# Guest-Induced Umpolung on a Protein Surface: A Strategy for Regulation of Enzymatic Activity

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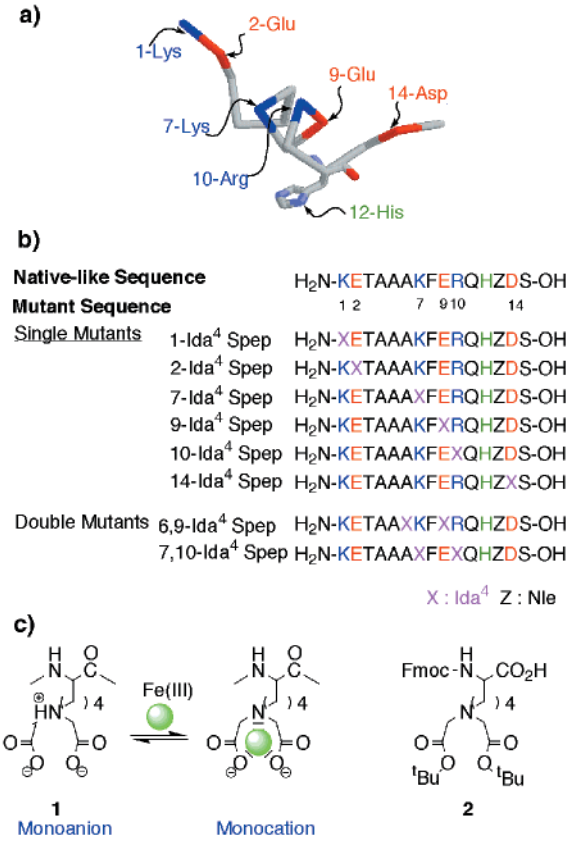
Recent developments in new bioorganic methodologies such as site-directed mutagenesis using expanded genetic codes, total- or semi-synthesis of enzymes, and protein splicing have enabled incorporation of unnatural amino acids into the framework of native proteins.[1] Although utilization of unnatural amino acids as valuable probes is now greatly facilitating biophysical understanding on proteins structure,[2] their contributions to chemistry which can modulate or expand enzyme activities are still limited.[3] To advance the new trend in protein engineering, it is desirable to establish chemistry-based general concepts for the regulation of enzymatic activities using unnatural amino acids. We propose herein that guest-induced Umpolung on a protein surface is a promising strategy for regulation of enzymatic activity. Surface charge inversion is induced upon complexation or decomplexation of transition metal ions with a site-specifically incorporated unnatural amino acid, and it can cause the protein to reversibly regulate the activity.

In signal transduction cascades of living cells, it is well-established that phosphorylation and/or dephosphorylation at specific sites on a protein surface is often involved as one of the key events.[4] Surprisingly, net activities of responsible enzymes are dramatically controlled by such subtle modification of surface charges (i.e., addition or deletion of anionic charges in these cases). These findings gave us an important clue for rational introduction of a sharp on−off switch into enzyme molecules.

Semisynthetic ribonuclease S′ (RNase S′), an RNA hydrolyzing enzyme, was employed as a suitable model.[5] As a surface charge modulator, an unnatural amino acid bearing iminodiacetic acid group (Ida[4], **1**) was incorporated into the *S*-peptide region of RNase S′ by solid-phase peptide synthesis (SPPS). The mutant *S*-peptides (Scheme 1) were combined to *S*-protein in a self-assemble manner.[6,7] The charge of the side chain of **1** is a mono*anion* at neutral pH and it is inverted to a mono*cation* upon complexation with a trivalent metal cation such as Fe(III) at its iminodiacetic acid moiety (see Scheme 1c).[8]

**Scheme 1**[a]




[a] The native sequence has methionine instead of norleucine at position 13. It is established that this mutation does not affect the structure and the activity of native RNase S′.

To examine the effect of surface charge inversion, we initially replaced charged amino acids located on the *S*-peptide fragment of RNase S′ (Lys1, Glu2, Lys7, Glu9, Arg10, Asp14) with Ida[4] and monitored the Fe(III)-induced activity change using initial rates of the cyclic-C hydrolysis catalyzed by RNase S′. Figure 1 shows the Fe(III)-response efficiency ($[V_{ini}(Fe^{3+}) - V_{ini}(non)]/V_{ini}(non)$) of the enzymatic activity of these mutants. It is clear that the response depends on the charge of the original amino acid, that is, the Fe(III)-induced activity enhancement occurs at the positively charged Lys or Arg site and the activity suppression occurs at the negatively charged Glu or Asp site. Conceivably, the replacement of Lys or Arg with the negatively charged **1** causes the charge imbalance on the protein surface to lessen the activity. The imbalance can be repaired upon Fe(III) cation complexation to restore the activity. In contrast, the replacement of Glu or Asp with **1** retains the surface charge, which is inverted when **1** binds to Fe(III) so as to decrease the activity.

Next, a double mutant having two Ida[4] at 7 and 10 positions (7,10-Ida[4]-RNase S′) was semisynthesized as a model equipped with the sharper on−off switch. Regardless of double incorporation of unnatural Ida[4], the mutant *S*-peptide was shown to form a stable complex (RNase S′) with *S*-protein by monitoring

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(6) **1** was properly protected to afford **2** so as to fit the SPPS. The crude peptides thus obtained were purified by HPLC and characterized by Tof-mass spectroscopy. Hamachi, I.; Yamada, Y.; Matsugi, T.; Shinkai, S. *Chem. Eur. J.* **1999**, *5*, 1503.
(7) Titration experiments of *S*-protein with the mutant *S*-peptides were monitored by CD spectroscopy or the conventional activity assay.
(8) A binding constant of iminodiacetic acid to Fe(III) was reported to be $10^{10.7}$. Napoli, A. *J. Inorg. Nucl. Chem.* **1972**, *34*, 987.

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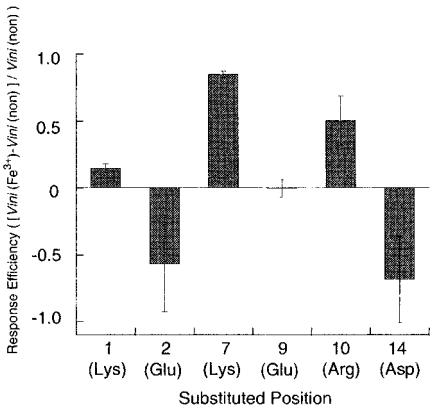


**Figure 1.** Comparison of response efficiency of single mutants having Ida[4]. The initial rates were measured for each mutant in the presence or absence of iron(III) ion, and the response efficiency was calculated according to the equation shown in the text. Reaction conditions are given in Supporting Information.

conventional circular dichroism (CD) or activity titration.[9] Conformational change of the semisynthetic 7,10-Ida[4]-RNase S′ upon Fe(III) complexation was clearly detected by CD spectroscopy (data shown in the Supporting Information). The $\theta$ value at 222 nm, characteristic of α-helix, was slightly lessened relative to that of native RNase S′. It is intensified by addition of Fe(III) and saturated to a value comparable to the native one at a 2:1 stoichiometry of Fe(III) over RNase S′. The magnesium cation is not effective for such structural change. These indicate that the slightly unwound α-helix of 7,10-Ida[4]-RNase S′ was effectively rewound upon noncooperative binding of 2 mol of Fe(III) at two Ida[4] sites.[10]

The Fe(III)-induced structural changes of RNase S′ apparently affect the enzymatic activity (Figure 2a). In the absence of metal cations, the activity of 7,10-Ida[4]-RNase S′ is very low. Upon Fe(III) addition, the initial rate is enhanced gradually and shows clear saturation at 2 equiv of Fe(III). The maximum rate (12-fold greater than that without metals) is comparable to that of native RNase S′. Interestingly, this is a sharp contrast to 6,9-Ida[4]-RNase S′ which was previously prepared by us. The initial rate of 6,9-Ida[4]-RNase S′ without metal ions is moderate and is suppressed by 4-fold upon 2 equiv of Fe(III) ion binding.

We then compared kinetic parameters for the hydrolysis reaction catalyzed by 7,10-Ida[4]-RNase S′ in the absence or presence of Fe(III). Both are proved to obey a typical Michaelis−Menten-type kinetics against cyclic-C concentration (see the Supporting Information). The curve fitting analyses give us Michaelis−Menten parameters (i.e. $k_{cat}$ and $K_m$ values) as follows: $k_{cat} = 0.36 \times 10^{7}$ min$^{-1}$, $K_m = 1.30 \times 10^{-4}$ M in the absence of Fe(III), and $k_{cat} = 6.63 \times 10^{7}$ min$^{-1}$, $K_m = 2.71 \times 10^{-4}$ M in the presence of Fe(III). It is clear that the catalytic efficiency ($k_{cat}$) is enhanced by 20-fold upon Fe(III) binding, whereas the binding affinity for substrate ($1/K_m$) is not considerably affected (0.5-fold relative to the value without Fe(III)). Therefore, the activity change in response to Fe(III) can be mainly ascribed to the Fe(III)-induced enhancement of $k_{cat}$. In addition, the $k_{cat}/K_m$ value ($2.45 \times 10^{10}$ M$^{-1}$ min$^{-1}$) in the presence of Fe(III) is proved to be comparable to that of native RNase S′ ($2.54 \times 10^{10}$ M$^{-1}$ min$^{-1}$, $k_{cat} = 17.2 \times 10^{7}$ min$^{-1}$, $K_m = 6.78 \times 10^{-4}$ M), indicating that the enzymatic activity of 7,10-Ida[4]-RNase S′, which is suppressed without metal ions, is completely restored upon Fe(III) complexation.

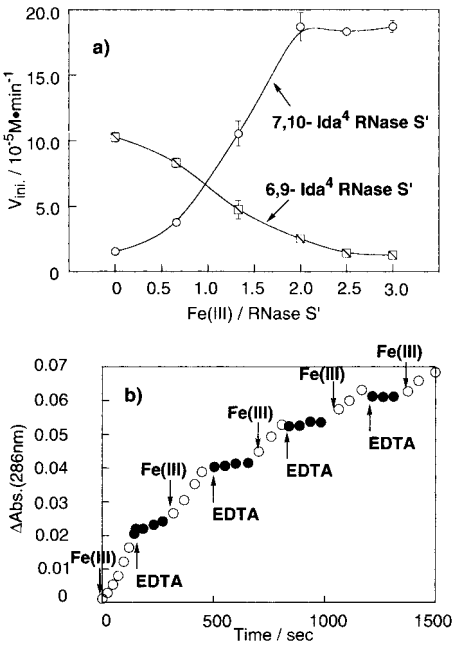


**Figure 2.** (a) Dependence of the enzymatic activity of two distinct double mutants upon iron(III) concentration. (b) The conventional on−off switching experiment sensitive to iron(III) concentration. Reaction conditions are given in Supporting Information.

The net activity of 7,10-Ida[4]-RNase S′ can be controlled depending on Fe(III) concentration so efficiently that one can demonstrate reversible switching in response to Fe(III) ion as shown in Figure 2b. Acceleration of hydrolysis was induced by Fe(III) and suppression was caused by addition of EDTA (a strong chelator for Fe(III)). The activity change was rapidly repeated. The opposite type of response against Fe(III) (i.e. deactivation upon Fe(III) complexation, and activation upon Fe(III) decomplexation) was observed for 6,9-Ida[4]-RNase S′ (data not shown).

In conclusion, we demonstrated that the inversion of point charges at a protein surface by external signal (i.e., "guest-induced Umpolung") can confer slight but crucial winding or unwinding of a specific α-helix structure, so as to carry out an on/off-type of switching of the enzymatic activity. To our knowledge, this is the first indication that Umpolung, which has been widely recognized as a versatile concept in the field of organic synthesis, is useful in the protein chemistry.[11] The present result also points out the compact function of unnatural amino acids. That is, introduction of only one unnatural amino acid is sufficient for construction of a metal binding site, whereas conventional mutagenesis usually requires several numbers of natural amino acids to form an appropriate metal binding motif.[12]

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**Supporting Information Available:** The experimental details on Figures 1 and 2 and CD titration and Michaelis−Menten curves (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) *S*-Peptide/*S*-protein titration shows saturation curves, giving the complexation constants (log $K_{com}$) as follows: 5.7 ± 0.2 in the absence of c-CMP, more than 7.0 in the presence of c-CMP (for 7,10-Ida[4]-RNase S′). These values are comparable to those of native ones (log $K_{com}$ 5.4, and more than 7.0 without and with the substrate, respectively) and do not practically change upon Fe(III) complexation.

(10) Since we conducted all of the experiments below 25 °C, the fraction of the denatured RNase S′ is negligible.

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