Photoswitching of the Enzymatic Activity of Semisynthetic Ribonuclease S' Bearing Phenylazophenylalanine at a Specific Site

Itaru Hamachi,* Takashi Hiraoka, Yasuhiro Yamada, and Seiji Shinkai
Department of Chemistry & Biochemistry, Graduate School of Engineering, Kyushu University, Hakozaki Fukuoka 812-8581

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Photoswitching of the enzymatic activity of ribonuclease S' was successfully carried out by site specific incorporation of phenylazophenylalanine into S-peptide skeleton by semisynthesis.

Development of useful methods for photoswitching of the activity of naturally occurring proteins and enzymes has been one of the major targets in the research field of recent protein engineering.[1] Modification of native enzymes with photochromic molecules seems a promising method for it. As a pioneering work, Aizawa and coworkers reported photocontrol of activities of spiropyrane-modified enzymes.[2] Recently, Willner and coworkers prepared azobenzene-appended papain, the activity of which changed by UV/visible light-irradiation.[3] These examples, however, used randomly-modified enzymes and thus, it is difficult to investigate how photoinduced conformational changes of the photochromic molecules affect the structure and activity of the enzymes in molecular terms. In order to generalize this methodology, it is now desirable to evaluate the availability of photochromic molecules using a site-specifically modified enzyme in atomic and/or molecular level. In this communication, we describe semisynthesis of Ribonuclease S' bearing phenylazophenylalanine at a specific site and photoswitching of the enzymatic activity.

Ribonuclease S (RNase S), a RNA hydrolyzing enzyme, is consisted of two peptide fragments, S-peptide and S-protein. It is well established that S-peptide variant is readily rebound to S-protein in self-assembly manner (RNase S').[4] We noticed that this enzyme is an appropriate model for investigation of the effect of photochromic molecules incorporated into a specific site of the polypeptide skeleton.

Phenylazophenylalanine (Azo) was synthesized according to Goodman's method[5] and converted to Fmoc-protected form (N-Fluorenylmethyloxycarbonyl-Azo) by a reaction with Fmoc-succinimide (Figure 1). Figure 2 shows the amino acid sequences of the mutant S-peptides. To investigate how the incorporation position of photoisomerizable Azo influences on the RNase activity, we prepared four mutants having an Azo unit. Phe8 or Nle13 in hydrophobic region which is close to the active center (His12) was replaced to Azo, whereas N- or C-terminal residue was changed as the contrast mutant having the replacement far from His12. These mutants were synthesized using solid phase technique of Fmoc chemistry. Crude peptide was purified through reverse-phase HPLC (Hitachi LC system, water-acetonitrile gradient) to afford a pure S-peptide. The purified S-peptide was identified with MALDI-Tof (time of flight) mass spectroscopy (Perseptive Voyager RP or Shimazu KOMPACT). 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).

Figure 3 shows titration curves of S-protein with various S-peptides monitored by circular dichroism spectropolarimeter (CD, Jasco J-720W).[6] When S-peptide is bound to S-protein, the S-peptide conformation changes from random coil to α–helix. In all peptide mutants, the helix content increases by addition of S-peptide. The titration curves show a typical saturation behavior at 1:1 ratio for mutants **1** and **4** both in trans and cis form, like native S-peptide. Relatively loose saturation was observed for mutants **2** and **3**, due to the lessened binding constant. The saturated CD value for addition of **1** and **4** is almost identical with that of native S-peptide, whereas that for addition of **2** and **3** is smaller. The CD titration data is not practically different by trans/cis conformation of Azo moiety. These results suggest that mutant **1** and **4** is bound to S-protein in the conformation similar to native S-peptide, mutant **2** and **3** is complexed with S-protein in a slightly disturbed manner both in trans and cis isomer.

The enzymatic activity of the semisynthetic RNase S' was spectrophotometrically assayed by poly-uridiric acid (poly-U) hydrolysis according to the literature.[7] The initial rates of Azo-incorporated RNase S' mutants (**1** - **4** have trans-Azo or cis-Azo rich state) are summarized in Figure 4. For mutant **1**, the initial rate in trans-form is slightly smaller than that of naive one and the

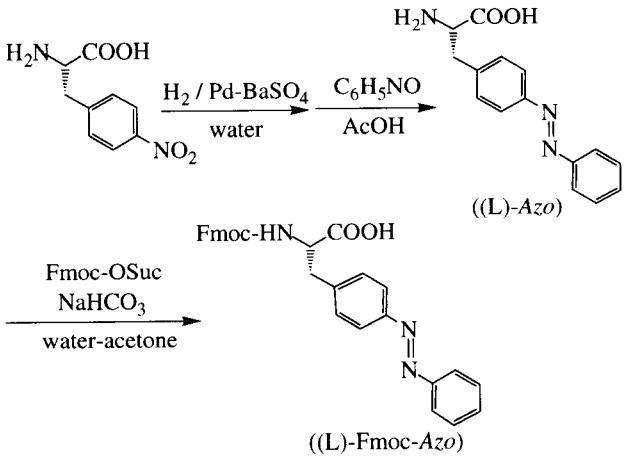

Figure 1. Reaction scheme.

	(N) 1 8 15 (C)
native	LysGluThrAlaAlaAlaLysPheGluArgGln**His**NleAspSer
Mutant 1.	*Azo*-LysGluThrAlaAlaAlaLysPheGluArgGln**His**NleAspSer
Mutant 2.	LysGluThrAlaAlaAlaLys*Azo*GluArgGln**His**NleAspSer
Mutant 3.	LysGluThrAlaAlaAlaLysPheGluArgGln**His***Azo*AspSer
Mutant 4.	LysGluThrAlaAlaAlaLysPheGluArgGln**His**NleAspSer*Azo*Thr

Figure 2. Modified S-peptide sequences.

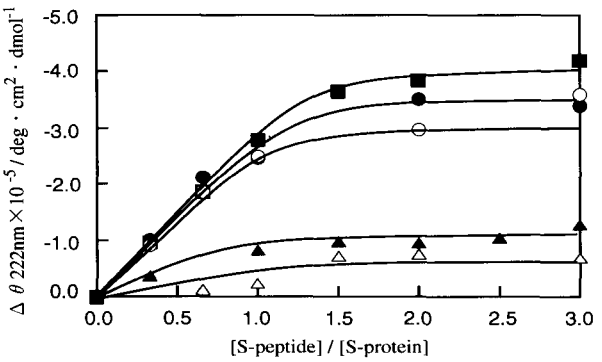

Figure. 3 S-peptide/S-protein titration curve monitored by CD-spectroscopy.: native(■), trans(●) and cis(○) of mutant **1**, trans(▲) and cis(△) of mutant **3**. S-protein concentration is constant (10 μ M), pH6.0, MES buffer at 25℃.

photoisomerization to cis-form does not change the activity. Since N-terminal site is completely exposed to the solvent phase, trans-cis conformational change does not effect the activity.[8] The mutant **2** scarcely shows the activity both in trans and cis isomer (the initial rate is 0.02-0.01 fold smaller than that of native one). This may be ascribed to that the replacement of phenylalanine to the bulky Azo induces an inactive complex of S-peptide with S-protein. The rate of trans-**3** mutant is 0.1-fold relative to that of native one, whereas that of cis-**3** is scarcely obsevable[9]. Figure 5 shows an activity-on-off experiment using mutant **3**. In the trans form (generated by visible-light irradiation (>400 nm)), poly-U hydrolysis occurs but it nearly stops in cis form generated by UV light irradiation. This process can be repeated by UV and visible light irradiation, indicating that we can switch the activity by light in an almost all-or-none type.[10] In contrast to others, the cis-isomer of **4** is more active than trans-**4** by 1.4-fold. It is clear that the effect of light-induced conformational change of azobenzene moiety on the activity is remarkably dependent on its

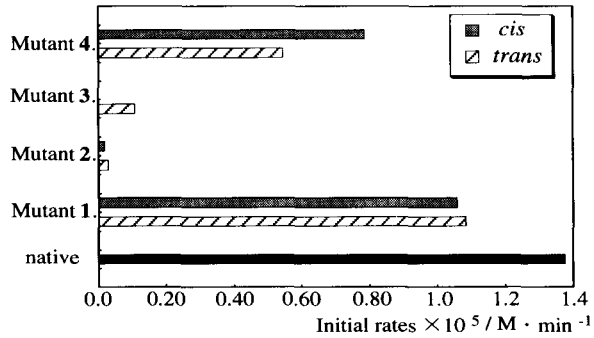

Figure 4. Initial rates of poly-U hydrolysis reaction catalyzed by various RNase S'.: All reaction were performed at 25 ℃ in 10mM MES bufer, pH 6.0, containing 0.1M NaCl.

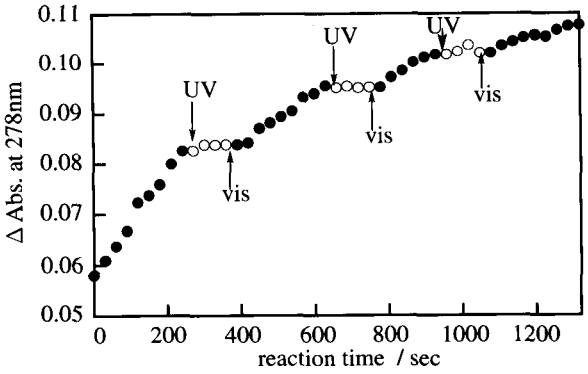

Figure. 5 Photoinduced ON-OFF experiment using mutant **3**.: Interval periods (150sec) for trans→cis or cis→trans photoisomerization were omitted for clarity. The rate becomes slower due to the consumption of Poly-U

incorporation site. The proximity of the photochromic molecule to the RNase active site (His-12) is one of the important factors for efficient response.[11]

In conclusion, we have clearly demonstrated in this study that single point modification with a photochromic molecule is sufficiently effective for photoswitching of the enzymatic activity, although random and multi-site modified enzymes were used in previous studies. Kinetic study in detail is now under progress in our laboratory.

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References and Notes

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