Single- or Dual-Mode Switching of Semisynthetic Ribonuclease S′ with an Iminodiacetic Acid Moiety in Response to the Copper(II) Concentration

Itaru Hamachi,* Yasuhiro Yamada, Tomoaki Matsugi, and Seiji Shinkai[a]

Abstract: Ribonuclease S′ bearing iminodiacetic acid as a metal-binding site was designed and semisynthesized by self-assembly of native S-protein with chemically modified S-peptide. Iminodiacetic acid-appended amino acid (Ida^4) was synthesized and incorporated into the S-peptide sequence by solid-phase peptide synthesis based on Fmoc chemistry at a single site or double sites of the solvent-exposed side of the S-peptide. Circular dichroism (CD) spectroscopy of these S-peptides confirmed that the Cu^{II} ion induced an increase or decrease of α-helix conformation depending on the replacement position. S-Peptide/S-protein titration monitored by conventional enzymatic activity and UV or CD spectroscopy demonstrated that various S-peptides form a stable complex (Ida^4–RNase S′) with S-protein, except when Met13 and Asp14 are replaced with Ida^4. In Cu^{II} titration and thermal denaturation experiments with single-site replacement mutants, Cu^{II} binding occurred at 1:1 stoichiometry of Ida^4/Cu^{II} with perturbation of the α-helix conformation. Both 2:1 and 2:2 stoichiometries were achieved by addition of Cu^{II} ions to double-site mutants, and were dependent on the Cu^{II} concentration. Most importantly, the A6/E9Ida^4–RNase S′ mutant shows cooperative binding of Cu^{II} ion with two Ida^4; holoenzyme stability is enhanced at 2:1 stoichiometry, but at 2:2 stoichiometry, two Ida^4 sites independently bind two Cu^{II} ions, and the mutant is destabilized. Other double mutants showed simple destabilization of 3D structure upon Cu^{II} binding. The response of the enzymatic activity of these Ida^4–RNase S′ to the concentration of Cu^{II} ion was evaluated by the hydrolysis of polyuridiric acid catalyzed by RNase S′ mutants. The Cu^{II}-induced activity change of single and double mutants agreed well with the structural response to Cu^{II}, that is, the activity of A6/E9Ida^4–RNase S′ was enhanced upon cooperative Cu^{II} binding at 2:1 stoichiometry and then suppressed at the 2:2 ratio. The activity of all other mutants was simply suppressed by Cu^{II} ions. These results represent successful switching of A6/E9Ida^4–RNase S′ activity in dual mode, that is, suppression (OFF) or enhancement (ON), depending on the environmental Cu^{II} concentration. Thus it has been established that rational design of a metal-binding site can confer the dual mode of response to a metal cation on the structure and activity of an enzyme.

Keywords: amino acids · copper · enzyme catalysis · ribonuclease · switchable enzymes

Introduction

Metal ions play crucial roles in various aspects of biological systems. In metal-containing proteins and enzymes, for example, metal ions act as an essential structural element in the regulation of the net enzymatic activity by metal-induced conformational change, and are components of active sites.[1] Native enzymes have adeptly utilized the valuable properties of reversible and strong coordination bonds in aqueous solution between metals and ligands consisting of side and main chains of the polypeptide matrix. This gives us an important clue for the artificial design of metal-ion-assisted semisynthetic enzymes.

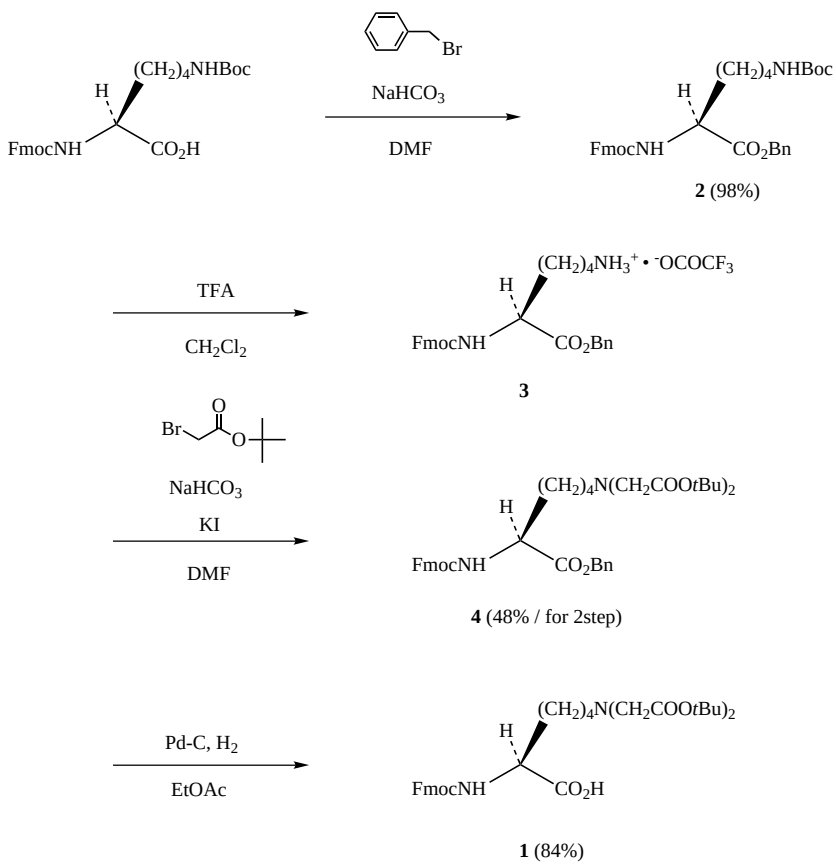
In the research field of de novo design of artificial peptides and proteins, such coordination chemistry has contributed towards the successful construction of various unique protein-like structures.[2] Three-helix bundles and DNA-binding protein mimics have been prepared by means of a transition metal complex with 2,2′-bipyridine or terpyridine as a template.[3] A short, single-strand helix structure has also been formed by bridging two iminodiacetic acids with a metal cation.[4] In spite of these successes, there have been few reports of enzyme engineering with the concept of coordination chemistry, and these have been limited to examples of site-directed mutagenesis based on DNA recombinant technology.[5] For example, a histidine cluster was introduced into the proximity of an active site so that chelation of a metal there involved the amino acid responsible for activity and thus suppressed the enzymatic activity. Aspartic acid or cysteine domains similar to the metal-binding motifs of other proteins have also been created as artificial binding sites for calcium ions or a cubic iron cluster, respectively.

[a] Prof. I. Hamachi, Y. Yamada, T. Matsugi, Prof. S. Shinkai
Department of Chemistry and Biochemistry
Graduate School of Engineering, Kyushu University
Hakozaki, Fukuoka 812-8581 (Japan)
E-mail: itarutcm@mbox.nc.kyushu-u.ac.jp

  0947-6539/99/0505-1503 $ 17.50+.50/0

In order to establish a rational design for sophisticated novel biocatalysts assisted by coordination chemistry, it is desirable to link valuable findings obtained from de novo peptide studies to enzyme engineering. For this purpose, ribonuclease S (RNase S′), a RNA-hydrolyzing enzyme consisting of S-protein and a short S-peptide segment, is regarded as an appropriate model, because the S-peptide can be replaced with other peptides prepared by solid-phase peptide synthesis.[6] It is also known that various unnatural amino acids can be stably incorporated into the S-peptide of RNase S′.[7] The pioneering work by Hopkins and co-workers in model peptides demonstrated that transition-metal induced conformational change occurs in peptides with two iminodiacetic acid groups (Ida[4]) at the *i* and *i*+3 or *i*+4 positions. We planned to apply this concept to the RNase S′ enzyme as a model case and examine the efficacy of the metal-induced conformational change at the local site for the modulation of the structure and function of the net enzyme.

We describe herein the incorporation of a unnatural amino acid, with an iminodiacetic acid moiety (Ida[4]) as a transition metal binding site, into ribonuclease S′ by a semisynthetic method. The iminodiacetic acid-appended RNase S′ (Ida[4]–RNase S′) displayed Cu^{II}-responsive properties, both in the structure and the activity, which were dependent on the replacement position and number of Ida[4] moieties.



Scheme 1. Synthesis of Fmoc-Ida[4](tBu)$_2$-OH (**1**).

Abstract in Japanese:

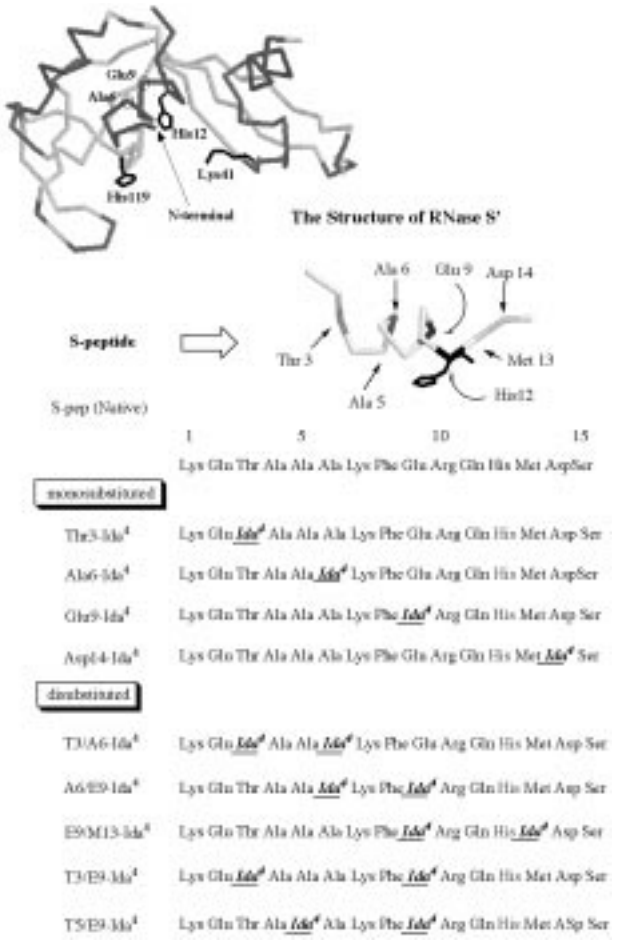
遷移金属イオンのレセプターとして機能するイミノ二酢酸型非天然アミノ酸を、S－ペプチド部分に導入したリボヌクレアーゼを半合成することに成功した。モノ置換体では銅イオンの添加によって、ヘリックス構造が乱され酵素が不安定化されることが分かった。これに対してジ置換体では6、9置換体において二つのイミノ二酢酸基が協同的に働くことによって銅イオンを補足し、それに伴って酵素が安定化された。またこの変異体では酵素活性も銅イオン一当量添加では活性化、二当量以上では不活性化した。これらの結果は人工レセプターの酵素の特定ペプチド部位への選択的な導入というコンセプトが、酵素に特定ゲストに対する応答機能を付与する有力な手段になることを物語っている。

Results

Design and synthesis of Ida^n-incorporated S-peptide by the Fmoc strategy: Although Hopkins used Boc chemistry to insert Ida^n into a model peptide,[8] we found Fmoc (fluorenylmethyloxycarbonyl) chemistry for mutant S-peptide synthesis to be more feasible. In order to allow Ida^n access to the solid-phase peptide synthesizer for the Fmoc method, we synthesized the unnatural amino acid **1** (Ida[4]), protected by a Fmoc group at the N-terminal and by *tert*-butyl groups at the iminodiacetic acid ends, as shown in Scheme 1.

Since iminodiacetic acid, a strong chelator for transition metal cations in aqueous solution, is hydrophilic, it is reasonable to expect that Ida[4] can favorably replace the amino acid residues positioned at the solvent-exposed side of the RNase S-peptide α-helix. According to the 3D-structure and activity data reported previously,[9] we selected four amino acid sites, all of which are expected to be exposed to the aqueous phase suggested by X-ray analysis of native RNase S (Scheme 2).[10] At first, four different peptides (Thr3Ida[4], Ala6Ida[4], Glu9Ida[4], Asp14Ida[4]), each of which have a single mutation in the native sequence of the S-peptide (1–15 residue sequence), were designed to check the accommodation capacity for Ida[4]. On the basis of the results from these single mutants, we next designed mutants with double-site replacement. Double-site replacement was conducted at several sets of the *i* and *i*+3 or *i*+4 positions (T3/A6Ida[4], A6/E9Ida[4], E9/M13Ida[4], A5/E9Ida[4]) in which two iminodiacetic acids may act cooperatively to bind transition metal

0947-6539/99/0505-1504 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 5



Scheme 2. Native and mutant S-peptide sequences.

cations so as to bridge one turn along the α-helix axis. T3/E9Ida[4] was prepared as a control sequence. These sequences are summarized in Scheme 2. All peptides were synthesized by a solid-phase peptide synthesizer, purified by reverse-phase HPLC, and characterized by MALDI-TOF mass spectroscopy.

Cu^{II}-induced conformational change of the Ida[4]-incorporated S-peptide in 30 % TFE aqueous solution: Before investigating the semisynthetic Ida[4]–RNase S′, we examined metal-induced conformational change of mutant S-peptides without S-protein by CD spectroscopy. In aqueous buffer solutions, neither native nor mutant S-peptides can form any 3D structures within the single component (that is, in the absence of S-protein). Under these conditions, no conformational change is observed by the addition of any metal cations. With 30 % of trifluoroethanol (TFE), all the S-peptides partially form α-helix conformation.[11] Figure 1 shows the Cu^{II}-induced CD spectral change of A6/E9Ida[4]. Two negative peaks at 222 and 208 nm, characteristic of an α-helix conformation, are intensified by the addition of up to 1 equivalent of copper sulfate, and subsequently reduced by further addition of Cu^{II}.[12] Other metal cations such as Ni^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} did not significantly induce conformational change (data not shown).[13] Titration curves for all of the mutants are summarized in Figure 2. In the case of all single mutants,

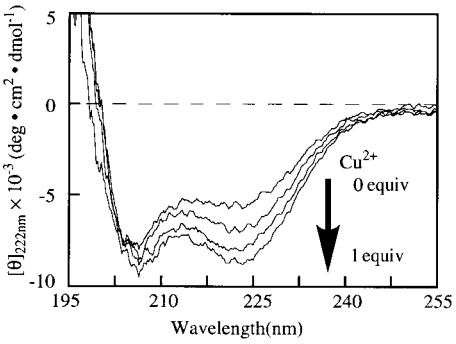


Figure 1. CD spectral change of A6/E9Ida[4]–S-peptide on addition of Cu^{II} cation in 30 % trifluoroethanol. A6/E9Ida[4]–S-peptide 60 μM in MES buffer (10 mM, pH 6.0, 0.1 M NaCl) containing 30 % trifluoroethanol at 25 °C.

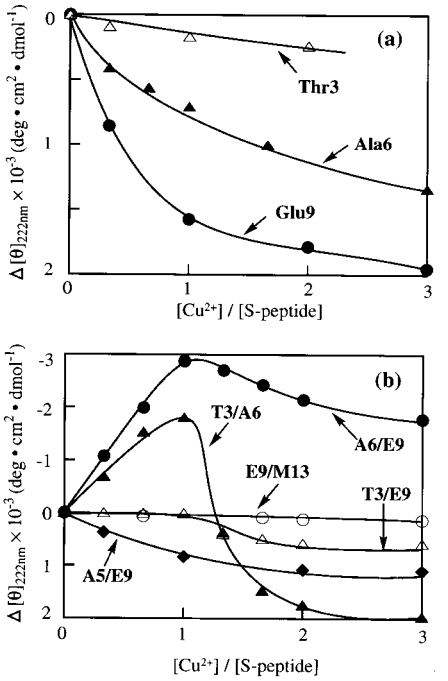


Figure 2. Cu^{II}/S-peptide titration curve monitored by CD-spectroscopy: a) single mutants: Glu9 (●), Ala6 (▲), Thr3 (△); b) double mutants: A6/E9 (●), T3/A6 (▲), E9/M13 (○), A5/E9 (◆), T3/E9 (△). S-peptide 60 μM in MES buffer (10 mM, pH 6.0, 0.1 M NaCl) containing 30 % trifluoroethanol at 25 °C.

these two peaks decrease upon Cu^{II} addition (Figure 2a). On the other hand, the double mutant, T3/A6Ida[4], displays a biphasic change similar to that of A6/E9Ida[4], that depends upon the Cu^{II} concentration (Figure 2b). The CD intensity of A5/E9Ida[4] and T3/E9Ida[4] mutants, however, steadily decrease up to the addition of 2 equivalents of Cu^{II}. Significant changes are not observed in the case of E9/M13Ida[4] peptide. It is clear that doubly replaced Ida[4] at the i and i+3 sites cooperatively bind a Cu^{II} ion in the S-peptide so as to increase the helix content, similar to Hopkins' model peptides, although the efficiency is greatly dependent on the mutation site. Double mutants at i and i+4 or i+6 did not show such a cooperative binding.[14] Noncooperative 1:1 binding of Ida[4] with Cu^{II} simply disturbs α-helix conformation.

Semisynthesis of RNase S′ by complexation of S-protein with mutant S-peptides: It is known that native self-assembled S-peptide/S-protein complex (so-called RNase S) restores the ordered 3D structure and the RNA hydrolysis activity identical to native RNase A. We confirmed the complex formation of the mutant peptides with S-protein by a conventional activity assay.[15]

Hydrolysis of polyuridiric acid (poly-U) is not catalyzed by the single components of S-peptide or S-protein. When S-protein was complexed with a mutant S-peptide, the hydrolysis reaction occurred. S-Peptide/S-protein titration monitored by this activity assay is a conventional method for the evaluation of complex formation. The initial rate was increased by the addition of most S-peptide mutants and saturated at almost a 1:1 ratio of S-peptide to S-protein, with the exception of Asp14Ida[4] and E9/M13Ida[4] (Figure 3).[16]

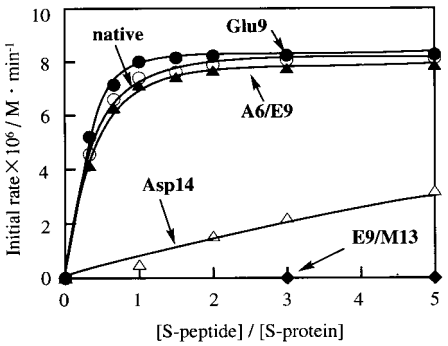


Figure 3. Initial rate of poly-U hydrolysis catalyzed by various RNase S′: native (○), Glu9 (●), A6/E9 (▲), Asp14 (△), E9/M13 (◆). S-protein 1 μM, poly-U 100 mM, 10 mM MES buffer, pH 6.0, containing 0.1 M NaCl, 25 °C.

This saturation behavior is the same as that of the native S-peptide, indicative that these mutant peptides are tightly bound to the S-protein to form the native-like RNase S′. Using this saturation curve, we estimated the association constant (K) of the corresponding S-peptide/S-protein complex.[17] The values for mutant S-peptides ($10^{7.4}$ – $10^{8.6}\,M^{-1}$) are comparable to that for native S-peptide ($10^{8.3}\,M^{-1}$),[18] suggesting that the mutation by Ida[4] at the 3, 5, 6, or 9 positions does not seriously affect the complexation. In the case of Asp14Ida[4] – RNase S′, the initial rate is slightly increased at more than 10 equiv of excess peptide, owing to its low binding affinity to the S-protein ($K < 10^4\,M^{-1}$). Since no reaction occurred for E9/M13Ida[4] – RNase S′, we cannot estimate the binding constant.

UV and CD spectroscopies were also utilized to monitor the S-peptide/S-protein complexation.[19] The absorbance at 287 nm was intensified when a mutant S-peptide (either single or double mutants) was added to the S-protein solution, as is the case for native S-peptide (data not shown). CD spectral titration of S-protein with S-peptide shows that the helix content increases upon S-protein – S-peptide binding (Figure 4). The CD spectral change is practically saturated at a 1:1 ratio of S-peptide to S-protein. Asp14Ida[4] and E9/M13Ida[4] are, once again, exceptions, and have shallow curves as a result of their low association constants. The saturated θ value for mutant S-peptide/S-protein complexes is almost identical to that of native species. This data implies that all mutant S-peptide/S-protein complexes are successfully formed in a manner similar to the native RNase S, apart from Asp14Ida[4] and E9/M13Ida[4], which is in good agreement with the data from the activity titration. We also checked the association constant in the presence of Cu^{II} for the following discussion on the Cu^{II} response of Ida[4] – RNase S′. The CD titration of S-protein with A6/E9Ida[4]-S-peptide suggested that the association constant ($10^{8.4}\,M^{-1}$) barely changes upon the addition of Cu^{II}, that is, $10^{8.5}\,M^{-1}$ at 1 equiv of Cu^{II} and $10^{8.6}\,M^{-1}$ at 2 equiv of Cu^{II}. Other mutants displayed the same behavior, indicating that Cu^{II} binding does not affect the tight complex of S-protein with S-peptide.

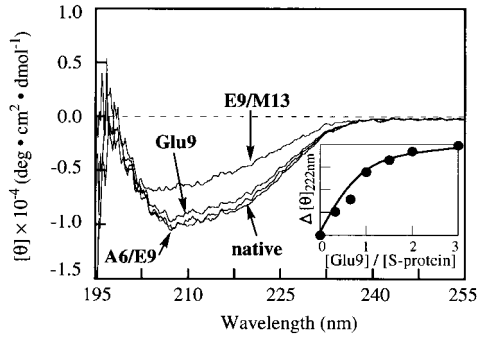


Figure 4. CD spectra of Glu9, A6/E9, E9/M13, native Ida[4] – RNase S′ (S-peptide/S-protein = 1:1), inset: Glu9 S-peptide/S-protein titration curve. S-protein 10 μM, 10 mM, MES buffer, pH 6.0, containing 0.1 M NaCl 25 °C.

Cu^{II}-induced structural change of semisynthetic Ida[4] – RNase S′: It was found that the two negative peaks from α-helix formation (208 nm and 222 nm) in the CD spectra of the single-mutant RNase S′ varieties were reduced by addition of Cu^{II}. Figure 5 depicts plots of the CD intensity (222 nm) against Cu^{II} concentration. The order of the Cu^{II}-induced θ value decrease is Glu9 > Ala6 > Thr3Ida[4] – RNase S′ (Figure 5a), which implies that the perturbation of the α-helix domain takes place upon Cu^{II} binding and it depends on the incorporation position of Ida[4]. This titration was not conducted for Asp14Ida[4] – RNase S′ because of its low association constant between S-peptide and S-protein.[16]

On the other hand, for A6/E9Ida[4], a double mutant, the peaks became more pronounced upon addition of up to 1 equiv of Cu^{II} and were then reduced upon furhter addition of Cu^{II} (Figure 5b). This suggests that Ida[4] units positioned on the S-peptide bind the Cu^{II} cation in two different modes at 2:1 and 2:2 stoichiometry.[20] For other double mutants (T3/A6, A5/E9, and T3/E9Ida[4]), the two peaks simply decreased upon addition of Cu^{II}.[21]

The subtle conformational change of the A6/E9Ida[4] mutant upon Cu^{II} addition was also probed by fluorescence of the tyrosine residues, shown in Figure 6.[22] The fluorescence at 307 nm due to Tyr residues, which are located only in the S-protein portion of RNase S′, was lessened at 1 equiv of Cu^{II} and gradually recovered to the original value at more than 1 equiv for the A6/E9Ida[4] mutant. Such changes were minimal

0947-6539/99/0505-1506 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 5

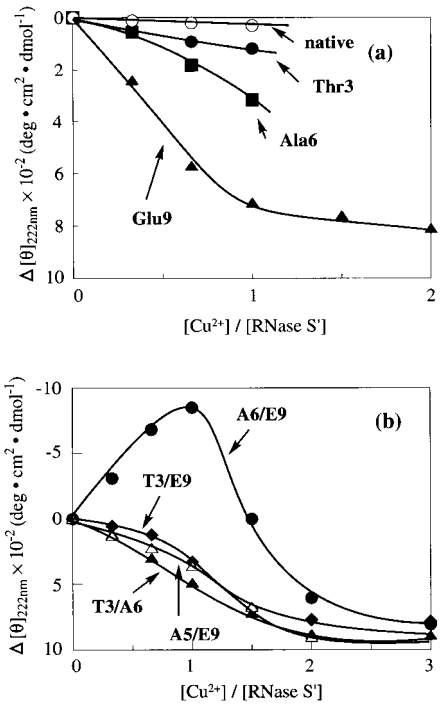


Figure 5. CD spectral change ([θ] 222 nm) of Ida^4–RNase S′ by addition of Cu^{II}: a) single mutants: native (○), Glu9 (▲), Ala6 (■), Thr3 (●); b) double mutants: A6/E9 (●), T3/A6 (▲), A5/E9 (△), T3/E9 (◆). Ida^4–RNase S′ 10 μM, 10 mM MES buffer, pH 6.0, containing 0.1 M NaCl, 35 °C.

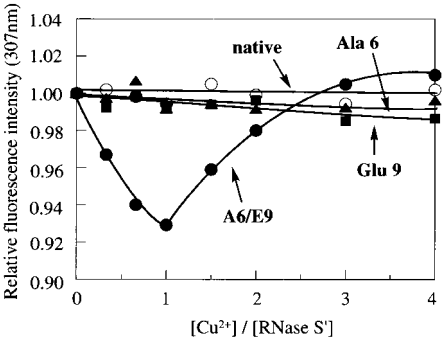


Figure 6. Plots of relative fluorescence intensity (307 nm) by addition of Cu^{II}: native (○), A6/E9 (●), Glu9 (■), Ala6 (▲). Each point is plotted with averaged volumes in three experiments. Ida^4–RNase S′ 1 μM, 10 mM MES buffer, pH 6.0, containing 0.1 M NaCl, 25 °C.

for other single and double mutants. This suggests that the structure of the S-protein was also affected by the cooperative Cu^{II} binding in the A6/E9Ida^4 mutant complex.

Thermal denaturation experiments clearly supported the above-mentioned effect of Cu^{II} on the net RNase S′ structure. Figure 7a shows the dependence of the thermal denaturation curves of Glu9Ida^4–RNase S′ upon the Cu^{II} concentration. Without the Cu^{II} ion, the denaturation temperature (T_d) was 36.1 °C. In the presence of 1 equiv of Cu^{II}, T_d shifted to 31.2 °C. T_d decreased similarly for Thr3Ida^4–RNase S′ (36.2 → 34.8 °C), or Ala6Ida^4–RNase S′ (35.4 → 33.1 °C) upon addition of 1 equiv of Cu^{II}. There was no significant shift in T_d for the native RNase S′ (37.1 → 36.3 °C). Clearly, the mutant RNase S′ enzymes with an iminodiacetic acid at a single site were destabilized upon Cu^{II} binding. The order of Cu^{II}-induced destabilization was in accord with the above-mentioned CD-titration data. In the case of A6/E9Ida^4–RNase S′, a double mutant, the Cu^{II} effect on the denaturation temperature is greatly dependent on the stoichiometry of Cu^{II} to RNase S′ (Figure 7b). At a 2:1 stoichiometry of Cu^{II} to A6/E9Ida^4–RNase S′, T_d rose by 3.1 °C (39.7 °C), and at 2:2 stoichiometry it fell by 7.4 °C (29.2 °C), relative to the T_d value without metals. It is noteworthy that the A6/E9Ida^4 mutant is more stable by about 3 °C with regard to the native RNase S′ through cooperative binding at 2:1 stoichiometry. In contrast, for the other double mutants, including T3/E6Ida^4, a simple decrease in the denaturation temperature was caused by the addition of Cu^{II} [35.8 (0 equiv) → 34.2 (1 equiv) → 32.1 °C (2 equiv) for T3/E6Ida^4, 35.4 (0 equiv) → 33.6 (1 equiv) → 30.9 °C (2 equiv) for T5/E9Ida^4, 36.0 (0 equiv) → 34.6 (1 equiv) → 31.7 °C (2 equiv) for T3/E9Ida^4].

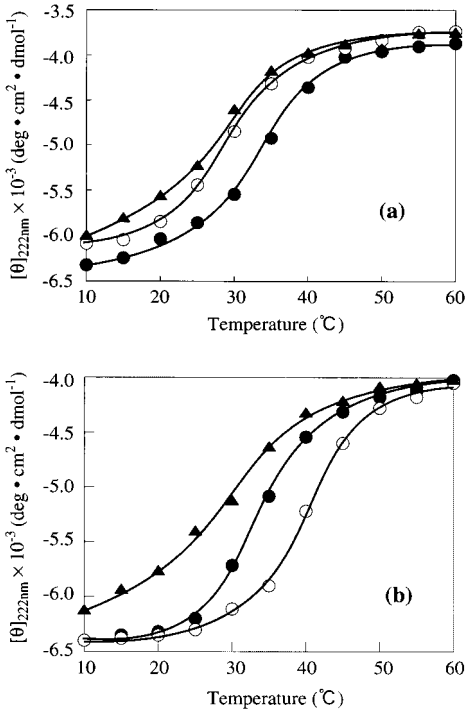


Figure 7. Thermal denaturation curve monitored by CD at [θ]222 nm of RNase S′: a) Glu9Ida^4–RNase S′; b) A6/E9Ida^4–RNase S′; Cu^{II} 0 equiv (●), 1 equiv (○), 2 equiv (▲). Ida^4–RNase S′ 10 μM, 10 mM MES buffer, pH 6.0, containing 0.1 M NaCl.

Cu^{II}-responsive activity change of semisynthetic Ida^4–RNase S′: The addition of Cu^{II} also affected the net activity of the semisynthetic RNase S′. The initial rate of the poly-U hydrolysis catalyzed by Glu9Ida^4–RNase S′ was reduced by an increase in Cu^{II} (Figure 8a).[23] A similar suppression was caused by Cu^{II} ions for other single mutants to different extents, but not for the native RNase S. The efficiency of the Cu^{II}-induced suppressions (the ratio of the initial rate with 1 equiv of Cu^{II} over that without Cu^{II}) is in the order of Glu9Ida^4 > Ala6Ida^4 > Thr3Ida^4–RNase S′ (the relative activities based on the initial rate are 62 %, 87 %, and 94 %, respectively). This order is coincident with the Cu^{II}-induced structural changes estimated by CD and thermal denaturation experiments. Figure 8b shows the titration curves for the

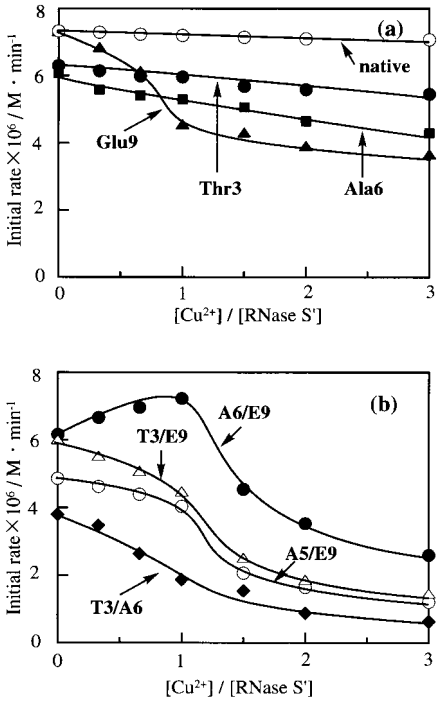


Figure 8. Initial rate of poly-U hydrolysis catalyzed by various RNase S′, a) single mutants: native (○), Glu9 (▲), Ala6 (■), Thr3 (●), b) double mutants: A6/E9 (●), T3/E9 (△), A5/E9 (○), T3/A6 (◆). S-protein 1 μM, poly-U 100 mM, 10 mM buffer, pH 6.0, containing 0.1 M NaCl, 25 °C.

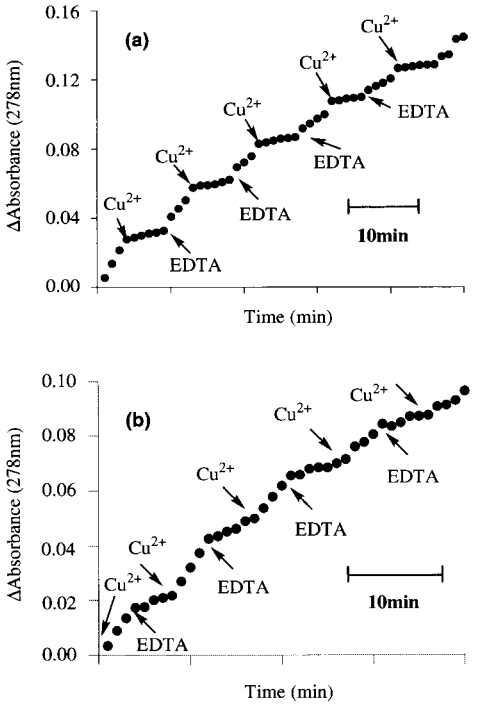


Figure 9. Switching of A6/E9Ida⁴ – RNase S′ activity depending on the environmental Cu^{II} concentration: a) inhibition of activity by Cu^{II}; A6/E9Ida⁴ – RNase S′ 1 μM, poly-U 100 μM, Cu^{II} 5 equiv (5 μM) and EDTA 5 equiv (5 μM) for every arrow, pH 6.0, 10 mM MES buffer, containing 0.1 M NaCl, 25 °C; b) enhancement of activity by Cu^{II}; A6/E9Ida⁴ – RNase S′ 1 μM, poly-U 300 μM, Cu^{II} 1 equiv (1 μM) and EDTA 1 equiv (1 μM) for every arrow, pH 6.0, 10 mM MES buffer, containing 0.1 M NaCl, 35 °C.

series of double mutants. In sharp contrast to the single mutants, the initial rate of A6/E9Ida⁴ – RNase S′ increased at 1 equiv of Cu^{II} cation (120 %) and then decreased after the addition of more than 1 equiv of Cu^{II} (relative initial rate: 60 %), indicating that the cooperative binding of two Ida⁴ of the A6/E9-mutant directly influences the enzymatic reactivity. Interestingly, such a dual response, that is, enhancement and suppression dependent on copper concentration, was not observed for other double mutants: T3/A6Ida⁴ (50 % at 2:1, 25 % at 2:2), A5/E9Ida⁴ (85 % at 2:1, 38 % at 2:2), and T3/E9Ida⁴ – RNase S′ (75 % at 2:1, 15 % at 2:2); that is, their activity is only suppressed by Cu^{II} binding. These experiments apparently demonstrated that the sensitivity for Cu^{II} concentration is less than 1 μM for all mutants.[24] This activity modulation by Cu^{II} in both single and double mutants accords well with the above-mentioned structural data. Addition of other metal cations such as Ni^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} did not influence the initial rates of the reaction catalyzed with RNase S′ (data not shown).[25]

Using both kinds of the Cu^{II}-induced reactivity change, we attempted to switch the RNase activity in two different patterns, that is, Cu^{II}-induced suppression and Cu^{II}-induced enhancement. Figure 9 shows the switching of the activity of A6/E9Ida⁴ – RNase S′ by changing the environmental concentration of the Cu^{II} cation. The poly-U hydrolysis, which is catalyzed by RNase S′ in the absence of Cu^{II}, is quickly suppressed upon addition of more than 2 equiv of Cu^{II} cation (5 equiv Cu^{II} used in Figure 9a). Subsequently, when 5 equiv of EDTA (ethylenediamine tetracetic acid) was added to the solution to remove the bound Cu^{II}, the reaction was resumed. These processes can be repeated several times; this indicates that the reversible change between the active state and the less active state of A6/E9Ida⁴ – RNase S′ is carried out upon the binding or release of Cu^{II} ions. Following this, we used only a small amount of Cu^{II} (less than 1 equiv to Ida⁴) to conduct the reverse type of switching. When 1 equiv Cu^{II} cation was added to A6/E9Ida⁴ – RNase S′, the reaction was not suppressed but accelerated as shown in Figure 9b. Under these conditions, addition of 1 equiv of EDTA caused the suppression of the reaction. Repeated addition of 1 equiv of Cu^{II} reactivated the A6/E9Ida⁴ – RNase S′ once again. Clearly, this is a switching mode completely opposite to that in Figure 9a.[26]

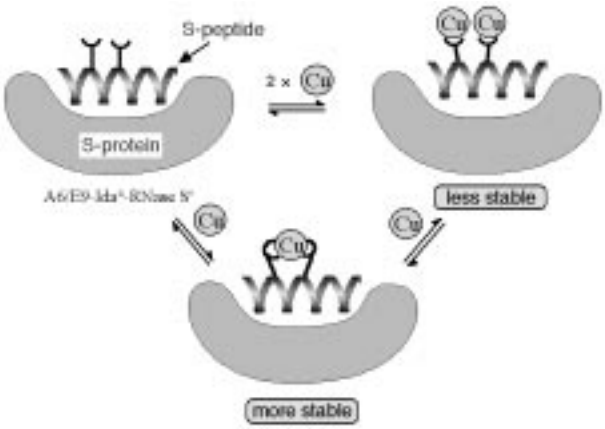
Discussion

In the study of the single mutants of Ida⁴ – RNase S′, it was clear that changing the free iminodiacetic acid to a Cu^{II}-complexed iminodiacetate induced destabilization (decrease of T_d) and deactivation (the decreased initial rate of poly-U hydrolysis). This is probably due to the bulkiness of the metal chelate and the introduction of positive charge upon Cu^{II} complexation. The responsive efficiency to the Cu^{II} ion was in the order of Glu9Ida⁴ > Ala6Ida⁴ > Thr3Ida⁴. This is reasonably explained by the following considerations: i) a chelation position close to the center of the α-helix is the most effective for structural perturbation of the S-peptide, and ii) since the order is also coincident with the distance (12 ⇔ 9 5.4 Å, 12 ⇔ 6 10.0 Å, 12 ⇔ 3 14.1 Å) between the incorporated Ida⁴ and the

0947-6539/99/0505-1508 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 5

enzymatic active site (His 12), a chelation position close to His 12 is the most influential.

With the double mutants, it has been demonstrated that Hopkins' strategy shown in a de novo designed peptide can be principally applied to an enzyme, that is, A6/E9Ida[4]–RNase S′ displays cooperative binding of a Cu^{II} ion with two iminodiacetic acids on the mutant enzyme. This transition metal binding at specific sites by the artificial chelator can cause the perturbation not only of the local α-helix structure, but also of the net structure and activity change in the holoenzyme (Scheme 3). A correct spatial arrangement of the



Scheme 3. Schematic illustration of the dependence of dual-mode modulation of A6/E9Ida[4]–RNase S′ on Cu^{II} concentration.

two receptors is essential for the dual response to Cu^{II} ions. However, we found several points of difference with respect to the Hopkins' peptide, owing to the structural specificity of the enzyme: i) the incorporation of Ida[4] at the i and $i+3$ positions does not always result in a cooperative binding site (for example, T3/A6-mutant), or rather, there are only a few positions (a so-called hot spot) which can cooperatively bind Cu^{II}; ii) we did not find cooperative binding at the i and $i+4$ position in our study (for example, A5/E9 or E9/M13-mutants); iii) the incorporation position of Ida[4] was also limited by the accommodation capacity of the holoprotein molecule (for example, Met13 or Asp14 mutants); iv) interestingly, metal-ion selectivity was found in the A6/E9Ida[4]–S-peptide and -RNase S′, that is, Cu^{II} is the most effective ion, whereas Zn^{II}, Co^{II}, and Ni^{II} showed almost no effect. Despite some limitations, the present study shows that a number of findings accumulated in the research on de novo designed peptides could also be applicable to native protein/enzyme engineering.

So far, there are only a few examples of synthetic metal binding sites which have been incorporated into native protein frameworks by site-directed mutagenesis.[5] Unnatural amino acid incorporation is expected to make these approaches more flexible. We have previously proposed, using myoglobin as a model template, that rational introduction of artificial receptor molecules into naturally occurring proteins and enzymes is one of the promising approaches for the design of environment-sensitive semisynthetic enzymes.[27] Generation of negative or positive charges upon binding with a corresponding guest close to an active site was a guiding principal in these examples. The present structure and activity switching, in contrast, is attributed to the fastening or unfastening of a specific α-helix domain by coordination chemistry, which is envisaged as a novel strategy for modulation of the enzyme activity.[28]

Experimental Section

Materials: Ribonuclease A (bovine pancreas) and subtilisin (Nagarse) were purchased from Sigma Chemical and used without further purification. The S-Protein of RNase (21 or 22–124 amino acid sequence of RNase A) was purchased or prepared by the subtilisin-catalyzed cleavage of RNase A followed by ion exchange and gel chromatography, according to the literature method.[29] Fmoc-Lys(Boc)-OH and other chemicals for peptide synthesis were purchased from Watanabe Chemicals (Hiroshima, Japan). HATU was purchased from PerSeptive Biosystems.

Synthesis of Fmoc-Ida[4](tBu)$_2$OH: Fmoc-Lys(Boc)-OH (10.0 g, 20.5 mmol), benzyl bromide (6.4 mL, 53 mmol), and anhydrous sodium bicarbonate (1.8 g, 20.5 mmol) were dissolved in dry DMF (dimethylformamide; 75 mL) and stirred at room temperature under a nitrogen atmosphere. DMF was removed under reduced pressure after 45 h. The residues were suspended in 75 mL of water and extracted with dichloromethane (1 × 125 mL, and then 3 × 50 mL). The organic layer was dried over anhydrous magnesium sulfate and concentrated. The crude residue was purified by silica-gel chromatography (4.5 diam. × 8 cm length, eluent: ethyl acetate/hexane = 1:5 → 5:2 gradient) to afford a colorless solid, Fmoc-Lys(Boc)-OBn (**2**; 8.4 g, 71% yield). IR (KBr): $\tilde{\nu}$ = 3320, 1730, 1690 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.3–1.9 (m, 15H), 3.10 (d, 2H), 4.10 (m, 1H), 4.39 (m, 3H), 4.58 (m, 1H), 5.26 (s, 2H), 5.45 (m, 1H), 7.30 (t, J = 7.1 Hz, 2H), 7.39 (t, J = 7.6 Hz, 2H), 7.49 (d, J = 8.3 Hz, 2H), 7.57 (d, 2H), 7.77 (d, J = 7.3 Hz, 2H), 8.19 (d, J = 8.4 Hz, 2H); m.p. = 87–89 °C.

The protected amino acid **2** (8.4 g, 14.6 mmol) was dissolved in dry dichloromethane (60 mL), and trifluoroacetic acid (TFA, 11.3 mL) was added to the solution. The reaction mixture was stirred at room temperature for 21 h before removal of the solvent under reduced pressure. The oily residue was dried in vacuo for several hours. Deprotection of the Boc group was confirmed by ^{1}H NMR ($CDCl_3$; i.e., disappearance of a singlet peak at δ = 1.3) and this crude product was used without further purification.

Crude Fmoc-Lys-OBn (**3**; 10.3 g, 16.3 mmol) thus obtained was dissolved in dry DMF (88 mL), and sodium bicarbonate (24.5 g, 0.28 mol), KI (3.7 g, 21.6 mmol), and *tert*-butyl bromoacetate (21.6 mL, 0.12 mol) were added to the solution. The mixture was stirred at room temperature for 84 h, and DMF was removed under reduced pressure. The residue was suspended in water (176 mL)and extracted with dichloromethane (90 mL and then 40 mL). The organic layer was dried over anhydrous magnesium sulfate and concentrated. The crude residue was purified by silica-gel chromatography (4.5 cm diam. × 7 cm length, eluent: ethyl acetate/hexane = 1:6–3:5 gradient) to afford a pale yellow oil, Fmoc-Ida[4](tBu)$_2$-OBn (**4**; 5.3 g, 52% yield for 2 steps). IR (KBr): $\tilde{\nu}$ = 3320, 1730, 1700 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.3–1.9 (m, 24H), 2.68 (m, 2H), 3.40 (s, 4H), 4.21 (t, 1H), 4.38 (m, 3H), 5.26 (s, 2H), 5.59 (d, 1H), 7.30 (t, J = 7.3 Hz, 2H), 7.39 (t, J = 7.2 Hz, 2H), 7.50 (d, J = 8.5 Hz, 2H), 7.60 (d, J = 7.2 Hz, 2H), 7.76 (d, J = 7.4 Hz, 2H), 8.19 (d, J = 8.5 Hz, 2H); anal. calcd for $C_{40}H_{50}N_2O_8$+ $2CH_3CO_2Et$: C 67.31, H 7.65, N 3.38; found: C 67.01, H 7.56, N 3.41%.

Fmoc-Ida[4](tBu)$_2$OBn (**4**; 1.0 g, 1.45 mmol) was dissolved in ethyl acetate (11 mL). Palladium charcoal (20 mg, Pd/C) was added to the solution. The suspended mixture was stirred under a hydrogen atmosphere at room temperature. After 11 h, Pd/C was filtered off with Celite and the filtrate was concentrated. The crude product was purified by silica-gel chromatography (4.5 cm diam. × 7 cm length, eluent: ethyl acetate/hexane = 3:5 → ethyl acetate only → ethyl acetate/ethanol = 5:1) to afford a colorless solid, Fmoc-Ida[4](tBu)$_2$-OH (**1**; 0.71 g, 72% yield). IR (KBr): $\tilde{\nu}$ = 3300, 1730, 1700, 1690 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.3–1.9 (m, 24H), 2.65 (m, 2H), 3.41 (s, 4H), 4.21 (m, 1H), 4.31 (m, 3H), 5.95 (m, 1H), 7.25–7.35 (m, 4H), 7.57 (d, 2H), 7.70 (d, 2H); m.p. = 52–54 °C; anal. calcd for

$C_{33}H_{44}N_2O_8+½\,CH_3CO_2Et$: C 65.61, H 7.50, N 4.50; found: C 65.61, H 7.55, N 4.37%.

Peptide synthesis: All S-peptide mutants were synthesized by peptide synthesizer (ABI, with NMP as a reaction solvent and DCC as a coupling reagent, or PerSeptive Pioneer, with DMF as a solvent and HATU as a coupling reagent) with solid-phase peptide synthesis based on Fmoc chemistry. The crude peptides were purified by reverse-phase HPLC (ODS column, eluent: water/acetonitrile gradient containing 0.1% of TFA, HITACHI-LC series). The purified peptides were characterized by MALDI time-of-flight (TOF) MS (α-cyano-4-hydroxycinnamic acid) as a matrix.

Preparation of RNase S′ S-protein:[18, 29] Subtilisin-catalyzed cleavage of RNase A and purification of the S-protein were conducted according to the literature method. We monitored the progress of the hydrolysis reaction by HPLC (ODS column, 4.6 mm diam., eluent: water/acetonitrile gradient containing 0.1% TFA, HITACHI-LC series). The S-protein was identified by MALDI-TOF mass spectroscopy.

CD and fluorescence measurements: CD spectra of the S-peptide mutants and S-peptide/S-protein complexes were measured by CD spectropolarimeter (JASCO J-720w). Fluorescence measurements of the RNase S′ mutants were conducted with a Hitachi fluorescence spectrophotometer F-4500.

Thermal denaturation: Thermal denaturation experiments were conducted by CD spectropolarimeter (JASCO J-720w spectropolarimeter, sensitivity: 10 mdeg; resolution: 0.2 nm; bandwidth: 2.0 nm; response: 0.5 s; scan speed: 200 nm min^{-1}) equipped with thermal controller; light-path length: 1 mm, temperature rose from 10 °C to 60 °C at 0.5 °C min^{-1}.

Activity assay:[30] Polyuridiric acid (poly-U) hydrolysis was catalyzed by RNase S′, according to the literature. The solution (MES buffer, pH 6.0, 0.1 M NaCl) containing S-protein (1 μmol) and S-peptide (1 μmol) was incubated at 25 °C for 5 min (in the case of Cu^{II} addition, an appropriate amount of $CuSO_4$ solution was mixed and incubated). The reaction was started by injection of poly-U solution (final concentration of 100 μmol) and the reaction was monitored by absorbance change at 278 nm (Hitachi U-3000 or JASCO UV/vis spectrophotometer, MES buffer, pH 6.0, 0.1 M NaCl). The initial rates were calculated with a differential molar extinction coefficient at 278 nm (1360 M^{-1} cm^{-1}).[28]

Acknowledgments

We thank Dr. Hisakazu Mihara and Seiji Sakamoto at Tokyo Institute of Technology for their helpful support and comments on peptide synthesis and TOF mass analysis. We also are grateful to Dr. Andrew Robertson (a JSPS foreign research fellow) for fruitful comments on this manuscript. This work is supported in part by a Grant-in-aid for the specially promoted area (Biotargeting, 10145245) from the Ministry of Education, Culture and Science of Japan and INAMORI Science Foundation.

[1] S. J. Lippard, J. M. Berg, in *Principles of Bioinorganic Chemistry*, University Science Books, Mill Valley, CA, **1994**.
[2] J. W. Bryson, S. F. Betz, H. S. Lu, D. J. Suich, H. X. Zhou, K. T. O'Neil, W. F. DeGrado, *Science* **1995**, *270*, 935.
[3] a) M. R. Ghadiri, C. Choi, *J. Am. Chem. Soc.* **1990**, *112*, 1630; b) M. R. Ghadiri, A. K. Fernholz, *J. Am. Chem. Soc.* **1990**, *112*, 9633; c) M. Lieberman, T. Sasaki, *J. Am. Chem. Soc.* **1991**, *113*, 1470; d) M. R. Ghadiri, C. Soares, C. Choi, *J. Am. Chem. Soc.* **1992**, *114*, 825; e) H. Mihara, N. Nishino, T. Fujimoto, *Chem. Lett.* **1992**, 1809; f) H. Mihara, N. Nishino, R. Hasegawa, T. Fujimoto, S. Usui, H. Ishida, K. Ohkubo, *Chem. Lett.* **1992**, 1813; g) B. Cuenoud, A. Schepartz, *Science* **1993**, *259*, 510; h) W. D. Kohn, C. M. Kay, B. D. Sykes, R. S. Hodges, *J. Am. Chem. Soc.* **1998**, *120*, 1124.
[4] F. Ruan, Y. Chen, P. B. Hopkins, *J. Am. Chem. Soc.* **1990**, *112*, 9403.
[5] a) R. Kuroki, Y. Taniyama, C. Seko, H. Nakamura, M. Kikuchi, M. Ikehara, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6903; b) J. N. Higaki, B. L. Haymore, S. Chen, R. J. Fletterick, C. S. Craik, *Biochemistry* **1990**, *29*, 8582; c) J. N. Higaki, R. J. Fletterick, C. S. Craik, *Trends Biochem. Sci.* **1992**, *17*, 100; d) D. J. Matthews, *Curr. Opin. Biotechnol.* **1995**, *6*, 419; e) V. A. Roberts, B. L. Iverson, S. A. Iverson, S. J. Benkovic, R. A. Lerner, E. D. Getzoff, J. A. Tainer, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 6654; f) B. L. Iverson, S. A. Iverson, V. A. Roberts, E. D. Getzoff, J. A. Tainer, S. J. Benkovic, R. A. Lerner, *Science* **1990**, *249*, 659; g) D. S. Gregory, A. C. R. Martin, J. C. Cheetham, A. R. Ress, *Protein Eng.*. **1993**, *6*, 29; h) W. S. Wada, J. S. Koh, N. Han, D. M. Hoekstra, R. A. Lerner, *J. Am. Chem. Soc.* **1993**, *115*, 4449; i) C. F. Barbas III, J. S. Rosenblum, R. A. Lerner, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6385; j) J. D. Stewart, V. A. Robberts, M. W. Crowder, E. D. Getzof, S. J. Benkovic, *J. Am. Chem. Soc.* **1994**, *116*, 415; k) A. L. Pinto, H. W. Hellinga, J. H. Caradonna, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 5562; l) C. D. Coldren, H. W. Hellinga, J. H. Caradonna, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 6635.
[6] R. T. Raines, *Chem. Rev.* **1998**, *98*, 1045 and references therein.
[7] a) B. Imperiali, R. S. Roy, *J. Am. Chem. Soc.* **1994**, *116*, 12083; b) R. S. Roy, B. Imperiali, *Protein Eng.*. **1997**, *10*, 691; c) I. Hamachi, T. Hiraoka, Y. Yamada, S. Shinkai, *Chem. Lett.* **1998**, 537.
[8] F. Ruan, Y. Chen, K. Itoh, T. Sasaki, P. B. Hopkins, *J. Org. Chem.* **1991**, *56*, 4347.
[9] H. W. Wyckoff, D. Tsernoglou, A. W. Hanson, J. R. Knox, B. Lee, F. M. Richards, *J. Biol. Chem.* **1970**, *245*, 305.
[10] a) E. E. Kim, R. Varadarajan, H. W. Wyckoff, F. M. Richards, *Biochemistry* **1992**, *31*, 12304; b) E. Scoffone, F. Rocchi, F. Marchiori, L. Moroder, A. Marzotto, A. M. Tamburro, *J. Am. Chem. Soc.* **1967**, *89*, 5450; c) F. M. Finn, K. Hoffman, *J. Am. Chem. Soc.* **1965**, *87*, 645.
[11] a) N. E. Zhou, C. M. Kay, R. S. Hodges, *J. Biol. Chem.* **1992**, *267*, 1992; b) R. Walgers, T. C. Lee, A. C. Goodwin, *J. Am. Chem. Soc.* **1998**, *120*, 5073.
[12] It was reported that the stability constant of iminodiacetic acid with the Cu^{II} cation is $10^{10.6}$ M^{-1}. A. E. Martell, R. M. Smith, in *Crystal Stability Constants, Vol. 1*, Plenum, New York, **1989**, p. 118.
[13] We confirmed that Ida^4-S-peptides do not show any change in the CD spectrum on addition of metal cations other than Cu^{II} under the same conditions.
[14] In contrast, Hopkins and co-workers reported that a synthetic peptide bearing two Ida^4 units at i and i+4 positions displayed cooperative binding to the Cu^{II} cation.
[15] a) U. W. Kenkare, F. M. Richard, *J. Biol. Chem.* **1966**, *241*, 3197; b) B. M. Dunn, C. Dibello, K. L. Kirk, L. A. Cohen, I. M. Chaiken, *J. Biol. Chem.* **1974**, *249*, 6295; c) M. Irie, K. Ohgi, M. Yoshinaga, T. Yanagida, Y. Okada, N. Teno, *J. Biochem.* **1986**, *100*, 1057.
[16] It has been reported that replacement of Met13 and Asp14 with hydrophilic residues markedly lowers the enzymatic activity. a) P. R. Connelly, R. Varadarajan, J. M. Sturtevant, F. M. Richards, *Biochemistry* **1990**, *29*, 6108; b) K. Hoffman, F. M. Finn, M. Limetti, J. Montibeller, G. Zanetti, *J. Am. Chem. Soc.* **1966**, *88*, 3633.
[17] All of the binding constants were calculated with a nonlinear curve fitting program.
[18] F. M. Richards, P. J. Vithayathil, *J. Biol. Chem.* **1959**, *234*, 1459.
[19] B. M. Dunn, C. Dibello, K. L. Kirk, L. A. Cohen, I. M. Chaiken, *J. Biol. Chem.* **1974**, *249*, 6295.
[20] Coordination structures of the copper(II) – Ida^4 complex were preliminarily investigated by EPR measurement. However, we did not obtain clear differences between the EPR signals for 1 equiv of Cu^{II} – A6/E9Ida^4 – RNase S′ and those of 2 equiv of Cu^{II} – A6/E9Ida^4 – RNase S′. Thus, the structures of Cu^{II} complexes are not clear so far.
[21] It may be interesting to point out that these decreases are sigmoidal (that is, 2 equiv of Cu^{II} is much more disruptive than 1 equiv of Cu^{II}).
[22] Tyr residues are located at positions 25, 73, 76, 92, 97 and 115 of the S-protein portion.
[23] We confirmed that the Cu^{II} cation does not show any RNase activity monitored by the hydrolysis reaction of poly-U under the present conditions.
[24] All measurements of the RNase activity assay were performed with 1 μM of RNase S′.
[25] We found that A6/E9Ida^4 – RNase S′ shows no activity change upon addition of metal cations (at least upon addition of 3 equiv) other than Cu^{II}.
[26] It was reported that the inhibition of native RNase S′ activity by Cu^{II} occurs at a high concentration of Cu^{II} rather than 10^{-4} M. a) T. Takahashi, M. Irie, T. Ukita, *J. Biochem.* **1967**, *61*, 669; b) T. D. Alger, *Biochemistry* **1970**, *9*, 3248. However, under the present condition

  0947-6539/99/0505-1510 $ 17.50+.50/0

(1 μM of Cu^{II} cation), Cu^{II} cation had no inhibition effect for RNase S′ (see the results of several control experiments with native RNase S′), clearly indicating that the present results with respect to the activity switching by Cu^{II} can be attributed to complexation of iminodiacetic acids with Cu^{II}.

[27] a) I. Hamachi, Y. Tajiri, S. Shinkai, *J. Am. Chem. Soc.* **1994**, *116*, 7437; b) I. Hamachi, Y. Tajiri, T. Nagase, S. Shinkai, *Chem. Eur. J.* **1997**, *3*, 1025; c) I. Hamachi, T. Nagase, Y. Tajiri, S. Shinkai, *Chem. Commun.* **1996**, 281; d) I. Hamachi, T. Nagase, Y. Tajiri, S. Shinkai, *Bioconjugate Chem.* **1997**, *8*, 862; e) I. Hamachi, T. Matsugi, K. Wakigawa, S. Shinkai, *Inorg. Chem.* **1998**, *37*, 1592.

[28] As other switchable enzyme systems, photoswitchable proteins have been reported; a) I. Willner, S. Rubin, *Angew. Chem.* **1996**, *108*, 419; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 367; b) I. Hamachi, S. Tanaka, S. Shinkai, *J. Am. Chem. Soc.* **1993**, *115*, 10458; c) I. Hamachi, S. Tanaka, S. Tsukiji, S. Shinkai, M. Shimizu, T. Nagamune, *Chem. Commun.* **1997**, 1735.

[29] M. S. Doscher, C. H. W. Hirs, *Biochemistry* **1967**, *6*, 304.

[30] S. B. delCardayre, R. T. Raines, *Biochemistry* **1994**, *33*, 6031.

Received: September 28, 1998 [F1365]