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Characterization of an artificial dimer of ribonuclease H using ^{1}H NMR spectroscopy

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Summary

The protein fusion technique was applied in the synthesis of an artificial dimer of ribonuclease H (305 residues). ^{1}H NMR spectroscopy was used to analyze the structure of this dimer. Spectral profiles and pK_a values of the histidine residues obtained using ^{1}H NMR indicate that the dimer retains the secondary and tertiary structures of the intact monomer. Selective spin-lattice relaxation measurements suggest that the two monomeric units in the dimer are in tight contact. Furthermore, the 2D ^{1}H NMR and paramagnetic relaxation filter results show that the two monomers bind together through interactions between the N- and C-terminal sites of the linked regions.

Introduction

Protein molecules with high molecular weight and high symmetry are favored in fabricating two-dimensional crystalline arrays, using the recently developed convective self-assembly (Nagayama, 1994). To meet these requirements, oligomeric protein molecules from ribonuclease H (RNase H) were created using genetic and chemical linking. The RNase H from *Escherichia coli* (155 amino acid residues, 17.6 kDa) is an enzyme with the specific function of cleaving the RNA moiety of an RNA–DNA hybrid duplex in the presence of Mg^{2+} (Crouch and Dirksen, 1982). The crystal and solution structures of this enzyme have been established using X-ray crystallography (Yang et al., 1990; Katayanagi et al., 1992) and NMR (Fujiwara et al., 1995), respectively, and the NMR resonances of individual protons have been completely assigned (Yamazaki et al., 1993). In an attempt to design oligomeric proteins, the artificial dimer of RNase H with 305 amino acid residues has been genetically formed by using a leucine amino acid as the connection between the C-terminus of one complete RNase H monomer and the N-terminus of one truncated RNase H, where six N-terminal residues had been deleted. Because it is interesting to understand how the monomeric units interact in the dimer, we pursued in this study the characterization of this dimer using ^{1}H NMR spectroscopy.

The applicable range of ^{1}H NMR spectroscopy in assigning individual resonances and in determining the tertiary structure of proteins is limited to molecules fairly smaller than 10 kDa (Wüthrich, 1986). If the assignments of proton resonances of the monomer have been previously completed, however, the quaternary structure of a multimer much larger than 10 kDa can be delineated by using ^{1}H NMR spectroscopy. Comparing ^{1}H NMR spectra and pK_a values for histidine residues of the dimer with those of the monomer, we examined the secondary and tertiary structures of the dimer. Selective spin-lattice relaxation measurements were used to determine whether the two monomeric units in the dimer are in tight or loose contact. We used a paramagnetic relaxation filter and 2D ^{1}H NMR spectroscopy to determine the binding regions in the artificial dimer. The results showed that the two monomers bind together through interactions between respective polypeptide regions of the connecting N- and C-termini of the dimer.

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Materials and Methods

All chemicals were reagent grade and used without further purification. Heavy water (D_2O) containing 99.9 atom % deuterium was purchased from Aldrich Chemical Company (Milwaukee, WI). Gadolinium acetate ($Gd(Ac)_3$) and diethelenetriamine pentaacetic acid (DTPA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka).

Sample preparation

The construction, expression, and purification of the artificial dimer of RNase H have been described elsewhere (Yamamoto et al., 1995). The construction of the plasmid was carried out by a polymerase chain reaction. To generate an artificial dimer of RNase H, the six residues from Met^1 to Glu^6 (i.e., Met^1, Leu^2, Lys^3, Gln^4, Val^5, and Glu^6) were deleted, then the N-terminus (i.e., Ile^7) was connected to a leucine amino acid, and finally the leucine was linked with the C-terminus of one complete RNase H monomer. We designate the entire monomer RNase H as A, and the truncated monomer with deletion of six residues as B. The paramagnetic reagent $[Gd(DTPA)]^{2-}$ was prepared by mixing equal molar quantities of $Gd(Ac)_3$ and DTPA in heavy water. The pH of the paramagnetic solution was adjusted to 5.5.

For the pH titration experiments, the protein was dissolved in heavy water with 100 mM deuterated sodium acetate at a protein concentration of about 3 mg/ml. The pH of the sample solution was adjusted by adding either DCl or NaOD. For the 2D experiments and relaxation measurements, the protein was dissolved in heavy water with 10 mM deuterated sodium acetate at pH 5.5. The protein concentrations were 14 and 7 mg/ml for the 2D experiments and relaxation measurements, respectively.

NMR methodology

The NMR experiments were done on a JEOL JNM-A500 NMR spectrometer at 500 MHz and 27 °C. The chemical shifts were measured relative to internal sodium 3-(trimethylsilyl)propionate (TSP). Proton chemical shifts are sensitive to protein folding (Wishart et al., 1991). They can be used to assess the structural difference between the dimer and monomer of RNase H. The pK_a values of the histidine residues were determined from the pH dependence of the chemical shifts of the $H^{\varepsilon 1}$ proton resonances in the pH range 4.1–10.7 at 27 °C. The data were analyzed by least-squares fitting. For macromolecules, the spin-lattice relaxation rates of protons suffer from spin diffusion (Sykes et al., 1978). Thus, only the initial recovery rate of the selective spin-lattice relaxation, R_1^S, can be used to evaluate the macromolecular dynamics (Boulat and Bodenhausen, 1993). We determined R_1^S by selective inversion-recovery, $180°(Q^3)$–t–$270°(G^1)$–Acq. We used a selective Q^3 Gaussian cascade (180°) (Emsley and Bodenhausen, 1992) to invert the longitudinal magnetization, and a selective Gaussian-shaped pulse (270°, G^1) (Emsley and Bodenhausen, 1989) to convert the partially recovered longitudinal magnetization to transverse magnetization. The durations of the Q^3 and G^1 pulses were 25 ms for the measurements of the histidine residues, and 5.5 ms for other residues.

HOHAHA and NOESY spectra were recorded in the pure-phase absorption mode. In the HOHAHA experiments, a mixing time of 30 ms was used, and in the NOESY experiments, mixing times of 50 and 75 ms were used for the dimer, and 100 and 150 ms for the monomer. The signal of residual water was suppressed by using preirradiation. The 2D spectra were acquired with 256 increments in the t_1 dimension and 1024 data points in the t_2 dimension. Before Fourier transformation, the time domain data were zero-filled to 512 points in the t_1 dimension, and multiplied by a phase-shifted sine-bell window function in both the t_1 and t_2 dimensions. The spectral widths were 7000 Hz for both dimensions.

Paramagnetic reagents can strongly relax the nuclear spins of molecules in their proximity (Jardetzky and Roberts, 1981). In the presence of paramagnetic reagents that cannot specifically bind at any sites on the protein surface, most signals from the protons that are located on the protein surface can be filtered out. In this way, by comparing the spectra of the dimer with those of the

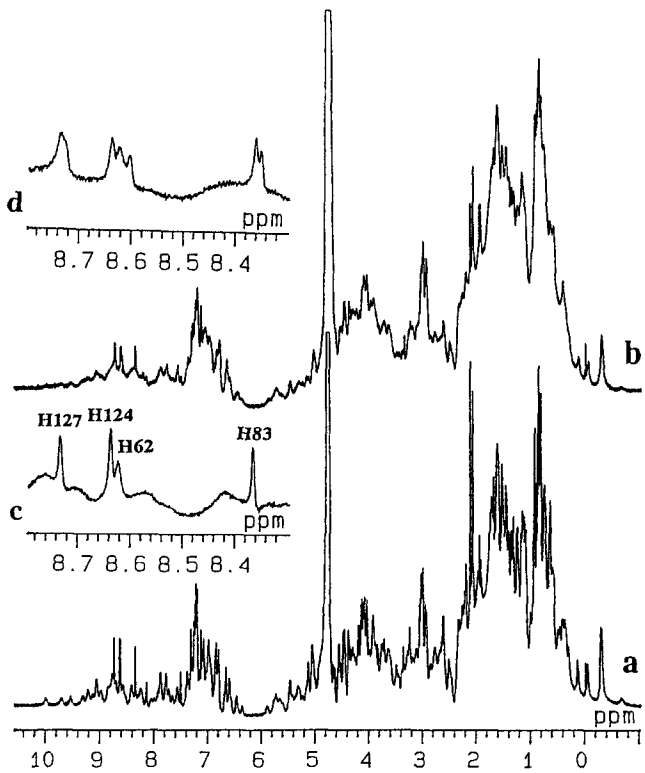

Fig. 1. ^1H NMR spectra of the monomer (a) and the dimer (b) of RNase H at 500 MHz, 27 °C, pH 5.5 in D_2O. (c) and (d) are the expanded spectra of Figs. a and b in the range 8.3–8.8 ppm, respectively.

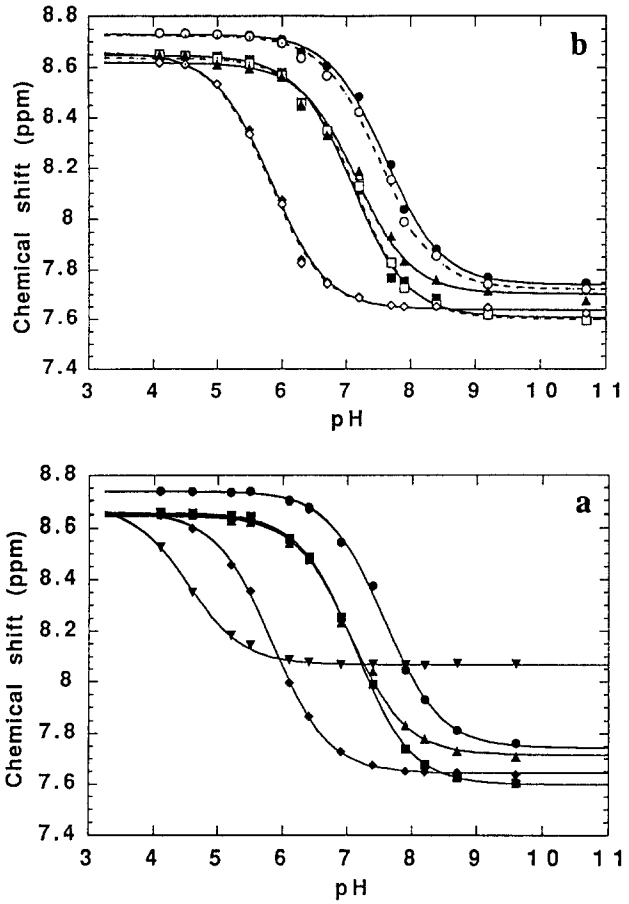

Fig. 2. The pH dependence of the chemical shifts of $H^{\varepsilon 1}$ protons at 27 °C. The fitted curves were analyzed by nonlinear square fits. (a) The monomer. The pH titration curves of all five histidine residues are shown: His^{127} (●), His^{124} (▲), His^{62} (■), His^{83} (◆), and His^{114} (▼). (b) The dimer. The pH titration curves of four pairs of residues are shown: His^{127} (● and ○), His^{124} (▲ and △), His^{62} (■ and □), and His^{83} (◆ and ◇). The fitted curves of each pair of histidines (in monomeric units A and B) are represented by solid and dashed lines, respectively.

monomer, we can find protons in the contact region of the dimer that are not accessible to the paramagnetic reagent. We recorded HOHAHA spectra for the dimer and monomer in the presence of $[Gd(DTPA)]^{2-}$.

Results

Figure 1 shows the 1D proton spectra of the monomer and dimer. For the dimer, the chemical shifts of the $H^{\varepsilon 1}$ protons for each pair of histidine residues in the two monomer units (A and B) are slightly different (Figs. 1c and d). Figure 2 shows the titration curves of all histidines, except His^{114} in the dimer, because this residue was located inside the protein and its $H^{\varepsilon 1}$ signal in the dimer was too broadened to be followed. The pK_a values are summarized in Table 1.

To know whether the two monomeric units of the dimer were in tight or loose contact, we measured the R_1^S values of some protons, i.e., of $H^{\delta 1}$ of Leu^{49} and $H^{\gamma 2}$ of

TABLE 1
pK_a VALUES OF HISTIDINE RESIDUES AS DETERMINED USING NMR TITRATION

Residue	pK_a value	
	Dimer[a]	Monomer
His^{62}	7.16, 7.14	7.0
His^{83}	5.80, 5.83	5.8
His^{114}		4.5
His^{124}	7.10, 7.13	7.1
His^{127}	7.62, 7.53	7.6

[a] Although only one digit after the decimal point is valid, the second digit is given to demonstrate the slight difference in the pK_a values for the two monomeric units.

Val^{98}, because the resonances of these protons are isolated from others. We also measured the R_1^S of the $H^{\varepsilon 1}$ protons of all the histidine residues. The signals that overlapped with $H^{\varepsilon 1}$ protons decayed during long pulses (25 ms duration). Thus, they did not interfere with the relaxation measurements. The values of R_1^S are listed in Table 2.

To find the binding region between the two monomeric units, we measured HOHAHA and NOESY spectra. We found several new cross peaks in the spectrum of the dimer in comparison with that of the monomer. The results are listed in Table 3. Figure 3 shows the HOHAHA spectra for the aromatic region. By comparing the NOESY spectrum of the dimer (mixing time 75 ms) with that of the monomer (mixing time 150 ms), we found four new cross peaks (i.e., 5.04 (ω_1)/7.08 (ω_2), 2.91/7.08, 4.40/7.06, and 3.10/7.06 ppm) in the spectrum of the dimer. These new cross peaks were still present when the mixing time was set to 50 ms, but the intensities decreased.

In the assignment of ^1H resonances, we assumed that only the chemical shifts of the protons located on the protein surface would change during the dimerization and that the change of the chemical shifts would not be very large. We can see that this assumption is reasonable (see below), because the dimer retains the secondary and tertiary structure of the intact monomer. Based on this assumption and the patterns of the new cross peaks (HOHAHA and NOESY), we assigned several scalar correlation cross peaks, as shown in Table 3. The cross peaks for the monomer (Yamazaki et al., 1993) are also shown in Table 3. From the crystal structure of the monomer (Katayanagi et al., 1992), we found that Asp^{94}, Thr^{149}, and Tyr^{151} were close to the C-terminus, and Tyr^{28} and

TABLE 2
INITIAL RELAXATION RATES OF PROTONS, R_1^S, MEASURED AT 27 °C USING THE SELECTIVE INVERSION-RECOVERY METHOD

R_1^S (s^{-1})	His^{62}	His^{83}	His^{114}	His^{124}	His^{127}	Leu^{49}	Val^{98}
Dimer	1.5	1.3	10.8	1.9	1.7	10.1	11.5
Monomer	0.8	0.6	5.2	1.00	0.8	5.8	6.6

TABLE 3
SCALAR CORRELATIONS OF RESIDUES THAT MAY BE INVOLVED IN THE BINDING REGION OF THE ARTIFICIAL DIMER OF RNase H

Residue	Correlation	Chemical shift (ω_1-ω_2 (ppm))		
		Dimer		Monomer
		A	B	
Tyr^{28}	H^{ε}-H^{δ}	6.86-7.26	6.80-7.08	6.86-7.26
Asp^{94}	$H^{\beta 1}/H^{\beta 2}$–H^{α}	2.68-4.62	2.66/2.80-4.64	2.64/2.80-4.64
Ile^{116}	H^{ε}-H^{δ}	0.61-1.28	0.60-1.25	0.61-1.28
Thr^{149}	H^{γ}-H^{β}	1.26-4.34	1.34-4.38	1.34-4.38
Tyr^{151}	H^{ε}-H^{δ}	6.64-7.06	6.60-7.00	6.60-7.00
Val^{155}	H^{β}-H^{α}	2.07-4.15	2.08-4.04	2.08-4.04
Val^{155}	H^{γ}-H^{β}	0.95-2.07	0.88-2.08	0.88/0.90-2.08

Ile^{116} were close to the N-terminus. For Asp^{94}, Thr^{149}, Tyr^{151}, and the C-terminal residue Val^{155}, we simply assigned the correlations with different chemical shifts compared to those of the monomer to the A monomer. Similarly, for Tyr^{28} and Ile^{116}, the correlations with different chemical shifts compared to those of the monomer were assigned to the B monomer. The new NOE cross peaks resulted from the interactions of intraresidue protons (i.e., H^{α}-H^{δ} and H^{β}-H^{δ} in Tyr^{28} (B) and Tyr^{151} (A)). When the pH of the solution was changed from 5.5 to 3.0, the new cross peak at 2.68/4.62 ppm, which was assigned to the scalar correlation H^{β}-H^{α} of Asp^{94}, shifted to 2.82/4.69 ppm, while the correlation $H^{\beta 1}/H^{\beta 2}$-H^{α} of Asp^{94} for the monomer shifted from 2.64/2.80-4.64 ppm (Yamazaki et al., 1993) to 2.71/2.85-4.70 ppm. This similar pH dependence of the chemical shifts provides another piece of evidence in confirming the assignment for the dimer.

After adding 0.2 mM of the paramagnetic reagent $[Gd(DTPA)]^{2-}$ to both the monomer and dimer solutions (pH 5.5), we measured HOHAHA spectra with a mixing time of 30 ms. Many cross peaks that were present in the absence of $[Gd(DTPA)]^{2-}$ disappeared in the presence of this paramagnetic reagent. By comparing the spectrum of the dimer with that of the monomer, we found that two additional new cross peaks, at 1.43/3.00 and 1.63/3.24 ppm, appeared in the dimer spectrum (Fig. 4). These cross peaks were overlapped with others in the absence of $[Gd(DTPA)]^{2-}$. The cross peak 1.43/3.00 ppm was assigned as the correlation of H^{δ}-H^{ε} of Lys^{91}, and the cross peak 1.63/3.24 ppm as that of H^{γ}-H^{δ} of Arg^{31}.

Discussion and Conclusions

The peptide of the artificial dimer consisting of 305 residues has thousands of possible different folding combinations. The 1D and 2D NMR spectra of the monomer and dimer exhibited almost the same patterns, showing that the peptide of the artificial dimer consisted of two monomeric units with secondary and tertiary structures identical to those of the monomer. The pK_a values of the histidines in the dimer were very close to those in the monomer, indicating that the local environments around the histidines were nearly unchanged in the dimer when compared with the monomer. The two monomeric units, however, seemed to arrange asymmetrically, because the $H^{\varepsilon 1}$ signals of each pair of histidines in the dimer appeared in doublets rather than in a singlet as seen in the monomer. This reflects small differences between the environments around the pair of histidines in the two monomeric units. The slightly different pK_a values of the histidines in the two units (Table 2) further emphasize this asymmetric arrangement. The slight differences in the pK_a values and chemical shifts may also result from the deletion of six residues in B.

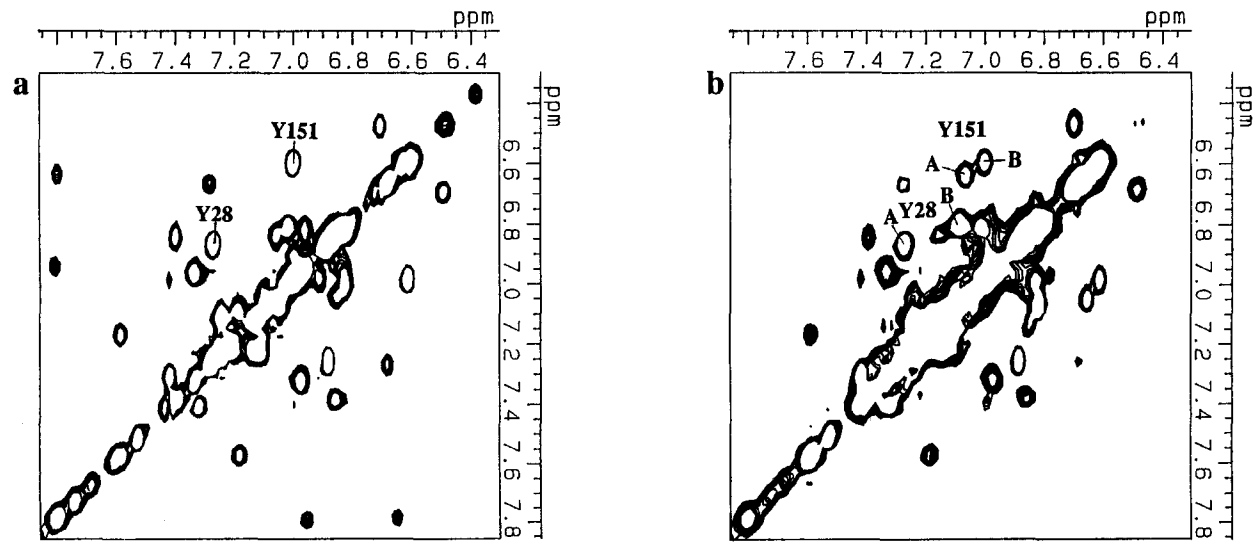

Fig. 3. Low-field regions of ^1H HOHAHA spectra of the monomer (a) and the dimer (b) of RNase H at 27 °C, pH 5.5 in D_2O. A and B in spectrum (b) indicate the monomeric units in the dimer.

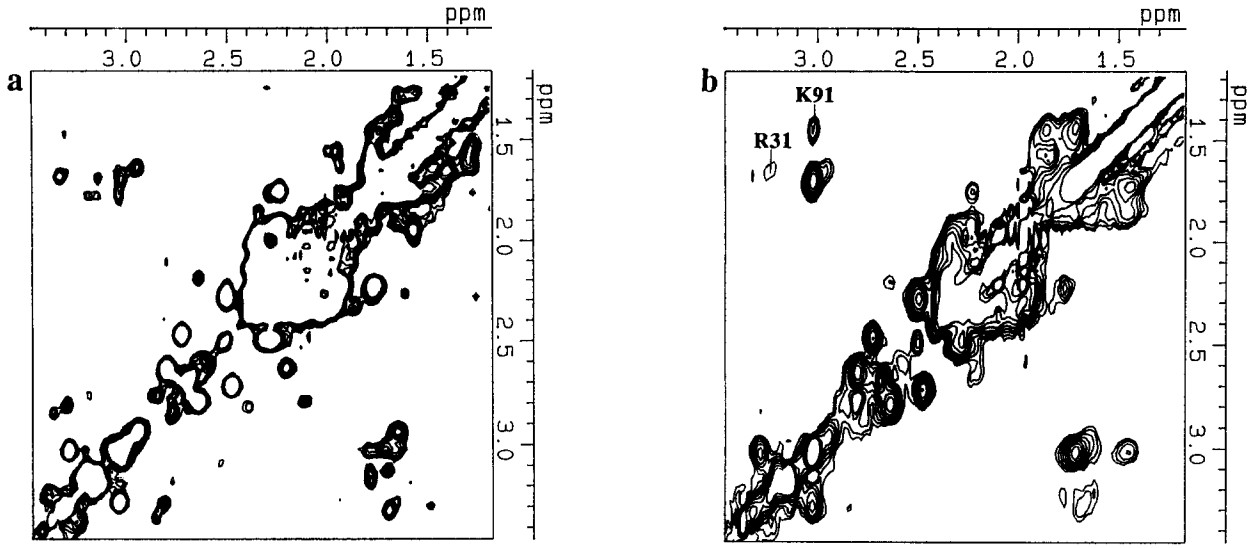

Fig. 4. ^{1}H HOHAHA spectra of the monomer (a) and the dimer (b) of RNase H in the presence of 0.2 mM $[Gd(DTPA)]^{2-}$ in D_2O.

From the crystal and NMR structures of RNase H, we found that the four C-terminal residues (i.e., Gln^{152}, Val^{153}, Glu^{154} and Val^{155}) are very flexible (they do not display any interactions with other residues). Thus, these four residues and the linking residue (i.e., leucine) can be considered as a spacer and we designate these five residues in the dimer as such. The spacer may be very flexible or rigid in the dimer. If A and B are very loosely linked (i.e., an unbound state, where there is no additional contact between A and B), then the monomer subunits can rotate as freely as the monomer, and thus the relaxation rates of the protons of the dimer would be close to those of the monomer. In contrast, if the units are in very tight contact (i.e., a bound state, where due to both the linking and the contact between the two units, no additional freedom is available), the dimer can be roughly considered as an axially symmetric ellipsoid with an axial ratio of 0.5. It has been found that the ratio of the spectral density function J(0) in an axially symmetric ellipsoid to that in a sphere of the same volume is in the range 1.1–1.5, depending on the orientation of the internuclear vector to the molecular axis (Woessner, 1962). If the internal motion of the side chains in the dimer and monomer is negligible, the initial relaxation rate of a proton, R_1^S, is proportional to the J(0) (Wang and Ikuta, 1989), because $\omega^2\tau^2 \gg 1$ for the rigid monomer and dimer, where ω is the proton Larmor frequency and τ is the correlation time. Therefore, the ratios of the proton relaxation rates of the rigid dimer to those of the rigid monomer should be in the range 2.2–3.0. In this study, the 1D spectra (Fig. 1) show that the resonances of the dimer were broadened significantly more than those of the monomer. Furthermore, the ratios were in the range 1.7–2.2 (see Table 2). These ratios are smaller than the theoretical prediction for a rigid dimer (2.2–3.0). This could arise from internal motion of side chains (Lipari and Szabo, 1982). Although we cannot exclude the possibility of partial freedom between the two units, the ratios are much larger than those for a loosely linked dimer (i.e., ~1.0). Therefore, we conclude that the two units in the dimer are in rather tight contact.

The entropy difference between the bound and unbound states of the dimer can be roughly estimated by the equation $\Delta S = -0.75\, \nu\, R \ln(n'+3)$, which is analogous to the entropy change induced by a cysteine bridge (Schulz and Schirmer, 1979), where ν is twice the number of bridges that connect chains, n' is the number of statistical segments between bridges, and R is the gas constant. If we assume that A and B, excluding the spacer, are two segments and each residue of the spacer of the dimer is one segment, then $n' = 7$, $\nu = 2$, and $\Delta S \sim -26$ J/mol K. The formation of some salt bridges ($\Delta H \sim -20$ kJ/mol for one salt bridge) and/or a few hydrogen bonds ($\Delta H \sim -12$ kJ/mol for one hydrogen bond) can compensate the entropy change from the unbound to the tightly bound state at 27 °C. Therefore, it is not completely unexpected that the dimer we studied was in tight contact.

If the two units in the dimer are in rather tight contact, then the chemical environment in the contacting region should be different from that in the intact monomer. Chemical shifts are sensitive to the chemical environment. Therefore, in this study, the new cross peaks seen in the dimer spectra should be due to the protons in or around the contacting region. From the HOHAHA and NOESY spectra, we could identify only five residues (i.e., Tyr^{28}, Asp^{94}, Ile^{116}, Thr^{149}, and Tyr^{151}) involved in the binding region. From the relaxation filter spectra when using $[Gd(DTPA)]^{2-}$, we found that Lys^{91} and Arg^{31} in the dimer were less accessible to $[Gd(DTPA)]^{2-}$ than the same residues in the monomer. Although the pair of histidine proton resonances in the dimer has slightly different chemical shifts (the largest difference is about 5 Hz (0.01

ppm) at pH 5.5), the chemical shift differences are much smaller than those of the residues Asp^{94}, Tyr^{28}, Ile^{116}, Thr^{149} and Tyr^{151} (Table 3). The pK_a and chemical shift of the $H^{\varepsilon 1}$ proton of histidine are very sensitive to the charges around the histidine. Even if the local structure around each pair of histidines is the same in the two units of the dimer, the charges around the histidine residues in unit A may differ from those in unit B due to the asymmetric arrangement of the two units and/or the deletion of six residues in B. If a histidine residue is involved in the contacting region, the initial relaxation rate of the $H^{\varepsilon 1}$ proton should be close to that of the residue His^{114}, which is located inside the protein. Thus, from relaxation rates of the histidine residues (Table 2), we can see that these residues are not involved in the contacting region. From the crystal structure of the monomer, we found that Asp^{94}, Lys^{91}, Thr^{149} and Tyr^{151} were close to the C-terminus, and Tyr^{28}, Ile^{116}, and Arg^{31} were close to the N-terminus. These results suggest that the two monomeric units interact through the N- and C-terminal regions of the linking sites.

In summary, the artificial dimer of RNase H consists of two monomeric units that retain the secondary and tertiary structures of the monomer of RNase H. These two units are in contact with each other. The contacting regions between the two units are around the N- and C-termini of the linked sites.

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