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# Control of function of a small peptide by a protein

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**Abstract**—A peptide that functions only in the presence of a protein has been developed using reaction-based selection from peptide phage libraries. The peptide was not functional in the absence of the protein, but formed enaminones with 1,3-diketone derivatives when bound to the protein.


In nature, protein functions including enzyme activities are often regulated through conformational change triggered by ligand binding or posttranslational modification at specific sites.[1,2] Design of such systems should allow for regulation of function of a protein or peptide in a programmed manner. For example, systems for controlling protein–protein and peptide–protein interactions have been designed by introducing mutations into the protein at the interface of the interaction to reduce the binding affinity and by addition of small compounds to restore the binding.[3] Catalytic activity or binding activity of peptides was also regulated using a metal.[4,5] Although structured small peptides with functions including catalytic activity have been reported,[6] development of strategies to control functions of small peptides composed of natural amino acids in a programmed manner is a challenge.[5] Here we have developed a system that can be used to control the function of a small peptide based on interaction between the peptide and a second protein molecule. We report the development of a peptide that forms enaminones with 1,3-diketones in the presence of a protein that interacts with the peptide (Fig. 1).

We previously reported α-helical peptides that form enaminones with 1,3-diketones and catalyze aldol, retro-aldol, and Michael reactions via an enamine mechanism.[7–9] These peptides formed dimers or oligomers to function. As a particular folded state was required for the catalytic activities of these peptides, we reasoned that function of a small peptide could be controlled by its conformation. We hypothesized that noncovalent binding of a small peptide to a second protein could serve as a conformational trigger for the peptide. In the inactive state, the peptide would not have a fixed conformation, but interaction with a second protein would result in a change to the functional conformation. When the interactive protein is absent, the peptide will not be functional; however, once the interactive protein is added, the peptide will bind to the protein and adopt a fixed and active conformation. The interactive protein may provide residues required for the function, although the protein alone cannot function. Only when the peptide and its interactive protein bind to each other, the complex can function. In order to realize this system, we have developed small peptides that form enaminones with 1,3-diketones only in the presence of the RNase S-protein.

RNase S is a product of cleavage of RNase A by the protease subtilisin.[10] RNase S consists of two tightly associated fragments, S-peptide (residues 1–20) and S-protein (residues 21–124). A complex of S-protein with only the 15 N-terminal amino acids of S-peptide (S15) is essentially identical in structure to that of RNase S.[11] S-peptide maintains its folded structure in the absence of S-protein at low temperatures and adopts this conformation when it binds to S-protein at room temperature.[12–14] To select a peptide that forms enaminone with 1,3-diketones only when the peptide interacts with S-protein, peptide libraries were developed that included a modified S-peptide sequence.

S15 includes an α-helical conformation that is stabilized by two salt bridges, Glu9-Hisl2 and Glu2-Argl0. There are two lysine residues, Lys1 and Lys7, in S15 and the

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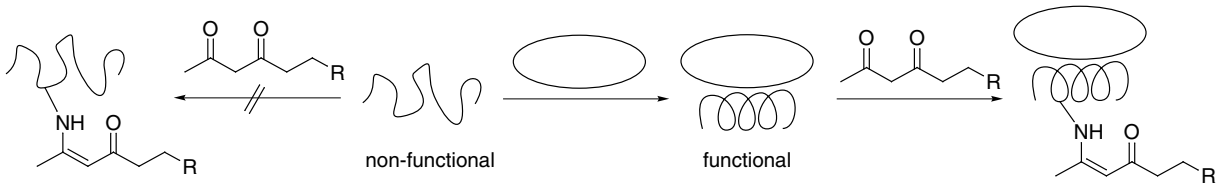
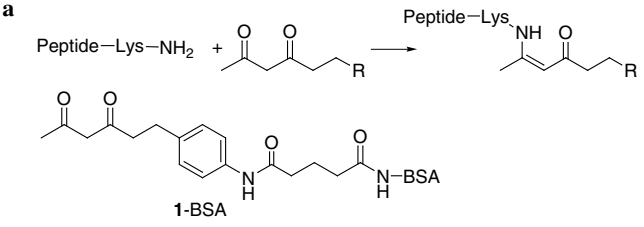



**Figure 1.** Schematic representation of the regulation of function of a peptide.

latter is in the α-helical structure. A partial sequence of S15 including Lys7 was used as a template scaffold for this study. His12 is one of the essential catalytic residues for the ribonucleolytic activity of RNase S. The salt bridge Glu9-His12 orients His12 for the ribonucleolytic activity and is critical for the activity. His12 was replaced by lysine and randomized amino acids were appended to the peptide. The mutation of His12 should suppress ribonuclease activity that would otherwise be a problem during selection using peptide phage. The ε-amino group of Lys12 is expected to form a salt bridge with Glu9 and the Glu-Lys salt bridge also stabilizes the helical conformation.[15] We expect that this mutant will have similar helical stability to wild-type S15: that is, the mutant peptide alone will not form the helical conformation at room temperature.

Libraries shown in Figure 2 were prepared and were subjected to selection through multiple rounds of phage panning with 1,3-diketone **1**-BSA (BSA = bovine serum albumin) in the presence of S-protein (2 μM) using the phagemid pComb3X system.[9,16,17] Library FT-S-T-NNK6 had a short linker sequence, SRS, between the S-peptide scaffold sequence and C-terminal 6-residue randomized region. Libraries FT-NNK6-HTH-S-1, -2, and -3 had the linker sequence, HLSQP, between the N-terminal randomized sequence and the S-peptide sequence and the lysine at the first position of the S-peptide was removed. In the libraries FT-NNK6-HTH-S-2 and FT-NNK6-HTH-S-3, a lysine residue was incorporated with in the randomized region.




b

1 71 9 2
S-peptide (S15): KETAAAKFERQHMDS

Libraries:

FT-NNK6-S: XXXXXXETAAAKFERQKMDS
FT-NNK-PRO-S: XXXXXXPETAAAKFERQKMDS
FT-S-NNK6:KETAAAKFERQKMDXXXXXX
FT-S-T-NNK6 KETAAAKFERQKMDSRSXXXXXX

FT-NNK6-HTH-S-1XXXXXXHLSQPETAAAKFERQKMD
FT-NNK6-HTH-S-2 XKXXXLHLSQPETAAAKFERQKMD
FT-NNK6-HTH-S-3 XXKXXLHLSQPETAAAKFERQKMD

Selected:

FT-RPF1310: RWQKSKHLSQPETAAAKFERQKMD

**Figure 2.** (a) Enaminone formation with 1,3-diketones. (b) Libraries used for the selection of peptides that form enaminones with 1,3-diketones and a selected peptide sequence. X, any of the natural 20 amino acids. The sequence of S-peptide (S15) is also shown.

After the panning, individual peptide phage clones were analyzed by enzyme-linked immunosorbent assay (ELISA) using **1**-BSA in the presence and absence of S-protein (2 μM). The ELISA showed that some of the clones bound to **1**-BSA both in the presence and absence of S-protein. Since we were interested in peptides that could be regulated by the addition of S-protein, the clones that bound **1**-BSA in the presence of S-protein but did not significantly bind to **1**-BSA in the absence of S-protein were chosen. The sequence, named as FT-RPF1310, showed the greatest difference in binding to **1**-BSA in the presence and in the absence of S-protein in the ELISA of the clones evaluated.

Peptide FT-RPF1310 was chemically synthesized and analyzed in detail. The enaminone formation of peptide FT-RPF1310 (100 μM) and 2,4-pentanedione (4 mM) was analyzed in aqueous buffer (pH 7.5) by measuring the absorption at 318 nm[18] in the presence and absence of S-protein (100 μM). Increase in absorption at 318 nm over time was detected in the presence of S-protein as shown in Figure 3, but peptide FT-RPF1310 (100 μM) alone or S-protein (100 μM) alone showed no increase in the absorption at 318 nm. These results indicate that using this regulated system, diketone derivatives can be covalently incorporated into the peptide only when the peptide is bound to S-protein.

The relative initial velocity of the enaminone formation reaction was also analyzed using a lower concentration of peptide FT-RPF1310 (50 μM) and 2,4-pentanedione (500 μM). The results are summarized in Table 1. Addition of S-protein (50 μM) or RNase S (50 μM) accelerated the enaminone formation, whereas RNase alone, S-protein alone, or FT-RPF1310 alone did not form an enaminone with 2,4-pentanedione under the same conditions. Peptide FT-RPF1310 also formed an enaminone with 2,4-pentanedione in the presence of RNase S (S-protein and S-peptide), indicating that peptide FT-RPF1310 either binds to S-protein with higher binding affinity than natural S-peptide or binds to S-protein with a different mode or in a different area from that of natural S-peptide. For an amino group to become a nucleophile, the amino group must be in a hydrophobic microenvironment or

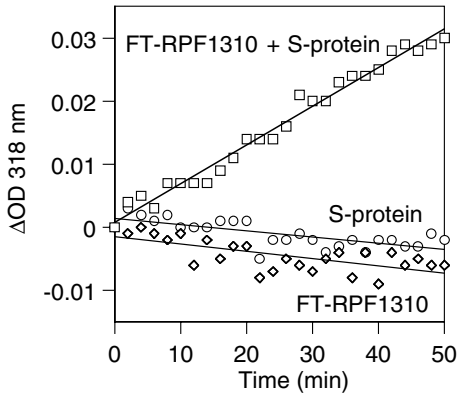



**Figure 3.** Time course of enaminone formation of peptide FT-RPF1310 with 2,4-pentanedione in the presence and absence of S-protein. Changes in absorption at 318 nm were monitored using a microplate spectrophotometer. Conditions: FT-RPF1310 + S-protein, [peptide FT-RPF1310] 100 μM, [S-protein] 100 μM, [2,4-pentanedione] 4 mM; FT-RPF1310, [peptide FT-RPF1310] 100 μM, [2,4-pentanedione] 4 mM; S-protein, [S-protein] 100 μM, and [2,4-pentanedione] 4 mM; all reactions in 4% $CH_3CN$–38 mM sodium phosphate buffer (pH 7.5) at 25 °C.

**Table 1.** Relative initial velocity of enaminone formation[a]

| Peptide and additive | Relative initial velocity |
|---|---|
| FT-RPF1310 + S-protein | 1.0 |
| FT-RPF1310 + RNase | 1.3 |
| FT-RPF1310 | <0.01[b] |
| S-Protein | <0.01[b] |
| RNase | <0.01[b] |

[a] The relative initial velocity of the enaminone formation with 2,4-pentanedione was measured by increase in absorption at 318 nm on a microplate spectrophotometer using 100 μL of a solution in 5% $CH_3CN$–42.5 mM sodium phosphate buffer (pH 7.5) at 25 °C. Concentrations of 2,4-pentanedione, peptide FT-RPF1310, and additives were as following: [2,4-pentanedione] 500 μM, [FT-RPF1310] 50 μM, [S-protein] 50 μM, and [RNase S] 50 μM.

[b] Increase in absorption at 318 was not detected.

must have an electrostatic interaction with a positively charged residue.[19,20] When peptide FT-RPF1310 binds to S-protein, it must form a fixed conformation and an amino group of the peptide must be activated either due to the stabilized conformation of peptide FT-RPF1310 or through interaction with S-protein.

In summary, we have developed a peptide whose function can be regulated by interaction with a second protein molecule. The enaminone formation activity of the peptide was turned-on in the presence of an interactive protein, but was off in the absence of the protein. We have demonstrated that reaction-based selection in the presence of an interacting protein can afford a peptide that is regulated by the presence of the protein. Since small peptides that selectively bind to a certain targeted protein molecule can be developed, it should be possible to convert such peptides to regulated peptides by the presence of their interacting proteins. Our strategy for the creation of the regulated peptide described here is the first step toward control of small peptide function in a programmed manner.

## Acknowledgment

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17. Chemically synthesized oligonucleotides (names of f-oligos and b-oligos for each library and their sequences are given below) were amplified by PCR using 5′-primer SfiI-f (5′-GAGGAGGAGGAGGCCCAGGCGGCC-3′) and 3′-primer Sfil-b (5′-GAGGAGGAGGAGGCCGG CCT GGCC-3′). The PCR conditions were as follows:SfiI-f, 1 μg; SfiI-b, 1 μg; f-oligo, 50 ng; b-oligo, 50 ng; 0.2 mM each dNTP; 10 μL of 10× PCR buffer; 2.5 U of Ampli Taq DNA polymerase (Roche) in total volume 100 μL; a program of 94 °C 1 mm; 94 °C 15 s, 52 °C 15 s, 72 °C 30 s (25 times); and 72 °C 10 min was used for amplification. The PCR products were purified, digested with *Sfi* I, and ligated to *Sfi*I-digested pComb3X. The phage libraries were prepared using previously reported procedures.[9,16] Binding selection against the 1,3-diketone was performed using the procedures described previously,[9] but in the presence of S-protein (2 μM). ELISA was also performed using the previously reported procedures[9] except in the presence of S-protein (2 μM).

| Library | f-oligo | b-oligo |
|---|---|---|
| FT-NNK6-S | FT-NNK6-S-f | FT-S-b |
| FT-NNK6-Pro-S | FT-NNK6-Pro-S-f | FT-S-b |
| FT-S-NNK6 | FT-S-f | FT-S-NNK6-b |
| FT-S-T-NNK6 | FT-S-f | FT-S-T-NNK6-b |
| FT-NNK6-HTH-S-1 | FT-NNK6-HTH-S-1-f | FT-HTH-S-b |
| FT-NNK6-HTH-S-2 | FT-NNK6-HTH-S-2-f | FT-HTH-S-b |
| FT-NNK6-HTH-S-3 | FT-NNK6-HTH-S-3-f | FT-HTH-S-b |

Oligonucleotide sequences were as follows:FT-NNK6-S-f (5′-GAGGAGGCCCAGGCGGCCNNKNNKNNKNNKNNKNNKGAGACAGCAGCCGCAAAGTTTGAG-3′), FT-S-b (5′-CTCTCTGGCCGGCCTGGCCACTATCCATCTTCTGACGCTCAAACTTTGCGGCTG-3′), FT-NNK-Pro-S-f (5′-GAGGAGGCCCAGGCGGCCNNKNNKNNKNNKNNKNNKCCAGAGACAGCAGCCGCAAAGTTTGAG-3′), FT-S-f (5′-GAGGAGGCC-CAGGCGGCCAAAGAGACAGCAGCCGCAAAGTTTGAGCGTCAGAAGATGGAT-3′), FT-S-NNK6-b (5′-GGAGGCCGGCCTGGCCMNNMNNMNNMNNMNNMNNATCCATCTTCTGACGCTC-3′), FT-S-T-NNK6-b (5′-GGAGGCCGGCCTGGCCMNNMNNMNNMNNMNNMNNGCTACGAGAATCCATCTTCTGACGCTC-3′), FT-NNK6-HTH-S-l-f (5′-GGCCCAGGCGGCCNNKNNKNNKNNKNNKNNKCACTTATCTCAACCAGAGACAGCAGCCGCAAAGTTTGAG-3′), FT-NNK-HTH-S-2-f (5′-GGCCCAGGCGGCCNNKAAANNKNNKNNKCTGCACTTATCTCAACCAGAGACAGCAGCCGCAAA GTTTGAG-3′), FT-NNK-HTH-S-3-f (5′-GGCCCAGGCGGCCNNKNNKAAANNKNNKCTGCACTTATCTCAACCAGAGACAGCAGCCGCAAAGTTTGAG-3′), FT-HTH-S-b(5′-GGCCGGCCTGGCCATCCATCTTCTGACGCTCAAACTTTGCGGCTGCTG-3′).

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