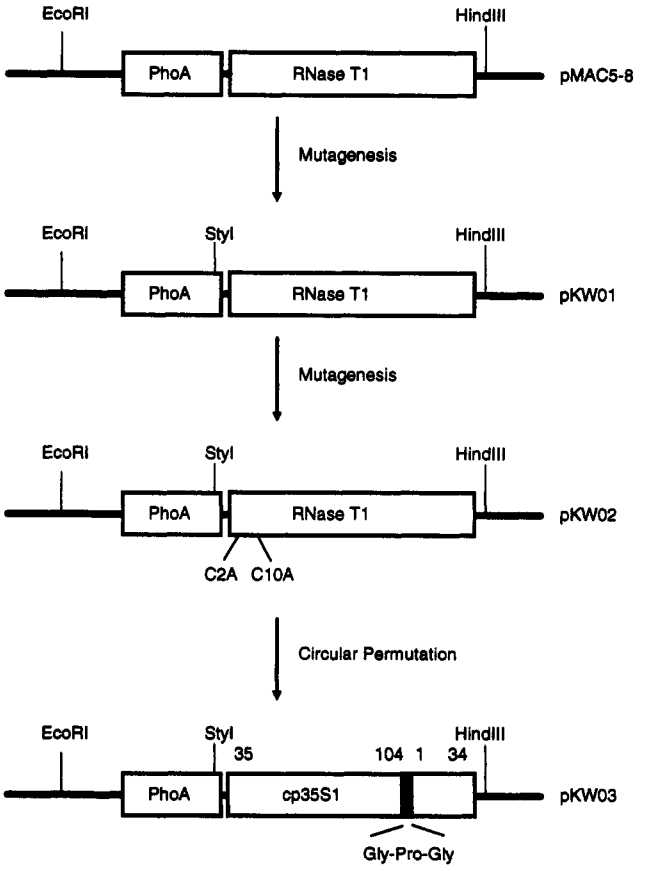

Figure 3. Structure of the plasmids utilized for the creation of the circularly permuted RNase T1.

Enzyme Purification and Assay. The plasmids pKW01, pKW02, and pKW03 were transformed into *E. coli* WK6 cells. The resulting proteins, RNase T1, (C2A, C10A), and cp35S1 were expressed and purified as described by Shirley and Laurents.[13] Specific activity was determined by the catalytic hydrolysis of RNA using the continuous assay of Oshmia

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Table 2. Characterization of the Circularly Permuted Variant of RNase T1

	protein		
	RNase T1	(C2A, C10A)	cp35S1
specific activity[a]	19 000 U/mg	17 000 U/mg	5400 U/mg
molecular mass (calculated)[b]	11 085	11 023	11 305
molecular mass (observed)[c]	11 088	11 004	11 303
stability (urea, pH 5.0)	10.1 kcal/mol	6.4 kcal/mol	4.2 kcal/mol
N-terminal sequence[d]		AADYTXGSNAYS	ASNSYPHKYNNY

[a] From the continuous assay described by Oshmia *et al.*[20] Units are defined as 0.01 change in absorbance at 298.5 nm/min at 37 °C. [b] Calculated molecular masses are based on average molecular weight for the individual amino acids. [c] Represents the molecular mass in the +1 protonation state. [d] These sequences were determined at the Biotechnology Support Services Laboratory of Texas A & M University.

et al.[14] Native and SDS polyacrylamide gel electrophoresis was performed at room temperature as described by Pace and Creighton.[15]

Thermodynamic Measurements. Urea denaturation curves were determined for RNase T1, (C2A, C10A), and cp35S1 by measuring the intrinsic fluorescence intensity (278-nm excitation and 320-nm emission) of solutions containing approximately 0.9 μM protein as a function of the urea concentration in a thermostated Perkin-Elmer MPF 44 B spectrophotometer. The solutions were kept at 25 °C, pH 5.0 (Na acetate/acetic acid), for 24 h before measurements were made. The free energy of unfolding was calculated by the linear extrapolation method.[16] The error in ΔG was ±0.6 kcal/mol.

Mass Spectrometry. The masses of the RNase T1 proteins were determined with matrix-assisted laser desorption ionization (MALDI) mass spectrometry on a home-built spectrometer constructed in the laboratory of Professor D. H. Russell of the Chemistry Department at Texas A & M University. The matrix solution was α-cyano-4-hydroxycinnamic acid (15 mg/mL) in methanol. The samples were ionized in a 20-kV electric field. The instrument was calibrated with 1 mg/mL hen egg lysozyme in 50% methanol. Each sample had a concentration of 1–2 mg/mL protein and was internally standardized to hen egg lysozyme (2 mg/mL, 50% ethanol).

Amino-Terminal Sequencing. The amino-terminal sequences of (C2A, C10A) and cp35S1 T1 were determined with an Applied Biosystems 470A sequencer in the Biotechnology Support Laboratory at Texas A & M University.

Results and Discussion

The three-dimensional structure of the native RNase T1 is shown in Figure 1A. The amino and carboxy termini are constrained in space with a separation of approximately 11 Å due to the presence of two disulfide bonds.[17] A large loop is formed between Cys-6 and Cys-103, while a smaller loop is made by the presence of a disulfide bond between Cys-2 and Cys-10. A peptide linker of five amino acids would be sufficient to bridge the distance between the amino and carboxy termini in the native oxidized protein without introducing any undo torsional strain.[18] However, it has previously been shown that when the disulfide bond between Cys-2 and Cys-10 is broken, the protein will remain folded and retain a conformation very similar to that of the native enzyme.[15] Therefore, removal of the disulfide bond between Cys-2 and Cys-10 would introduce greater flexibility at the amino terminus such that a peptide linker of approximately three amino acids would be of sufficient length to bridge the gap between the original N- and C-terminal ends of RNase T1.[17] The sequence of the peptide linker chosen for the construction of the circular permutation of RNase T1 was Gly-Pro-Gly because these residues have a relatively high propensity to accommodate β-turns.[19]

There are five loops in the tertiary structure of the native RNase T1 that link secondary structural elements. The most solvent exposed region of RNase T1 is the loop between the lone α-helix and the start of the central β-sheet (residues 28–39).[5] This loop has not been implicated in either substrate binding or catalytic activity, so it was chosen as the location for the new termination site.[5] The circularly permuted RNase T1, designed in this manner, has Ser-35 as the new amino terminus, Thr-104 linked to Ala-1 by Gly-Pro-Gly, and Gly-34 as the new carboxy terminus. To ensure correct processing of the circularly permuted variant of RNase T1 in the expression system, an additional alanine was added prior to Ser-35 at the amino terminus. A computer-generated model of this circular permutation of RNase T1, cp35S1, is shown in Figure 1B.

RNase T1, (C2A, C10A), and cp35S1 were characterized by gel electrophoresis (SDS and native), specific activity, matrix-assisted laser desorption (MALDE) mass spectrometry, amino-terminal sequencing, and thermodynamic stability. The results of these characterizations are presented in Table 2. The proteins were purified to greater than 95% homogeneity, as determined by SDS gel electrophoresis. The mobilities of RNase T1 and (C2A, C10A) were identical on 15% native gels, whereas cp35S1 migrated at a slower rate. The observed molecular weights of these proteins, as determined by laser desorption mass spectrometry,[20] are fully consistent with the values predicted by the expected amino acid composition. N-terminal protein sequencing verified the anticipated amino acid sequence of both (C2A, C10A) and cp35S1. The catalytic activities for the hydrolysis of RNA exhibited by (C2A, C10A) and cp35S1, relative to RNase T1, were 89% and 28%, respectively. Urea denaturation studies were conducted in order to more fully assess the effect of sequence transposition on the properties of the folded protein (Figure 4). The m values for wild-type RNase T1, (C2A, C10A), and cp35S1 were 1600, 1500, and 1650 cal mol^{-1} M^{-1}, respectively. The thermodynamic stabilities of (C2A, C10A) and cp35S1 were reduced relative to the native RNase T1 by 3.7 and 5.9 kcal/mol, respectively.

The specific catalytic activity and overall thermodynamic stability of the (C2A, C10A) RNase T1 is very similar to the Cys-2, Cys-10, reduced and carboxy-methylated RNase T1 previously described by Pace and Creighton.[15] The behavior of this mutant confirms that the presence of the disulfide bond between Cys-2 and Cys-10 in the native protein primarily imparts thermodynamic stability but has little overall effect on catalytic activity. The circularly permuted protein characterized in this study is a direct transposition of the amino acid sequence of (C2A, C10A) RNase T1. The use of this mutant protein as a starting point permitted a shorter linker sequence to be used to bridge the distance between the original amino and carboxy termini. The overall activity and stability of the circular variant indicates quite clearly that it has adopted an overall tertiary fold that is very similar to that of the native protein even though the α-helix has

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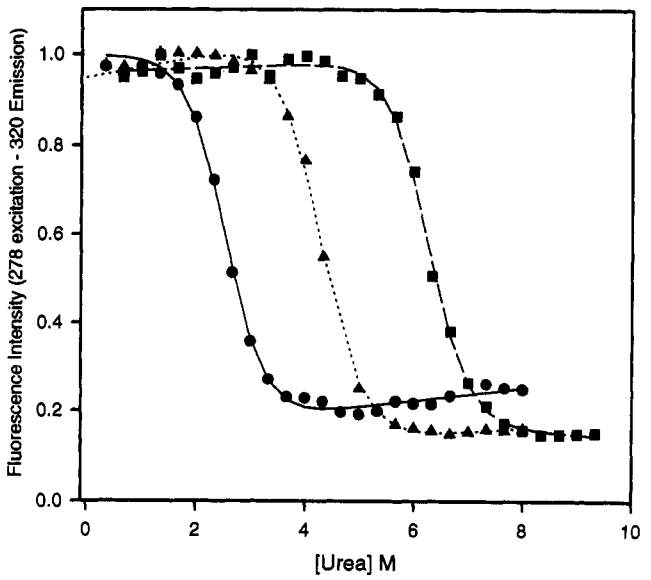

Figure 4. Urea denaturation curves for the unfolding of RNase T1 and mutants: native RNase T1 (■); (C2A, C10A) (▲); and cp35S1 (●). The assay conditions were 25 °C, 100 mM Na acetate, pH 5.0. Additional details are given in the text.

been relocated from the amino-terminus region to the carboxy-terminus region. Therefore, with regards to the final tertiary structure, the transposition of the first 34 amino acid residues to the C-terminus has little effect on overall folding. The real effect of this transposition, however, might become more evident as an effect on the kinetics of the specific folding pathway the protein follows to reach the final tertiary structure.

The design of a circularly permuted protein is governed by the location of the new termini and the choice of the linker peptide sequence connecting the original termini. Generally, the least disruptive locations for the new termini are to be found in loops between secondary structural elements and functional domains. The choice of the linker is, however, less well-defined. The length of the linker peptide is partially restricted by the geometrical constraints in the distance between the original amino and carboxy termini. Choices for the amino acid content of the linker sequence can be found by molecular modeling and data base comparisons with similar structural motifs. As more circular variants are characterized, the effect of the length and sequence of the peptide linker on the structure and stability of the circular variant will be better understood.

In conclusion, we have demonstrated the utility of a general PCR approach for the creation of circularly permuted proteins through the initial characterization of cp35S1. This circularly permuted variant of RNase T1 can be expressed and purified in a catalytically active form. The PCR procedure outlined in this paper can be applied to any cloned gene. This procedure will be used to make other circularly permuted variants of RNase T1 in order to investigate the impact of specific alterations in the amino acid sequence on the protein-folding pathways.

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