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Caged RNase: Photoactivation of the Enzyme from Perfect Off-State by Site-Specific Incorporation of 2-Nitrobenzyl Moiety

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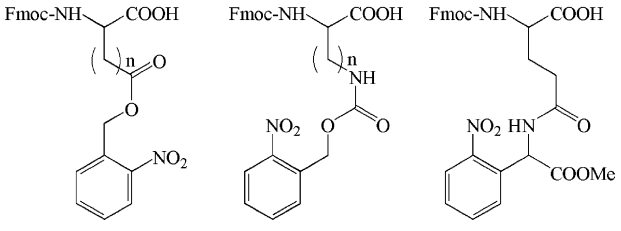
Abstract—Photo-triggered activation of semisynthetic Ribonuclease S′ from a perfect off-state was successfully carried out by incorporation of photo-labile caged moiety into a proximity to the active site.

The light-triggered release of biological small molecules called as caged compounds including nucleotides,[1] neurotransmitters,[2] second messengers,[3] and peptides[4] has been widely applied to explore many biological phenomena. Recently, besides these small molecules, caged biopolymers such as proteins and enzymes are also highly desirable for conducting a broad range of time-resolved experiments relevant to protein folding, protein–protein and protein–ligand interactions, catalytic mechanism, and protein conformational studies. So far, a few examples of light-triggered activation of natural proteins (proteinase,[5] T4 Lysozyme[6] and antibody[7]) were carried out by masking the catalytic residue using site-directed mutagenesis or chemical modification. Very recently, in addition to the active site, an allosteric regulation site is becoming a target for the modification of caged groups. Bayley's group, for instance, succeeded in incorporating a light-sensitive caged group (4-hydroxyphenacyl) into the regulation site (Thr 197) of cAMP-dependent protein kinase (PKA) by using thiophosphorylation reaction.[8] However, these examples do not enable one to *perfectly* suppress the catalytic activity before photolysis.

In this communication, we describe that the perfect off-state is produced by site-specific incorporation of *o*-nitrobenzyl (*o*-NB) unit into RNase S′, a RNA hydrolyzing enzyme. Four kinds of amino acids (Lys, Glu, Asp, Gln) localized at an α-helix region of RNase S were modified with photo-labile *o*-nitrobenzyl moiety. Among these mutants, a mutant bearing the caged amino acid at a proximity to the active site displayed the photo-triggered activation from the *perfect* off-state.

Ribonuclease S (RNase S) is consisted of two peptide fragments, S-peptide(1–20) and S-protein(21–124). It is well-known that many S-peptide variants can be readily rebound to S-protein in self-assembly manner (RNase S′) to restore the native-like structure and activity.[9–13] The active site is consisted of two His (His12 and His119) which are not appropriate to convert caged His, because of its instability. Thus, instead of His12, we attempted to incorporate the *o*-nitrobenzyl unit into the S-peptide fragment by solid phase peptide synthesis (SPPS).



n=1 caged Asp (**cD**)
n=2 caged Glu (**cE**)

n=4 caged Lys (**cK**)

caged Gln (**cQ**)

Caged Asp (**cD**), Glu (**cE**), and Lys (**cK**) which possess *o*-NB at the side chain were synthesized according to the literature.[14,15] Design and synthesis of caged Gln (**cQ**) was referred to Hess's report.[16]

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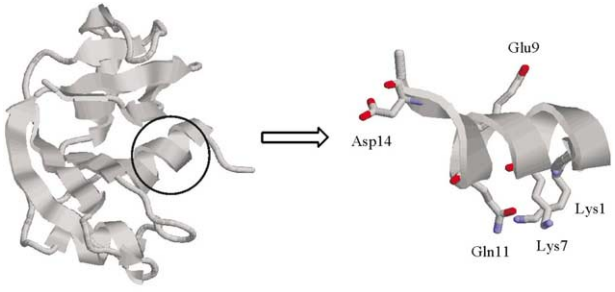
To screen a suitable site of the caged moiety, six mutants having single caged amino acid (**cD**, **cE**, **cK**, or **cQ**) were prepared. Figure 1 shows the amino acid sequence of the mutant S-peptides (Mutant **1**–**6**). The mutants **1**–**4** have the caged moiety far from the active site (His12), whereas the caged site is rather close to the His12 in the mutants **5** and **6**. These mutants were synthesized by SPPS based on Fmoc chemistry (peptide synthesizer ABI 433A). Crude peptides were purified through reverse-phase HPLC (Hitachi LC system, eluent: water/acetonitrile gradient) to afford pure S-peptides and identified with MALDI-TOF MS (PerSeptive Voyger RP).[17] S-Protein was purchased from Sigma Co. Ltd. and used without further purification.

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). Before photolysis, the helix content of mutants **1**–**6** (13–15%) is almost same as that of native RNase S′ (17%). This suggests that mutants **1**–**6** are stably complexed with S-protein in the conformation similar to native S-peptide regardless of steric bulkiness of the caged moiety.

Mutants **1**–**6** were photolyzed by Hg-lamp as a light source equipped with a filter cut off wavelength below 290 nm. MALDI-TOF MS after 20 min photo-irradiation showed disappearance of the peak due to the corresponding caged S-peptide and generation of the native peptide, indicating that the *o*-NB group is completely cleaved off in mutants **1**–**4** and **6**. In the case of mutant **5**, the cleavage of an amide-type of **cQ** required Nd-YAG laser as the more powerful light source (Quantel YG-571-10 Nd-YAG, 38-39 mJ/pulse), by which the mutant **5** is converted to native S-peptide, as shown in Figure 2.



Native	NH_2-KETAAAKFERQH-Nle-DS-OH
Mutant **1**	NH_2-**cK**-ETAAAKFERQH-Nle-DS-OH
Mutant **2**	NH_2-K-**cE**-TAAAKFERQH-Nle-DS-OH
Mutant **3**	NH_2-KETAAA-**cK**-FERQH-Nle-DS-OH
Mutant **4**	NH_2-KETAAAKF-**cE**-RQH-Nle-DS-OH
Mutant **5**	NH_2-KETAAAKFER-**cQ**-H-Nle-DS-OH
Mutant **6**	NH_2-KETAAAKFERQH-Nle-**cD**-S-OH

Figure 1. Replaced S-peptide sequences.

Subsequently, the enzymatic activity of these caged RNase S′ was spectrophotometrically assayed using poly-uridic acid (poly-U) as a substrate according to the literature.[18] The initial rates of caged RNase S' mutants before and after photolysis are summarized in Table 1.

Before photolysis, the initial rates of mutant **1**–**4** display the almost same values as native RNase S′, regardless of the bulky caged moiety. The values did not changed after photolysis, indicating that the caged effect was not

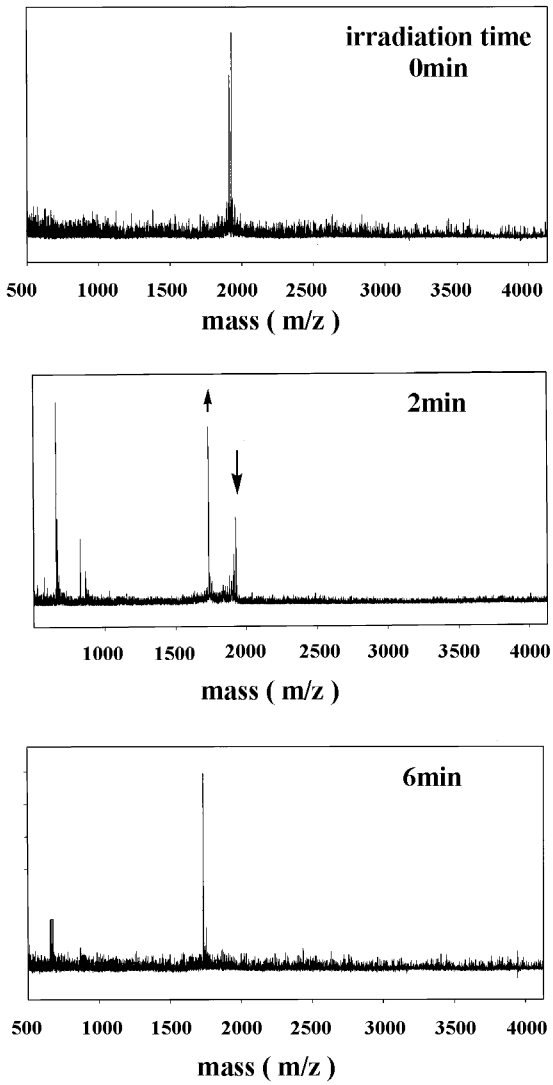


Figure 2. MS spectral change of mutant **5** by photolysis.

Table 1. Initial rates of native and mutant **1**–**6** before and after photolysis (10^5 M/min)

	Before	After
Native	1.02	
Mutant **1**	1.01	1.04
Mutant **2**	0.96	0.81
Mutant **3**	1.10	1.04
Mutant **4**	0.99	0.97
Mutant **5**	0	0.37
Mutant **6**	0.29	0.78

[S-protein] = [S-peptide mutants] = 1 μM, [Poly(U)] = 100 μM, 10 mM MES containing 100 mM NaCl, pH 6.0, 298 K.

observed for these mutants. In contrast, the initial rate of the mutant **6** is suppressed to 30%-fold, relative to that of native one and the activity of the mutant **5** perfectly suppressed before photolysis. Such a difference among the mutants implies that the caged effect is primarily dependent on the distance between the modified site and the active site (i.e., His 12). That is, the caged moiety is positioned too far from the active site to be effective in the mutants **1**–**4**, whereas the mutant **5** and **6** have the caged group close enough to be operated. Particularly, the side chain of Gln 11 in S-peptide is known to stabilize a phosphate anion of the RNA substrate in the active center via a hydrogen bond.[19] Therefore, it is reasonably expected that the caged moiety (*o*-NB) for mutant **5** efficiently masks the active site, so that the substrate can not gain access to the active pocket of RNase S′. This is supported by the kinetic analysis of the caged mutant **6**. The Michaelis-Menten parameters show that k_{cat} value (3.24×10^7 min^{-1}) is not considerably different from that of native one (4.07×10^7 min^{-1}), whereas K_m (1.13 mM) becomes predominantly greater by 4-fold relative to native one (0.27 mM). Thus, the suppression of the enzymatic activity can be reasonably attributed to the decrease of the substrate binding in the caged mutant **6**. The similar situation may be expected in the case of the mutant **5**, although one cannot analyze the kinetic parameters for the caged mutant **5**, because of its no activity. After photolysis, on the other hand, the initial rates were restored to the almost 80% for mutant **6** and to 37% for mutant **5** relative to the native one. The laser irradiation reduced the enzymatic activity even in the case of the native one by 20–30%. Such a photodamage might prevent from 100% recovery of the mutant **5** after photolysis.

In summary, we demonstrated in this paper that incorporation of a caged group at a single position which is not an active site, is effective to perfectly suppress the enzymatic activity by the careful site-selection.

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