DOI: 10.1002/chem.200600007

Activating an Enzyme by an Engineered Coiled Coil Switch

Satoshi Yuzawa, Toshihisa Mizuno,* and Toshiki Tanaka*[a]

Abstract: We designed a de novo protein based on a circular permutant of RNaseT1, in which the enzymatic activity can be manipulated by engineered peptide binding. The circular permutant of RNaseT1 was obtained by tethering the original C- and N-termini with a GPAG linker and cleaving the molecule between Glu^{82} and Asn^{83}. This mutant lacked enzymatic activity, due to the destabilization of entire protein structure. We previously reported the construction of ABC-type heterotrimeric coiled coil peptides, in which the A- and B-type peptides cannot form the folded trimeric structure without the C-type peptide. The introduction of the A- and B-type coiled coil peptides to the C- and N-termini of the circular permutant of RNaseT1, respectively, and the subsequent addition of the C-type coiled coil peptide enabled the RNaseT1 domain to refold properly, thus, restoring the enzymatic activity. The formation of the trimeric coiled coil structure should bring the cleaved sites of RNaseT1 close enough to refold the RNaseT1 domain spontaneously.

Keywords: circular permutation · de novo design · helical structures · proteins · RNaseT1

Introduction

The design of de novo functional proteins is an attractive target in protein engineering.[1] Especially, ligand-gated proteins with functions that can be manipulated by metal ions and specific ligands have great potential in various applications, and have attracted the attention of many scientists.[2] Their functions adjust to various natural biological processes.[3] Within signal transduction pathways in living cells, many kinds of signal mediators participate in signal-cascade systems, for example, inositol triphosphate for kinases, GTP for G-proteins, Ca^{2+} for nerve axons, and so on.[4] "Feedback inhibition" and "precursor activation", which regulate enzymatic activities, are also involved in ligand-dependent protein function.[5] For the de novo design of artificial ligand-gated proteins, introducing a ligand-binding site to the functional domain is necessary. However, the suitable creation of a ligand-binding site in a native functional protein is not straightforward.

Previously, fusing the ligand-dependent domain of a natural protein to other functional domains seemed to be an effective approach for de novo design. Quall et al. created a light-switchable gene expression system by tethering the GAL4 DNA binding domain (GDB) to the N-terminal domain of photosensitive Phytochrom (N-Phy) via a linker. Upon irradiation with light, the N-Phy isomerization increased the binding affinity to the PIF3 domain. Subsequently, the RNAP fused with PIF3 activated the gene downstream of the GAL4 binding element.[6] Chambon et al. designed a ligand-dependent enzyme catalyzing homologous recombination in eukaryotic cells, using the estrogen binding domain and the Cre recombinase.[7] The Cre recombinase domain dimerization was induced by ligand binding to the estrogen receptor domain, and it increased the homologous recombination activity. In these systems, however, the ligand binding does not directly affect the structure of the functional domain, so the versatility of designing artificial ligand-gated proteins is somewhat limited.

There are only a few examples of designed ligand-dependent functional proteins regulated by ligand binding that is accompanied by large structural changes. Muir et al. reported small molecule modulation of protein function. They combined cleaved intein fragments with a hetero-dimerizing domain governed by the binding of an antibiotic, rapamycin, and manipulated the intein activity (positive allosteric effect) following the ligand-assisted refolding of the cleaved intein domains.[8] Recently, Regan et al., Michnick et al. and

[a] S. Yuzawa, Prof. T. Mizuno, Prof. T. Tanaka
Graduate School of Material Science
Nagoya Institute of Technology
Gokiso-cho, Nagoya 466-8555 (Japan)
Fax: (+81) 52-735-5210
E-mail: toshitcm@nietch.ac.jp
ttanaka@nitech.ac.jp

Morii et al. also described the leucine zipper-assisted refolding of cleaved GFP (green fluorescent protein), DHFR (dihydrofolate reductase), β-lactamase and pleckstrin homology (PH) domain fragments.[9] Bringing the cleaved sites of the original proteins close together by the interaction between the leucine zipper peptides allows the cleaved proteins to refold spontaneously into the proper tertiary structure, thus restoring the protein functions.

The α-helical coiled coils have a representative amino acid sequence of $(defgabc)_n$ heptad repeats. The a-and d-positions are usually occupied by hydrophobic residues, which form a hydrophobic core.[10] We first designed a trimeric coiled coil peptide, IZ, [YGG(IEKKIEA)$_4$] (defgabc).[11] Subsequently, we created various types of triple-stranded α-helical coiled coils, based on IZ. By fine-tuning the hydrophobic core interactions among each chain, AAB- and ABC-type heterotrimeric coiled coils,[12] metal–ion gated coiled coils[13] and pH-responsive coiled coils[14] were successfully constructed. These designed coiled coils could potentially be utilized in functional proteins as a "structural modulator unit".

In this work, we chose RNaseT1 as a functional domain to examine the utility of the designed ABC-type coiled coil as a "structural modulator unit". RNaseT1 is an RNA hydrolysis enzyme that specifically cleaves the 3′-position of a guanosine phosphodiester.[15] RNaseT1 can refold spontaneously, thus facilitating the design of a ligand-gated protein. By cleaving the native RNaseT1 at the proper site and introducing the A- and B-type coiled coil peptides, the enzymatic activity could be modulated by interactions with the C-type peptide, as an "effector ligand". These design principle, the "introduction of a refolding-assisting domain to functional proteins" should provide flexibility and variety for the design of de novo ligand-gated proteins in protein engineering.

Materials and Methods

Taq DNA polymerase, restriction enzymes, T4 DNA ligase and alkaline phosphatase were purchased from Takara Shuzo, Japan. General chemicals, isopropyl β-D-thiogalactopyranoside (IPTG), bacto tryptone, agar, and yeast extract (Wako Chemicals, Japan), ampicillin (Meiji Seika, Japan), agarose ME (Iwai Chemicals, Japan), glycogen (Roche Diagnostics, Japan) and others were used without further purification.

Peptide synthesis and purification: The IZ(4)C peptide was manually synthesized on the Rink amide resin (substitution 0.37 mmol g^{-1}) by the standard Fmoc strategy. The side chain protection groups were Glu(O*t*Bu), Lys(Boc) and Tyr(*t*Bu). Fmoc-amino acid derivatives (1 mmol) were coupled to the resin after activation by HBTU/HOBt (1 mmol each). Deprotection of the side chain and cleavage from the resin were performed by a treatment with TFA containing 2.5% ethanedithiol and 2.5% H_2O (v/v) at room temperature for 1.5 h. The peptide was precipitated in cold ether. Purification was carried out by reverse-phase HPLC on a YMC-Pack ODS-AM column (10 mm i.d. × 250 mm, YMC Inc., Japan) with a linear gradient of 25 to 35% CH_3CN/H_2O containing 0.1% TFA, over the course of 30 min. The final products were characterized by analytical HPLC and confirmed by MALDI-TOF mass spectrometry, *m*/*z* 3616.3 (calcd for 3615.1) for IZ(4)C.

Expression plasmid construction: pET-AmRB containing the gene encoding IZ(4)A-m-RNaseT1-IZ(4)B was prepared as follows. The IZ(4)A gene, with NdeI and XhoI restriction enzyme sites at each end, was prepared by PCR using the following DNA oligomers: 5′-GCG CAT ATG ATT GAG GCA AAG ATA GAA GCA ATT GAA GCC A-3′, 5′-A GAA GCA ATT GAA GCC AAG GCG GAG GCC ATA GAG GCT AAA A-3′, 5′-AAT CTT CGC CTC CAT CGC TTC GAT TTT AGC CTC TAT GGC CT-3′ and 5′-G CGCT CGA GCC ACC AGC CTC AAT CTT CGC CTC CAT CGC-3′. The underlined bases indicate the DNA sequences of the NdeI and XhoI restriction enzyme sites, respectively. The IZ(4)B gene, with BamHI and HindIII restriction enzyme sites at each end, was prepared by PCR using the following DNA oligomers: 5′-GCG GGA TCC AGC GGT ATC AAA GCA GAG ATA GAG GCA ATT A-3′, 5′-CA GAG ATA GAG GCA ATT AAG GCC GAA ATC GAA GCC GCC AAG-3′, 5′-C TTT TAT AGC CTC TAT CTC AGC CTT GGC GGC TTC GAT TTC-3′ and 5′-GCG AAG CTT CGC CTC AAT TTC CGC TTT TAT AGC CTC TAT CT-3′. The underlined bases indicate the BamHI and HindIII restriction enzyme sites, respectively. The m-RNaseT1 gene was prepared by fusing two DNA fragments (N-RNaseT1 and C-RNaseT1), which were generated by PCR, using the RNaseT1 gene[16] (as a template) and the following DNA oligomers: 5′-GCG CTC GAG CGG TAA CAA CCA GCT CGC-3′ and 5′-GCG CTG CAG GAC CGG TGC ATT CTA CAA-3′ for N-RNaseT1 (N-RNaseT1 gene with XhoI and PstI restriction enzyme sites at each end), and 5′-GC GCT GCA GGT GCT AGC GAC TAC ACC TGC GGC AGC AAC AGC TAC TCT AGC-3′ and 5′-GCG GGA TCC GCC TTC GTT GAA TAC TAC-3′ for C-RNaseT1 (C-RNaseT1 gene with PstI and BamHI restriction enzyme sites at each end). The former primer includes the mutations of Cys^2 and Cys^{10} to Ser (double bars). All of the genes were assembled into the pET-32d vector (Novagen Corp., USA) by using the NdeI, XhoI, PstI, BamHI and HindIII restriction enzyme sites, to obtain the expression vector, pET-AmRB. The other plasmids for the various RNaseT1 variants, such as m-RNaseT1, IZ(3)A-m-RNaseT1-IZ(3)B, IZ(4)A-N-RNaseT1 and IZ(4)B-C-RNaseT1, were also constructed by the same procedures described above.

Expression and purification of the RNaseT1 variants: To prepare IZ(4)A-m-RNaseT1-IZ(4)B we followed the procedures described below. *E. coli* BL21(DE3) cells were transformed with pET-AmRB, and the protein expression was induced by IPTG (1 mM) for 3 h at 37°C. After the *E. coli* cells were disrupted, the target protein was obtained as the thioredoxin-fusion form in the supernatant fraction. The supernatant was applied to His-bind resin (Novagen Corp., USA) under native conditions at room temperature. After washing the column with six volumes of wash buffer (60 mM imidazole, 500 mM NaCl, and 20 mM Tris·HCl at pH 7.9), the target fusion protein was eluted with six volumes of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris·HCl at pH 7.9). The eluted fraction was dialyzed against 1000 volumes of de-ionized water for 2 d, and then the solvent was removed in vacuo. The protein was subjected to thrombin (Mochida Seiyaku, Japan) digestion in 50 mM Tris·HCl (pH 7.5) containing 100 mM NaCl, 1.6 M urea, and 10 mM β-mercaptoethanol, to remove the thioredoxin domain. This solution was subjected to reverse phase HPLC on a YMC-Pack ODS-AM column (10 mm i.d. 250 mm, 5 mm, YMC Inc., Japan), with a linear gradient of 40 to 60% CH_3CN/H_2O containing 0.1% TFA over the course of 30 min, to purify the target IZ(4)A-m-RNaseT1-IZ(4)B. The purity and molecular weight were confirmed by SDS PAGE analysis.

CD spectroscopy: CD measurements were performed at various temperatures on a Jasco-820 spectropolarimeter using a 2 mm cuvette. The peptide and protein concentrations were determined by measuring the tyrosine and tryptophan absorbance in 6 M guanidine hydrochloride using ε_{280} = 1300 and 5700 M^{-1} cm^{-1}, respectively.[17] The mean residue ellipticity, $[\theta]$, is given in units of deg cm^2 dmol^{-1}. Thermal transition curves were obtained by monitoring $[\theta]_{222}$ as a function of temperature, with a 2 mm path length cuvette. The total peptide concentration was 5 μM, and the temperature was increased at a rate of 1°C min^{-1}.

Synthesis of diribonucleotides (GpC, UpC, ApC and CpC): Various diribonucleotides (GpC, UpC, ApC, and CpC) were synthesized by conventional phosphoramidite chemistry on a solid support. Starting from Ac-C-

RNA-CPG (Glen Research Corp.), A-TOM-CE-phosphoramidite, C-TOM-CE-phosphoramidite, G-TOM-CE-phosphoramidite, and U-TOM-CE-phosphoramidite were condensed by using 1*H*-tetrazole in MeCN. Cleavage of the diribonucleotides from each support was performed by a treatment with NH_3 aq. (30%)/EtOH 3:1 at 60°C for 6 h. After separation from the resin, the combined solution was evaporated to dryness. To remove the 2-*O*-triisopropylsilyloxymethyl (TOM) group from the synthesized diribonucleotides, each residue was treated with tBu_4NF at RT for 12 h. Purification of each diribonucleotide was performed by reverse phase HPLC on a YMC-Pack ODS-AM column (5 mm i.d. × 250 mm, YMC Inc., Japan) with a linear gradient of 20 to 30% CH_3CN/H_2O containing 20 mM acetate/triethylamine buffer (pH 7.0), over the course of 30 min. The concentration of each diribonucleotide was determined by the UV absorbance at 260 nm.

Enzymatic reactions of RNaseT1 variants: The enzymatic activities of the RNaseT1 mutants fused with the IZ peptides were evaluated by monitoring the hydrolysis rates of diribonucleotides (GpC, UpC, ApC, and CpC). The hypochromicity at A_{260} upon diribonucleotide digestion was analyzed to determine the K_m and k_{cat} values. For IZ(4)A-m-RNaseT1-IZ(4)B, before and after the addition of IZ(4)C, the detailed experimental conditions are described below. The solution (50 mM Tris-HCl (pH 7.5), 100 mM NaCl), containing IZ(4)A-m-RNaseT1-IZ(4)B (250 nM) and IZ(4)C (25 μM), was incubated at 30 or 25°C for 10 min. The enzymatic reaction was started by the injection of diribonucleotide (0–75 μM). By monitoring the changes in A_{260} over time, the initial rates of the enzymatic reaction at various diribonucleotide concentrations were determined.[18] General Lineweaver–Burk analyses were used to determine the K_m and k_{cat} values.

Results

Design of ligand-gated RNaseT1 variants: To construct the ligand-gated RNaseT1, we had to introduce a peptidic ligand-binding site. However, it is usually quite difficult to place a ligand-binding site in a folded, single domain protein. Therefore, we utilized the "introduction of a refolding-assisting domain" design principle to create ligand-gated proteins. After cleaving the proper site of the functional protein, the refolding-assisting domain is introduced at the cleaved sites. This refolding-assisting domain has a random-coil structure in the absence of a ligand, but after the addition of the peptide ligand, it assumes a folded tertiary structure. If the folding of the refolding-assisting domain could induce the proper folding of the cleaved protein, then it would be a design principle for constructing artificial ligand-gated proteins.

We previously constructed various types of de novo designed trimeric α-helical coiled coils. Based on the de novo designed parallel homotrimeric α-helical coiled coil peptide, IZ, [YGG(IEKKIEA)$_4$] (defgabc),[11] the AAB- and ABC-type heterotrimeric α-helical coiled coils were successfully synthesized after adjusting the size complementarity of the amino acid side chains in the hydrophobic core and the electrostatic interactions between the neighboring strands (Table 1).[12] The design of hydrophobic packing is quite important for selective interaction between the IZ derivatives. Especially, combination of one Trp and two Ala residues are well packed in the hydrophobic core of the ABC-type heterotrimeric coiled coil. The specific interaction in the ABC-type coiled coils could be applied to the "refolding-assisting domain". We designed an antiparallel ABC-type trimeric coiled coil, IZ(4)A, IZ(4)B and IZ(4)C (Table 1), in which the numbers in the parentheses indicate the number of heptad repeats. In the parallel orientation of the coiled coil, the a-position of one peptide and the a′-positions of the other peptide chains contribute to the hydrophobic interaction (a–a′-positions) in the same layer, and the e- and g′-positions form an interhelical interaction (e–g′-positions). On the other hand, in the antiparallel orientation, those interactions correspond to the a–d′- or d–a′-positions for the hydrophobic interactions, and the g–g′- or e–e′-positions for the interhelical interactions. The Trp-Ala-Ala interaction at the a-positions in the hydrophobic core facilitates the formation of the parallel AAB- and ABC-types of heterotrimeric coiled coils. Therefore, we placed one Trp-Ala-Ala interaction in the antiparallel coiled coil, where the a-positions of two peptides have Ala residues and the d-position of the other peptide has a Trp residue. The amino acids at the e- and g-positions were chosen to selectively form the trimer in an antiparallel orientation (up-up-down) by the ion pair interaction. IZ(4)A, IZ(4)B and IZ(4)C can be wrapped together and folded in the ABC-type of antiparallel trimeric α-helical coiled coil structure. IZ(4)A and IZ(4)B inserted in this variant RNaseT1 work as the binding domain for IZ(4)C. These interactions should assist in the refolding of the RNaseT1 domain, resulting in the recovery of the enzymatic activity.

Table 1. Amino acid sequences of the IZ peptide derivatives.

Peptide		defgabc	defgabc	defgabd	defgabc
IZ	YGG	IEKKIEA	IEKKIEA	IEKKIEA	IEKKIEA
IZ(4)A		--A----	--A-A--	--A----	--A----
IZ(4)B		-KAEA--	-KAEA--	-KAEA--	-KAEA--
IZ(4)C		--Q----	--Q-W--	--Q----	--Q----
IZ(3)A		--A----	--A-A--	--A----	
IZ(3)B		-KAEA--	-KAEA--	-KAEA--	
IZ(3)C		--Q----	--Q-W--	--Q----	

The designed m-RNaseT1 variants for this study are illustrated in Figure 1. RNaseT1, consisting of 104 amino acid residues with a structure comprising four β-strands and one α-helix, is an RNA hydrolysis enzyme in which His^{40} and His^{92} are the vital residues for catalysis.[15c] RNaseT1 contains four Cys residues at the 2-, 6-, 10- and 103-positions, and they form two S–S bonds, Cys^2–Cys^{10} and Cys^6–Cys^{103}. The S–S bond of Cys^2–Cys^{10} does not affect the enzymatic activity, while that of Cys^6–Cys^{103} is essential for both the structural stability and enzyme activity.[19] Thus, we replaced Cys^2 and Cys^{10} with Ser and retained Cys^6 and Cys^{103} in this study, to reduce the complexity. N-RNaseT1 and C-RNaseT1 were obtained by cleaving this RNaseT1 mutant between Glu^{82} and Asn^{83}.

The placement of IZ(4)A and IZ(4)B (refolding-assisting domain) in RNaseT1 is quite important to regulate the enzymatic activity. By choosing the proper linking positions, the structure of the RNaseT1 domain should be destabilized before the addition of IZ(4)C, whereas it can be refolded properly after the addition of IZ(4)C. Studies on the

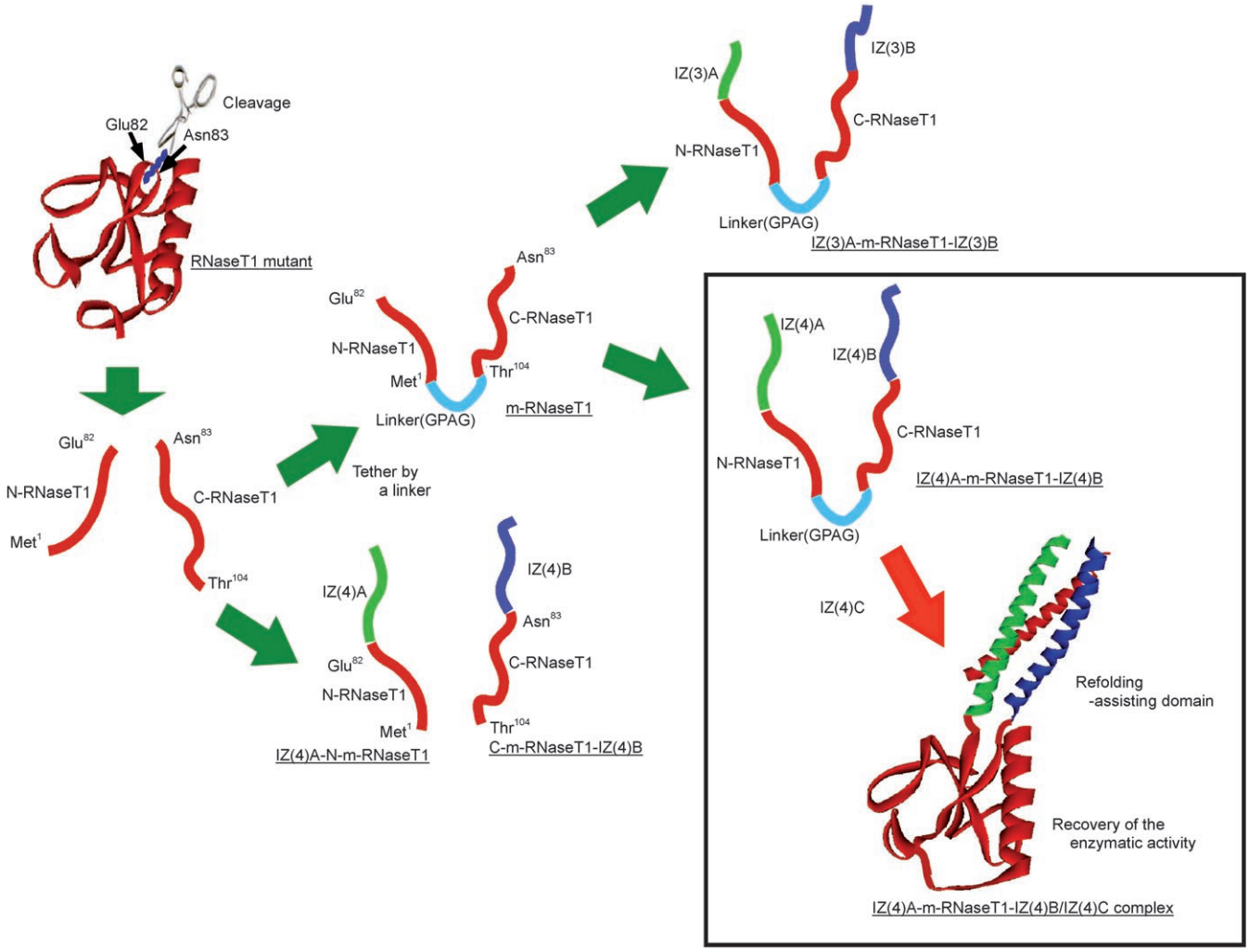

Figure 1. Schematic illustration of m-RNaseT1 variants and the recovery of the enzymatic activity of the RNaseT1 domain by coiled coil formation.

domain assembled proteins of the cleaved GFP, DHFR (dihydrofolate reductase) and β-lactamase fragments with leucine zipper peptides,[9] revealed that protein function recovery strongly depends on the cleaved sites of the native proteins, and thus it is important to optimize the site cleaved in RNaseT1. Mullins et al. reported that the circular permutation of RNaseT1, with the original C- and N-termini tethered by GPG as a linker and cleaved between Gly^{34} and Ser^{35}, had lower structural stability, but still retained about 29% enzymatic activity compared with that of the wild-type.[20] To abolish the enzymatic activity, we herein designed a different circular permutant, m-RNaseT1, in which the original C- and N-termini of the RNaseT1 mutant were tethered by a GPAG linker, and the protein was cleaved between Glu^{82} and Asn^{83} in the β-turn structure consisting of Asn^{81}-Glu^{82}-Asn^{83}-Asn^{84}, between β-strands 3 and 4. The cleavage between Glu^{82} and Asn^{83}, which separate the β-strands, should reduce the structural stability and cause the formation of a random structure. Furthermore, the essential residues (His^{40} and His^{92}) for the enzymatic reaction would become separated, thus destroying the enzymatic activity. The A- and B-type coiled coil peptides, IZ(4)A and IZ(4)B, were fused to the N- and C-termini of m-RNaseT1 , respectively, via a GGSSG linker, to form IZ(4)A-m-RNaseT1-IZ(4)B, as a model of the ligand-gated RNaseT1. After the trimeric coiled coil structure is formed by the addition of IZ(4)C, the cleaved sites within RNaseT1 should be close enough to refold the RNaseT1 domain spontaneously into the active tertiary structure.

To examine the neighboring effect on the affinity among the IZ derivative peptides in IZ(4)A-m-RNaseT1-IZ(4)B, we also prepared IZ(3)A-m-RNaseT1-IZ(3)B. IZ(3)A, IZ(3)B, and IZ(3)C contained only three heptad repeats instead of four, so they cannot form the trimeric structure (data not shown). We also examined the effect of a GPAG linker between the original N- and C-termini of RNaseT1 on the refolding of m-RNaseT1, by using the divided fusion proteins, IZ(4)A-N-m-RNaseT1 and C-m-RNaseT1-IZ(4)B, respectively (Figure 1).

Examination of the structure of RNaseT1 variants by CD spectroscopy: CD spectroscopy was performed to character-

ize the secondary structures of the RNaseT1 variants. As native RNaseT1 consists of four β-strands and one α-helix, it yields a mixed CD spectrum (Figure 2).[19] The RNaseT1 mutant, in which only Cys^2 and Cys^{10} were replaced by Asn and Ser, respectively, also has a similar CD spectrum (data not shown). On the other hand, the circularly permutated RNaseT1, m-RNaseT1, exhibited a random structure with a minimum less than 200 nm (Figure 2). By cleaving between Glu^{82} and Asn^{83}, the β-strand–β-strand interactions were reduced, thus destabilizing the entire protein structure. The CD spectrum of IZ(4)A-m-RNaseT1-IZ(4)B was not characteristic for random structure, but was rather similar to that of RNaseT1 as shown in Figure 3. On the other hand, IZ(3)A-m-RNaseT1-IZ(3)B showed a random structure (data not shown). These results might suggest that m-RNaseT1 dose not fold spontaneously, and the weak interaction between IZ(4)A and IZ(4)B effects in the folding of the m-RNaseT1 domain to some extent. As a result, IZ(4)A-m-RNaseT1-IZ(4)B might has a similar structure to that of RNaseT1 with loosing folding.

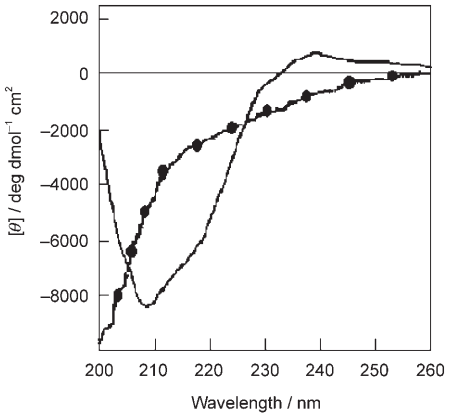

Figure 2. CD spectra of the native RNaseT1 (——) and the circular permutant, m-RNaseT1 (●); [protein] = 5 μM, 50 mM Tris-HCl buffer (pH 7.5), 25 °C.

The addition of IZ(4)C to IZ(4)A-m-RNaseT1-IZ(4)B caused the $[\theta]$ values at 208 and 222 nm to change (Figure 3a). A larger $[\theta]_{222}$ value implies an increase in the α-helical content, probably by the formation of an α-helical structure. At the 1:1 molar ratio of IZ(4)A-m-RNaseT1-IZ(4)B and IZ(4)C, the CD spectral changes had an inflection point, suggesting that IZ(4)A, IZ(4)B, and IZ(4)C formed a complex at a 1:1:1 ratio (Figure 4). We calculated a theoretical CD spectrum of the IZ(4)A-m-RNaseT1-IZ(4)B/IZ(4)C complex, based on the CD spectra of the original native RNaseT1 and ABC-type IZ peptides (Figure 3b). The theoretical and empirical CD spectra are quite similar, which indicates that the expected complex structure between IZ(4)A-m-RNaseT1-IZ(4)B and IZ(4)C was formed. Thus, the coiled coil structure should be formed after the addition of IZ(4)C, which interacts with IZ(4)A and IZ(4)B. As for IZ(3)A-m-RNaseT1-IZ(3)B, no CD

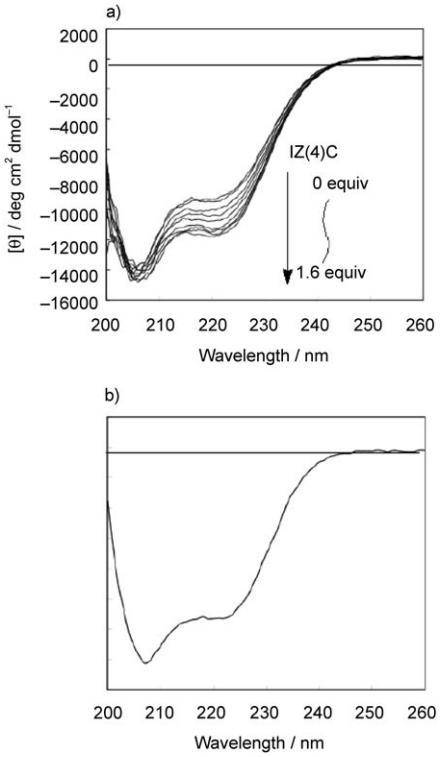

Figure 3. a) CD spectral changes of IZ(4)A-m-RNaseT1-IZ(4)B after the addition of IZ(4)C; [IZ(4)A-m-RNaseT1-IZ(4)B] = 5 μM, [IZ(4)C] = 0–8 μM, 50 mM Tris-HCl (pH 7.5), 25 °C. b) Theoretical CD spectrum of the IZ(4)A-m-RNaseT1–IZ(4)B/IZ(4)C complex.

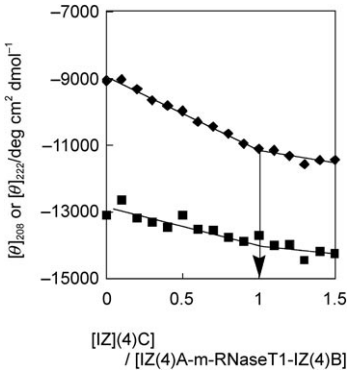

Figure 4. Changes of $[\theta]_{208}$ (■) and $[\theta]_{222}$ (◆) of IZ(4)A-m-RNaseT1-IZ(4)B after the addition of IZ(4)C; [IZ(4)A-m-RNaseT1-IZ(4)B] = 5 μM, [IZ(4)C] = 0–8 μM, 50 mM Tris-HCl (pH 7.5), 25 °C.

spectral changes were observed upon the addition of IZ(4)C, and thus the random structure was maintained (data not shown). These results indicate that the three heptad repeats of IZ(3)A and IZ(3)B were too short to interact with IZ(4)C.

The structural changes of the mixture of IZ(4)A-N-m-RNaseT1 and C-m-RNaseT1-IZ(4)B that occurred upon the addition of IZ(4)C were also examined. The mixture of IZ(4)A-N-m-RNaseT1 and C-m-RNaseT1-IZ(4)B yielded the CD spectrum of a random structure, indicating that the

cleaved circular permutant fragments of RNaseT1 did not spontaneously refold (Figure 5). Although obvious CD spectral changes were observed after the addition of IZ(4)C (Figure 5), only an α-helical structure might be induced based on the analyses of the subtracted CD spectra. These

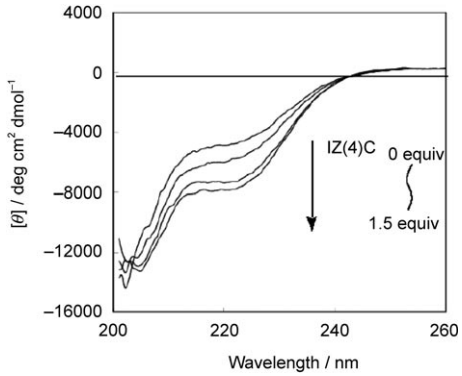

Figure 5. CD spectral changes of the mixture of IZ(4)-N-m-RNaseT1 and C-m-RNase T1-IZ(4)B after the addition of IZ(4)C; [IZ(4)-N-m-RNaseT1 and C-m-RNase T1-IZ(4)B] = 5 μM, [IZ(4)C] = 0–7.5 μM, 50 mM Tris-HCl (pH 7.5), 25 °C.

results suggested that the ABC-type of trimeric coiled coil structure was formed (Figure 6); however, the refolding of the m-RNaseT1 domain seemed to be limited. We concluded that the GPAG linker of IZ(4)A-m-RNaseT1-IZ(4)B is essential for the refolding of the m-RNaseT1 domain in this system.

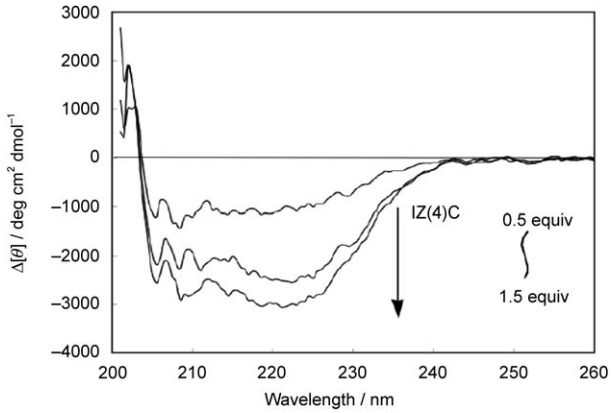

Figure 6. Subtracted CD spectra of the mixture of IZ(4)-N-m-RNaseT1 and C-m-RNaseT1-IZ(4)B after the addition of IZ(4)C; [IZ(4)-N-m-RNaseT1 and C-m-RNaseT1-IZ(4)B] = 5 μM, [IZ(4)C] = 0–7.5 μM, 50 mM Tris-HCl (pH 7.5), 25 °C.

The T_m values for IZ(4)A-m-RNaseT1-IZ(4)B before and after the addition of IZ(4)C were measured by using temperature-variable CD spectroscopy. The $[\theta]_{222}$ values were monitored as a function of the temperature (Figure 7). The T_m value of the ABC-type coiled coil, consisting of IZ(4)A, IZ(4)B and IZ(4)C, is 48 °C based on our previous work (unpublished results). Before the addition of IZ(4)C, the

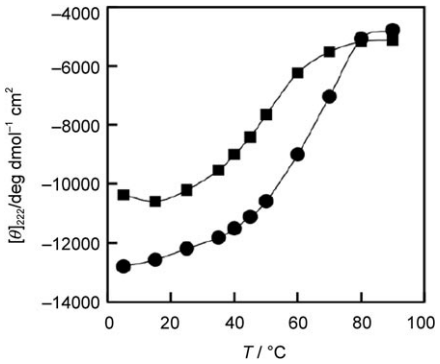

Figure 7. Comparison of thermal stabilities by analyses of the $[\theta]_{222}$ values before (■) and after (●) the addition of IZ(4)C to IZ(4)A-m-RNaseT1-IZ(4)B; [IZ(4)C] = 5 μM, [IZ(4)A-m-RNaseT1-IZ(4)B] = 5 μM, 50 mM Tris·HCl (pH 7.5), 0–90 °C.

midpoint of the structural transition of IZ(4)A-m-RNaseT1-IZ(4)B was observed at 49 °C. On the other hand, after IZ(4)C was added, the T_m value of IZ(4)A-m-RNaseT1-IZ(4)B increased to 63.5 °C, and there only seemed to be a single structural transition. These results suggest that the two domains, m-RNaseT1 and the coiled coil domains (IZ(4)A, IZ(4)B, and IZ(4)C), work cooperatively. Interestingly, the T_m value of the IZ(4)A-m-RNaseT1-IZ(4)B/IZ(4)C complex is higher than that of wild-type RNaseT1 (59.3 °C).[15c]

Enzymatic activities of RNaseT1 variants before and after the peptide ligand addition: RNaseT1 specifically cleaves RNA at the 3′-position of GpX, to form Gp and X. We examined the enzymatic activity of various RNaseT1 variants by using diribonucleotides (GpC, CpC, ApC, and UpC) as substrates. The enzymatic reaction progress was monitored by the increase in the absorbance at 260 nm, and each reaction was performed at 30 °C. As described previously, m-RNaseT1 has a random structure, due to the destabilization by cleaving between Glu^{82} and Asn^{83}, even though the cleaved fragments are tethered by a GPAG linker (Figure 1), and thus m-RNaseT1 was almost inactive (k_{cat} was negligible) for all of the diribonucleotide substrates (data not shown). As for IZ(4)A-m-RNaseT1-IZ(4)B, although the CD spectrum was similar to that of RNaseT1, but no enzymatic activity was observed (Table 2 and Figure 8). After the addition of IZ(4)C to IZ(4)A-m-RNaseT1-IZ(4)B, an obvious increase in the enzymatic activity was observed. This increment of enzymatic activity was saturated by the addition of 100 equivalents of IZ(4)C (Figure 8). The k_{cat}/K_m value was $3.3 \times 10^4\ s^{-1}$ at 30 °C, which is almost 1/600 of the value for the native RNaseT1. The low enzymatic activity might be due to the absence of the S–S bond between Cys^4 and Cys^{103}, and therefore we analyzed the percentage of S–S bond formation between Cys^4 and Cys^{103} by an improved Ellman's test, as reported by Gruber and colleagues.[21] However, the S–S bond was formed (over 95%) even before the addition of the C-type IZ peptide. On the other hand, when the temperature was

Table 2. k_{cat} and K_m values for various RNaseT1 variants.[a]

	k_{cat} [s^{-1}]	K_m [M]	k_{cat}/K_m [$M^{-1}s^{-1}$]
wild-type RNaseT1[b]	249	1.3×10^{-4}	1.9×10^{7}
	210[c]	6.8×10^{-5}[c]	3.1×10^{7}[c]
RNaseT1 mutant (C2S, C10N)[b]	258	1.5×10^{-4}	1.7×10^{6}
m-RNaseT1	n.d.	n.d.	n.d.
IZ(4)A-m-RNaseT1-IZ(4)B	n.d.	n.d.	n.d.
IZ(4)A-m-RNaseT1-IZ(4)B +IZ(4)C	0.4	1.2×10^{-5}	3.3×10^{4}
	4.0[c]	9.4×10^{-6}[c]	4.3×10^{5}[c]
IZ(3)A-m-RNaseT1-IZ(3)B	n.d.	n.d.	n.d.
IZ(3)A-m-RNaseT1-IZ(3)B +IZ(4)C	n.d.	n.d.	n.d.
IZ(4)A-N-m-RNaseT1 + C-m-RNaseT1-IZ(4)B+IZ(4)C	n.d.	n.d.	n.d.

[a] All data were evaluated from the time course changes of A_{260} at 30°C, following the digestion of the GpC substrate, by a standard Lineweaver–Burk analysis. The concentration of each protein was 0.25 μM. On case of the addition of IZ(4)C, the concentration of IZ(4)C was 25 μM. The "n.d." stands for "not detected". [b] These data were cited from ref. [17]. [c] The enzymatic reaction was carried out at 25°C.

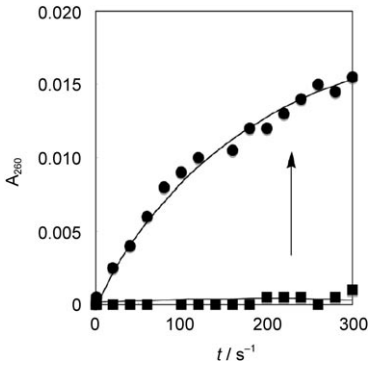

Figure 8. GpC hydrolysis by IZ(4)A-m-RNaseT1-IZ(4)B before (■) and after (●) the addition of IZ(4)C monitored by A_{260}; [IZ(4)A-m-RNaseT1-IZ(4)B] = 250 nM, [IZ(4)C] = 25 μM, [GpC] = 50 μM, 50 mM Tris-HCl (pH 7.5), 30°C.

reduced to 25°C, the k_{cat}/K_m value increased 10-fold, to $4.3\times10^5\ s^{-1}$, which is 1/100 of the value for native RNaseT1 (Table 2). These results indicated that the addition of IZ(4)C induces the proper refolding of the m-RNaseT1 domain to restore the enzymatic activity. This complex did not hydrolyze CpC, ApC, or UpC, and thus the substrate selectivity was not affected by either the circular permutation or the fusion of the IZ peptides to m-RNaseT1.

IZ(3)A-m-RNaseT1-IZ(3)B had no enzymatic activity in both the presence and absence of IZ(4)C. This result was supported by the CD spectral analyses, which showed random structures under both conditions. The mixture of IZ(4)-N-m-RNaseT1 and C-m-RNaseT1-IZ(4)B with IZ(4)C also lacked enzymatic activity for all of the substrates. The CD spectral analyses revealed that IZ(4)C induced the α-helical structure of the mixture of IZ(4)-N-m-RNaseT1 and C-m-RNaseT1-IZ(4)B. The formation of the coiled coil structure would bring the cleaved sites of the RNaseT1 domain close together, but the enzymatic activity would not spontaneously recover. Therefore, the GPAG linker plays a crucial role for the proper refolding of the m-RNaseT1 domain in IZ(4)A-m-RNaseT1-IZ(4)B to exert the enzymatic activity.

Discussion

In nature, coiled coils play important roles in assembling different proteins and/or protein domains. We applied the selective interaction between the ABC-type coiled coil peptide to construct an artificial ligand-gated protein, where the C-type peptide behaves as an "effector ligand". By attaching the A- and B-type IZ peptides via GGSSG linkers to the N- and C-termini of the circularly permutated RNaseT1, we obtained IZ(4)A-m-RNaseT1-IZ(4)B, which might has a similar structure to RNaseT1 but did not show the enzyme activity. The addition of the C-type IZ peptide induced proper folding of the m-RNaseT1 domain, and an obvious increase in its enzymatic activity was detected, although it was still lower than that of the native RNaseT1. The structure of the RNaseT1 domain might still be flexible after coiled coil formation. At the lower temperature (25°C), the enzymatic activity was 10-times higher than that at 30°C. This result also supports the flexibility of the RNaseT1 domain. The structure of the linker between the coiled coil and the cleaved RNaseT1 domain does not completely reflect the β-turn structure, and thus it may still be flexible. This defect might be improved by further modifications: i) bringing Glu^{82} and Asn^{83} closer by selecting a suitable linker or ii) choosing the position of the IZ peptide tethering with the C- and N-termini of the circularly permutated RNaseT1.

On the other hand, increasing the thermal stability of a protein is an important goal of protein engineering. Many researchers have sought to increase thermal stability by mutagenesis, including random selection strategies. The tertiary structures of heat-resistant proteins from various heat-resistant bacteria, such as thermophiles and hyperthermophiles, have been analyzed to understand their stability under high-temperature conditions. However, the detailed mechanisms have not yet been clarified. In this study, we fused the coiled coil to the circular permutant RNaseT1, and the RNaseT1 variant showed higher thermal stability than the native RNaseT1. This abnormal stability is attributed to the coiled coil domain. The designed IZ(4)A-m-RNaseT1-IZ(4)B/IZ(4)C complex is considered to consist of two domains (circularly permutated RNaseT1 and coiled coil domains). However, a single two-state transition was observed, and therefore, each domain works cooperatively. Coiled coils are considered as a de novo design framework in protein engineering, but their ability to increase thermal stability will also be useful for de novo protein design.

In this system, the coiled coil formation is obviously linked to the enzymatic activity. The principle that the folding of the ligand binding domain, triggered by the peptide ligand, induces the refolding of the enzymatic domain

should provide a versatile and flexible methodology to design novel artificial ligand-gated proteins. Thus far, we have successfully designed various types of IZ peptides that can be triggered by pH and metal ions. Some organic ligands also have the potential to trigger the assembly of coiled coil peptides. By using these IZ peptides as refolding-assisting domains for various functional proteins, the rational design of artificial ligand-gated proteins will be expanded to further applications.

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Received: January 4, 2006
Revised: March 22, 2006
Published online: July 25, 2006

