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DESIGN AND SEMISYNTHESIS OF SPERMINE-SENSITIVE RIBONUCLEASE S'

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Abstract: Spermine-sensitive stabilization of semisynthetic Ribonuclease S' was successfully carried out by sequence specific incorporation of a poly-anion domain into α–helix region of S-peptide.

Introduction

Development of rational design for stimuli-responsive enzymes and proteins has been one of the major targets in the research field of recent protein engineering.[1] It has potential applications for novel type of biosensors, sophisticated biocatalysts and protein drugs. We recently proposed the site-specific incorporation of artificial receptors is one of the promising approaches.[2-6] In order to generalize this methodology, it is now desirable to explore a sort of useful receptors and to evaluate the effect upon host-guest interactions which occur on protein surfaces using a site-specifically modified enzyme in detail. In addition to nonnatural receptors, it is known that peptide segments of native proteins act as a molecular recognition site.[7] Loop regions, for instance, may catch some metal cations. As an elegant example, Sasaki and coworkers showed that a helix region of *de novo* peptides bearing oligo-glutamic (or aspartic) acids with a specific arrangement is capable of binding spermine, a bioactive oligoamine.[8] We would like to extend the pioneering results to the field of protein engineering by incorporating a poly-anion domain into a small protein. In this communication, we describe semisynthesis of Ribonuclease S' bearing poly-anion charges at a specific site of α–helix of S-peptide and spermine-induced stabilization of the net enzyme structure.

Results and Discussion

Ribonuclease S (RNase S), a RNA hydrolyzing enzyme, is consisted of two peptide fragments, S-peptide and S-protein.[9] It is well established that S-peptide variant is readily rebound to S-protein in self-assembly manner (RNase S').[10,11] We noticed that this enzyme is a suitable model for investigation how the spermine-binding event affects the net structure of the enzyme bearing a poly-anionic domain at an α–helix

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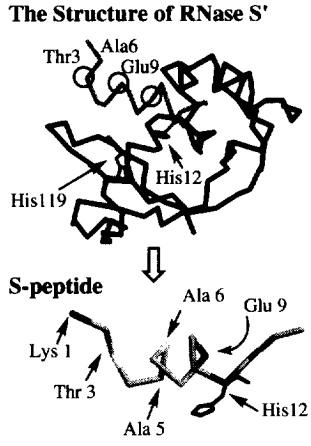

	1 5 10 15
native	Lys Glu Thr Ala Ala Ala Lys Phe Glu Arg Gln His Met Asp Ser
mutant **1**	Lys Glu **Glu** Ala Ala **Glu** Lys Phe Glu Arg Gln His Met Asp Ser
mutant **2**	**Glu** Glu Thr Ala **Glu** Ala Lys Phe Glu Arg Gln His Met Asp Ser
mutant **3**	Lys Glu **Glu** Ala Ala Ala Lys Phe Glu Arg Gln His Met Asp Ser
mutant **4**	Lys Glu **Asp** Ala Ala **Asp** Lys Phe Glu Arg Gln His Met Asp Ser

Figure 1. Replaced S-peptide sequences.

region.[12] Figure 1 shows the amino acid sequences of the mutant S-peptides. To investigate influences of the spatial arrangement of poly-anion charges on the spermine-response, we prepared four mutants having poly-anion charge, in addition to native sequence. Several amino acids located in the solvent-exposed side were replaced by Glu or Asp.[13] In mutant **1**, for example, 3-Thr and 6-Ala were altered to Glu. Since S-peptide has a Glu residue at 9 position, three Glu residues are arranged in i, i+3, i+6. In mutant **2**, on the other hand, i, i+4, i+8 arrangement is prepared. A T3-Glu mutant **3** (i, i+6) was used as a control sequence. As mutant **4**, 3 and 6 position are replaced by Asp. These mutants were synthesized using solid phase technique of Fmoc chemistry (PerSeptive Pioneer). Crude peptides were purified through reverse-phase HPLC (Hitachi LC system, eluent: water-acetonitrile gradient), and identified with MALDI-TOF mass spectroscopy (PerSeptive Voyager RP).[14] S-Protein was obtained by subtilisin-catalyzed cleavage of RNase A, followed by cation-exchange (CM- 52, phosphate buffer, NaCl gradient) and then, by gel-chromatography (Sephadex G-50, 50 % aqueous acetic acid).

When S-peptide is bound to S-protein, the S-peptide conformation changes from random coil to α–helix. This is conventionally monitored by circular dichroism spectropolarimeter (CD, Jasco J-720W). The CD titration curve of S-protein with mutant S-peptides **1** displays a typical saturation behavior at 1:1 ratio, like native S-peptide (data not shown), indicating that mutant **1** is associated to S-protein, with high affinity comparable to that of native S-peptide. Nonlinear curve fitting analysis estimated the association constant (log$K$) to be 7.6 for native S-peptide, 6.9 for **1**, 6.5 for **2**, 7.1 for **3** and 7.1 for **4**, indicating that replacement of amino acids at the position of 1, 3, 5, or 6 does not significantly reduce the S-peptide/S-protein association.

In CD spectra of the S-peptide **1**/S-protein complex (i.e., T3/A6Glu-RNase S'), the peaks at 220 and 208 nm were intensified upon the addition of spermine and the spectral changes were saturated with 10 eq. of spermine (See Figure 2). This indicates that spermine is bound to T3/A6Glu-RNase S' so as to cause the conformational change of an α–helix domain. The binding constant (log$K$) can be estimated by the saturation curve to yield 5.1, which is comparable to the value of a *de novo* peptide measured in 30 % trifluoroethanol aqueous solution. Addition of spermidine increased the helix content (log$K$ = 5.4), although the changing

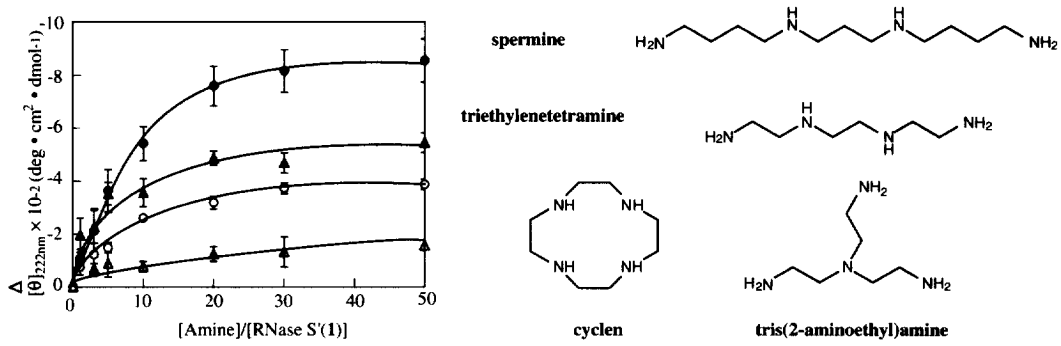

Figure 2. Tetramine titration curves monitored by CD-spectroscopy.; addition of spermine(●), triethylenetetramine (▲), tris(2-aminoethyl)amine(○), cyclen(△). RNase S'(**1**) 10 μM, 10mM MES buffer, pH6.0(10mM NaCl).

amplitude is a half-fold smaller than that for the spermine addition. The CD spectral changes are not induced by addition of methylamine or 1,3-diaminopropane even in 30 eq. excess. Next, we tested the binding affinity of a series of tetramines to T3/A6Glu-RNase S' as shown in Figure 2. The changing amplitude is in the order of spermine > triethylenetetramine > tris(2-aminoethyl)amine, while the binding constant (log*K*) of the corresponding amines are in the range of 5[15]. Cyclen, a cyclic tetramine, in contrast, is found to be a weak binder (log*K* = 3.7) to T3/A6Glu-RNase S'.

The same changes induced by spermine were observed in T3/A6Asp-RNase S', whereas these changes did not take place both in K1/A5Glu-RNase S' and T3Glu-RNase S'. This suggests that the spatial fitting between anionic charges of the peptide domain and cationic charges of polyamines is critical for the tetramine binding on the enzyme surface.

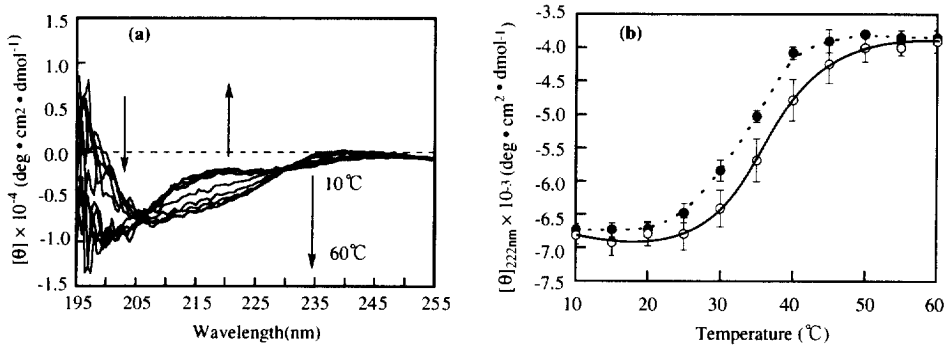

Figure 3. Thermal denaturation experiment; (a) CD spectral change of T3A6Glu-RNase S' (b) thermal denaturation curves at $[\theta]_{222nm}$ T3A6Glu-RNase S'; in the absence (●) and the presence (○) of spermine: T3/A6Glu-RNase S' 10μM, spermine 100μM, 10mM MES buffer, pH6.0(10mM NaCl).

The net stability of the structure of semisynthetic RNase S' was evaluated by thermal denaturation curve monitored by CD spectroscopy. The α–helix regions which are characteristic of native RNase S' cooperatively decreases with increase of temperature. Figure 3a shows a typical CD change of T3/A6Glu-RNase S'. The denaturation temperatures (T_d) were estimated from the denaturation curve as shown in Fig. 3b. It is clear that T_d

of T3/A6Glu-RNase S' is enhanced by 1.9 ±0.2 °C upon binding with spermine. While methylamine and 1,3-diaminopropane do not affect the T_d, spermidine is moderately effective (delta T_d of 0.8 °C). In agreement with the order of the changing amplitude in CD spectra, the increase of T_d is in the order of spermine (1.9 °C) > triethylenetetramine (0.8 °C) > tris(2-aminoethyl)amine (0.5 °C) > cyclen (0.2 °C). Among the above-mentioned mutants, the similar stabilization induced by spermine was observed only in T3/A6Asp-RNase S' (T_d increase by 1.3 °C), whereas both K1/A5Glu-RNase S' and T3Glu-RNase S' did not show any spermine-induced stabilization.

In conclusion, we have clearly demonstrated in this study that spermine-binding on an α–helix of RNase S' can stabilize the net enzyme structure. The spatial matching of oligo-anion charges located on the helix to oligo-cations of spermine is essential for the specific binding. Most importantly, our results imply that a valuable finding obtained in the research of *de novo* peptides can be successfully linked to the protein engineering.[16]

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16. In preliminary assay of the enzymatic activity of mutant **1**, the initial rate of the hydrolysis of 2',3'-cCMP was enhanced by 120±5% in the presence of spermine ([Spro]=1μM, [mutant **1**]=2 μM, [spermine]=10 μM, [2',3'-cCMP]=100 μM 10mM MES buffer, pH6.0 (10mM NaCl), A_{obs}=286nm, at 37 °C)