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Analysis of Internal Motions of RNase T1 Complexed with a Productive Substrate Involving ^{15}N NMR Relaxation Measurements

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The backbone dynamics of RNase T1 in the presence of *exo*-guanosine 2′,3′-cyclophosphorothioate (*exo*-cGPS isomer), which is a productive substrate, and in the presence of 3′-guanylic acid (3′GMP), which is an nonproductive substrate, were examined using ^{15}N nuclear magnetic resonance. Although the X-ray crystal structure suggests that the modes of binding of these substrates to the active-site cleft are very similar, the order parameters in a number of regions in RNase T1 complexed with *exo*-cGPS isomer were different from those with 3′GMP. Moreover, the chemical exchange in line width observed for RNase T1 complexed with *exo*-cGPS isomer was also different from that observed for RNase T1 complexed with 3′GMP. From these results, we concluded that the internal motions in RNase T1 complexed with a productive substrate were not always identical to those in RNase T1 complexed with a nonproductive substrate.

Key words: internal motion, NMR, productive substrate, RNase T1.

The recognition of a substrate by an enzyme is a critical component of function of a majority of biological processes. The "induced fit" model is widely accepted to explain the efficiency of an enzyme reaction for a substrate. Among the methods available for characterizing a protein conformational change in solution, NMR can provide detailed experimental information regarding the conformational change and the mobility of each amino acid residue in the protein. Analyses of the internal motions of a few enzymes, namely, 4-oxalocrotonate tautomerase (*1*), hen lysozyme (*2*), and human lysozyme (*3*) involving NMR suggested that the immobilization of some residues in enzymes caused by binding to a substrate analogue is compensated for by the mobilization of other residues. These observations indicate a new aspect of induced fit or the enzyme-substrate binding mode. It has been considered that such internal motions of proteins may be very important for their biological functions, as has been shown for enzyme catalysis and protein–ligand interactions. Although we initially hoped in these experiments to study an enzyme in a complex with a substrate, a nonproductive substrate or an enzyme inhibitor was actually used because a productive substrate was rapidly hydrolyzed by the enzyme during NMR relaxation experiments.

RNase T1, an extracellular enzyme consisting of 104 amino acids and originally isolated from *Aspergillus oryzae*, is a typical model system for the study of enzyme structure and function. It catalyzes the depolymerization of RNA through cleavage of the bond between phosphorus and the 5′-oxygen at the 3′ end of the guanine base. Eckstein *et al*. (*4*) have determined the stereochemistry of the catalytic mechanism of RNase T1 using guanosine 2′, 3′-cyclophosphorothioate (cGPS), a cyclic nucleotide in which one of the exocyclic oxygens on the phosphate is replaced by sulfur. Moreover, RNase T1 has been co-crystallized with *exo*-cGPS isomer, which is an analog of the natural cyclic substrate for the hydrolysis (*5*). It is hydrolyzed very slowly by RNase T1, guanosine 3′-thiophosphate (3′GPS) being produced, which in turn is rapidly desulfurized to 3′-guanylic acid (3′GMP) (*5, 6*). Thus, in this study, we analyzed the backbone dynamics of RNase T1 complexed with *exo*-cGPS isomer (productive substrate) by means of ^{15}N relaxation measurements and compared them with those of RNase T1 complexed with 3′GMP (nonproductive substrate). This report is the first discussion of the differences between the internal motions of an enzyme in the presence of a productive substrate and that of a nonproductive substrate.

MATERIALS AND METHODS

Sample Preparation—Expression and purification of ^{15}N-labeled RNase T1 were carried out as described previously (*7*). The culture supernatant was diluted with distilled water to decrease the salt concentration. The dilute solution was adsorbed on a column (1 × 50 cm) of Super Q Toyopearl (TOSOH, Tokyo), which had been pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The column was eluted with a gradient of 300 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, and then 300 ml of the same buffer containing 0.3 M NaCl. After the protein fraction obtained on ion-exchange chromatography had been diluted again with distilled water and the solution had been adsorbed on a column of resource

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Q (1×5.0 cm; Amersham Bioscience K.K.) in a HPLC, which was pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. The column was eluted with a gradient of 40 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, and then 40 ml of the same buffer containing 0.3 M NaCl at a flow rate of 1.0 ml/min. Finally, we obtained approximately 10 mg of ^{15}N-labeled RNase T1 from 1 liter of culture.

Guanosine 2′,3′-Cyclophosphorothioate Preparation—*Exo*-Guanosine 2′,3′-cyclophosphorothioate (cGPS) isomer was prepared according to a previous report (*5*).

NMR Measurements—An NMR sample comprising 0.3 mM protein in 90% H_2O/10% D_2O (v/v) was prepared, and the pH was adjusted to 5.5. For preparation of RNase T1 complexed with *exo*-cGPS isomer or 3′GMP, these compounds were added to the protein solution to give a slight excess of substrate relative to the enzyme, respectively. NMR experiments were performed at 10°C on a Varian Inova 600-MHz spectrometer equipped with a triple-resonance, pulse-field gradient probe with an actively shielded z gradient and a gradient amplifier unit. The 3D ^{15}N-edited NOESY-HSQC spectra (*8*) with a mixing time of 150 ms were collected with a time domain data size of 96 ($t1$) × 32 ($t2$) × 1,024 ($t3$) complex points. Pulse sequences for the measurement of ^{1}H-^{15}N NOE values, and the R1 and R2 relaxation times were given previously (*9*, *10*).

The ^{15}N R1 values were determined from a series of ^{1}H-^{15}N correlation spectra with different relaxation durations, namely, 32.94, 92.94, 172.94, 332.94, 652.94, 1,292.94, and 1,812.94 ms, while the R2 values were determined from spectra with durations of 4, 30, 58, 86, 130, 172, and 282 ms. A recycle delay of 4.0 s was used for R1 relaxation and one of 2.4 s for the R2 relaxation. The ^{1}H-^{15}N steady-state NOE values were determined from pairs of spectra, *i.e.*, ones recorded with and without proton saturation. A recycle delay of 4.0 s was used for each NOE experiment. The spectra widths of the $F1$ and $F2$ dimensions were 3,647 Hz and 9,611 Hz, respectively. Each collected data set comprised 256 ($t1$) × 1,152 ($t2$) complex data points.

Calculation of R1 and R2 Relaxation Parameters—R1 and R2 values were obtained by fitting the intensities of peak heights in each spectrum to a single exponential curve using a nonlinear least-squares fitting program, CURVEFIT, available from the website of Palmer's group. Errors in R1 and R2 were estimated by Monte Carlo analysis using CURVEFIT. ^{1}H-^{15}N steady-state NOE values were obtained by recording spectra with and without ^{1}H saturation for 3 s, and by calculating the ratios of the intensities of peak heights. Errors in NOE values were estimated using the root-mean-square of background noise (*10*).

Relaxation Data Analysis—The overall rotational correlation times for the backbone NH bond vectors of RNaseT1 complexed with 3′GMP or *exo*-cGPS isomer were calculated from R2/R1 ratios using a program, R2R1_TM, available from the website of Palmer's group. $S2$, τe, Rex, and S_f^2 were calculated using a program, Modelfree4.0 (*11*), available from the same site, by applying the isotropic diffusion model with the obtained overall rotational correlation time.

RESULTS AND DISCUSSION

Stability of Exo-*cGPS against RNaseT1 at 10°C*—The rate of hydrolysis of *exo*-cGPS isomer by RNase T1 was examined by HPLC and ^{1}H NMR spectroscopy at various temperatures. Namely, after the incubation of *exo*-cGPS with RNase T1 at millimolar concentrations for 20 h, which corresponded to the NMR measurement time, the levels of the *exo*-cGPS isomer and its product were measured by ion-exchange HPLC. The *exo*-cGPS isomer and its product on the ion-exchange HPLC were identified by ^{1}H NMR spectroscopy. As a result, the cleavage of *exo*-cGPS isomer amounted to only 1% at temperatures below 10°C (data not shown). These results suggested that the *exo*-cGPS isomer was sufficiently stable for relaxation measurements at 10°C.

^{1}H-^{15}N Chemical Shift Assignments—The backbone NH proton assignments of RNase T1 complexed with *exo*-cGPS isomer or 3′GMP were carried out by comparing cross-peak resonances in 3D NOESY-HSQC NMR spectra by referring to published assignments for RNase T1 (*12*). The changes in the chemical shifts of resonances between RNase T1 complexed with *exo*-cGPS isomer and its free state are shown in Fig 1. Residues Y38, H40, K41, Y42, N43, E46, F48, E58, R77, H92, and F100 were markedly affected by the *exo*-cGPS isomer. It has been reported that Y38, H40, E58, R77, H92, and F100 are located in the binding site of the substrate (*5, 6*). These findings suggested that the productive substrate, *exo*-cGPS isomer, had certainly bound to the active site of RNase T1. On the other hand, the chemical shift difference between RNase T1 complexed with *exo*-cGPS isomer and that complexed with 3′GMP was insignificant (data not shown). As shown in Fig 2, the X-ray crystallographic structures of RNase T1 complexed with *exo*-cGPS isomer and 3′GMP, respectively, indicated that the binding sites of these substrates are the same (*5, 6*), and that their binding modes are similar. It is therefore reasonable that the chemical shifts between RNaseT1 complexed with *exo*-cGPS isomer and that complexed with 3′GMP were insignificantly different. However, some residues could not be used for relaxation analyses due to signal broadening derived from substrate binding.

Comparison of Internal Motions between RNaseT1 Complexed with Exo-*cGPS Isomer and That Complexed with 3′GMP*—We measured the relaxation parameters of RNase T1 in the presence of *exo*-cGPS isomer or in that of 3′GMP according to our previous report (*7*). These parameters were obtained by fitting the peak heights of a series of spectra recorded with different delay times, T, to single exponential curves, as described in detail under "MATERIALS AND METHODS." The R1, R2, and ^{1}H-^{15}N NOE values are shown in Fig 3. The quality of the fitting for either R1 or R2 data was poor for some residues. Under these circumstances, the relaxation parameters could be obtained for 56 and 59 of 104 amide groups of RNase T1 complexed with *exo*-cGPS isomer and those of RNase T1 complexed with 3′GMP, respectively. The overall rotational correlation times, τm, of RNase T1 complexed with *exo*-cGPS isomer and those of RNase T1 complexed with 3′GMP were determined to be 9.305 ns and 9.2649 ns, respectively, from the R2/R1 values.

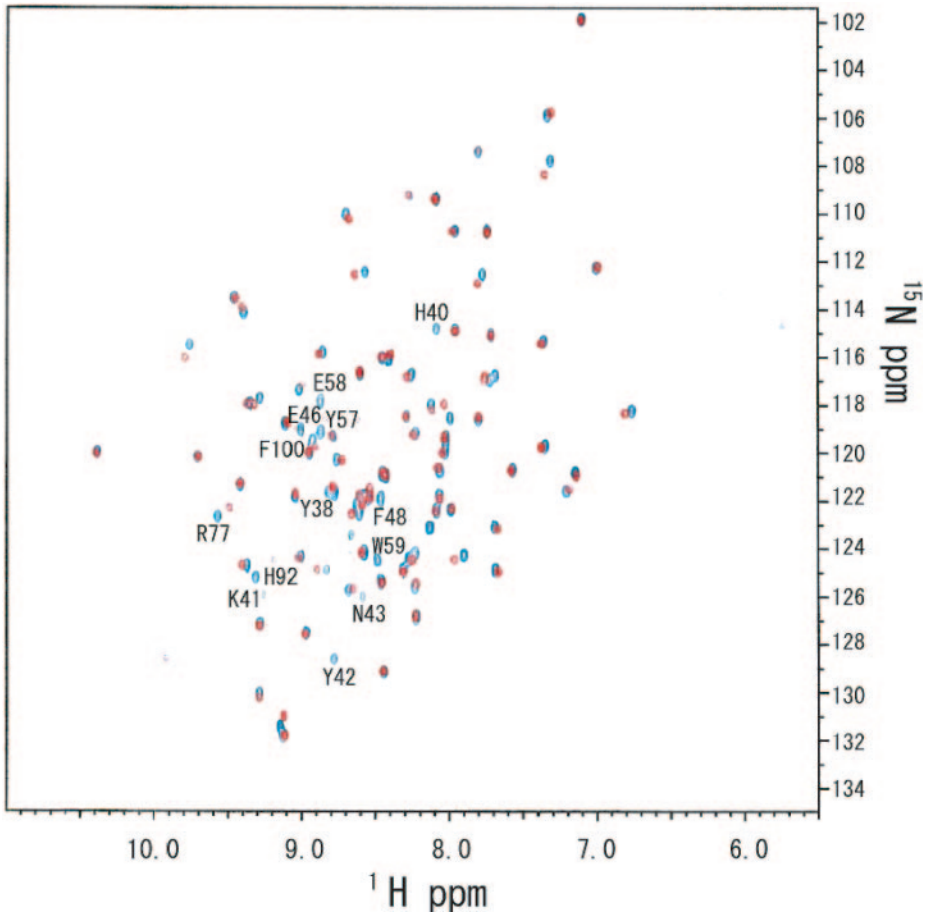

Fig. 1. **Superposing ^{1}H-^{15}N HSQC spectra of ^{15}N labeled RNase T1 in the free state and RNase T1 in the presence of exo-cGPS isomer.** The cross-peaks of RNase T1 in the free state are shown in blue, and those in the presence of *exo*-cGPS isomer are shown in red. The peaks markedly affected by complex formation are labeled.

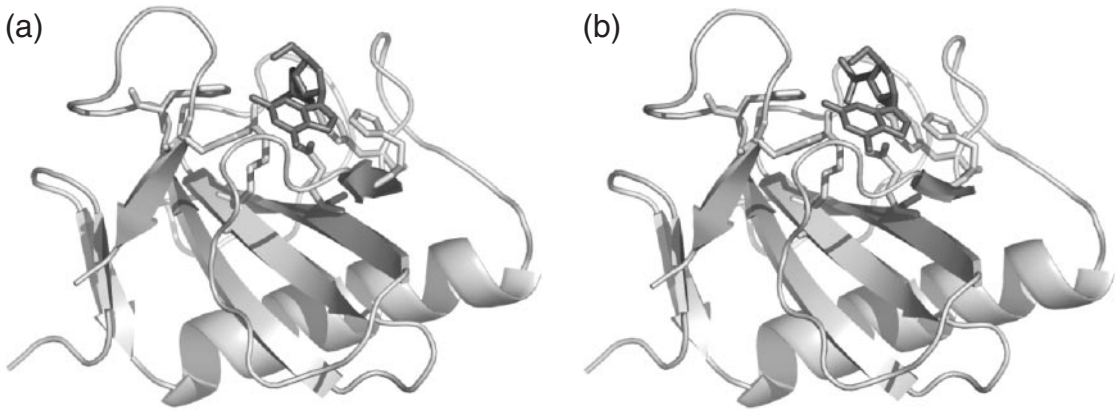

Fig. 2. **Crystal structure of RNase T1.** (a) RNase T1 complexed with *exo*-cGPS isomer, cited from Zegers *et al.* (1998) (*5*). (b) RNase T1 complexed with 3′GMP, cited from Zegers *et al.* (1994) (*6*). The substrate and amino acids in the active site are shown as a stick model.

The motional parameters were obtained from these relaxation data, *i.e.*, R1, R2, and ^{1}H-^{15}N NOE values, according to Lipari-Szabo model-free formalism of the spectral density function (*13, 14*). Model-free analysis, a widely accepted method for evaluating the internal motions of a molecule, includes a generalized order parameter such as a measure of the spatial restriction of ^{15}N-^{1}H bond vectors (S^2), which contributes to small amplitude motions on the pico-to-nanosecond time-scale. Namely, the increases in the order parameter (S^2) values imply restriction of the internal motions of a protein. In this analysis, we applied five types of model-free spectral density functions, as shown in Table 1, and optimized them by fitting the functions to the experimental data.

Most of the residues in RNase T1 complexed with *exo*-cGPS isomer or 3′GMP showed order parameters (S^2) in the range of 0.9–1.0, indicating that the internal motions of the enzyme in the complex were essentially restricted

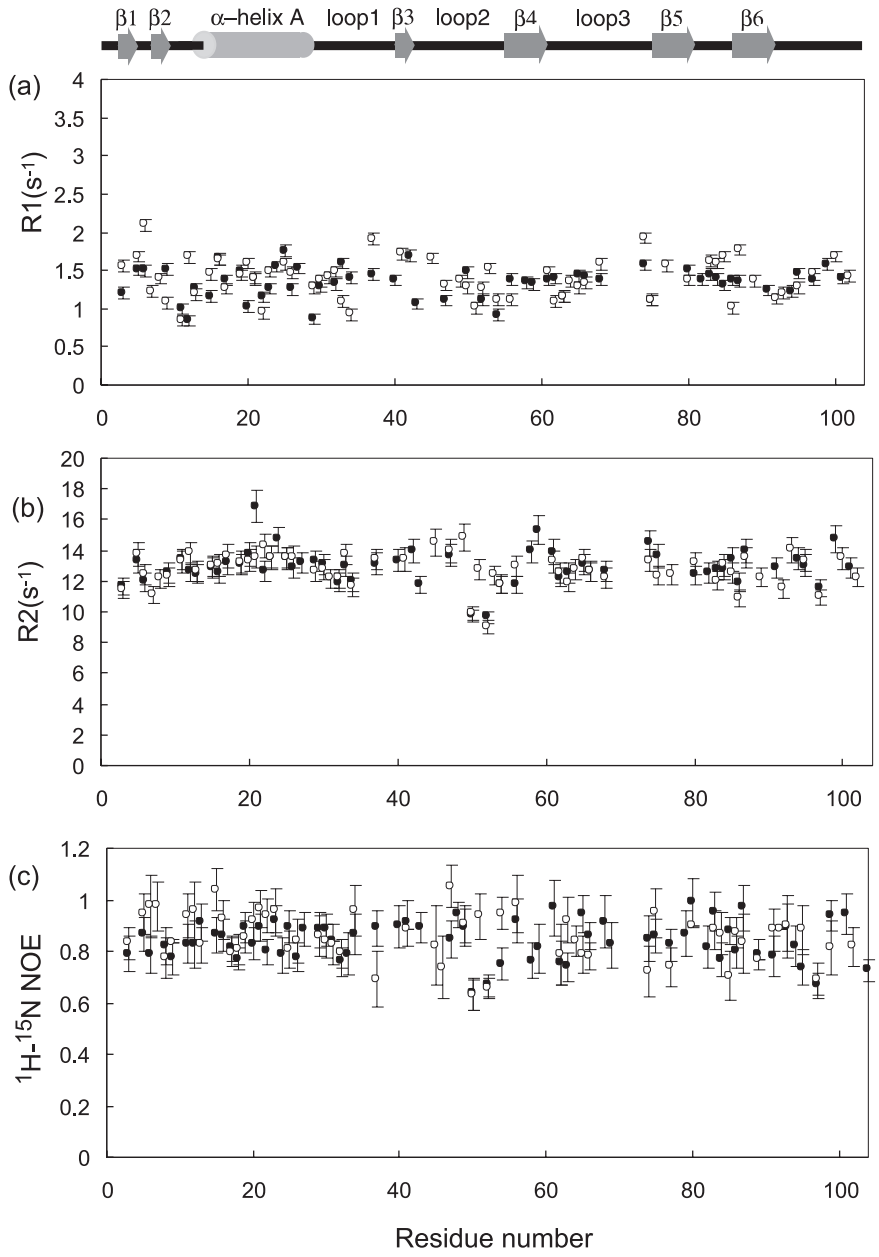

Fig. 3. **Measured relaxation parameters.** (a) ^{15}N R1, (b) R2, and (c) ^{1}H-^{15}N NOE. Values for RNaseT1 complexed with *exo*-cGPS isomer (open circles) or 3′GMP (closed circles).

(Fig 4a). Lower-order parameters (below 0.8) for RNase T1 complexed with *exo*-cGPS isomer were observed for T5, Y11, S12, A22, G34, S37, K41, G47, F50, S51, V52, S54, Y56, L62, G74, A75, Q85, L86, A87, and G97, and ones for RNase T1 complexed with 3′GMP were observed for Y11, S12, Q20, D29, G47, F50, V52, and S54 (Fig. 4a). Significant differences in the order parameters between the RNase T1 complexed with *exo*-cGPS isomer and that complexed with 3′GMP were observed in several regions. The low order parameters at a number for regions in the RNase T1 complexed with *exo*-cGPS isomer were more than those for that complexed with 3′GMP, reflecting increased motion in all of these regions except for a few on the pico-to-nanosecond time scales.

In order to obtain information regarding the dynamics for the two kinds of complexes in terms of their tertiary structures, we plotted the residues with lower order parameters observed only for RNase T1 complexed with *exo*-cGPS isomer in the structure of RNase T1 when we compared lower order parameters (below 0.8) between RNase T1 complexed with *exo*-cGPS isomer and that complexed with 3′GMP (Fig 5a). In particular, residues S37 and G74 were located around the active site and formed hydrogen bonds via H40 and R77, respectively, which are important for catalytic reactions (*15*), although some residues located at the active site could not be used for relaxation analysis due to signal broadening and overlapping with other peaks. Such internal motions might be linked to the slight difference in the mode of binding to the active-site cleft in RNase T1 between *exo*-cGPS isomer and 3′GMP. On the other hand, increased flexibility was observed for several residues not located at the active

site in RNase T1 complexed with *exo*-cGPS isomer (Fig. 5a). This finding was consistent with a previous one (*16*). These observations may be explained by the concomitant compromise of a decrease in the Gibbs free energy to stabilize the bound state through on increase in the entropic effect in a part of the protein, which is far away from the region interacting with the substrate analogue. Regardless, it was found that the internal motions on the pico-to-nanosecond time scale in RNase T1 complexed with *exo*-cGPS isomer were different from those in that complexed with 3′GMP, even in regions other than the active site in RNase T1. In Fig. 5b, the chemical exchange in line width (Rex) observed for RNase T1 complexed with *exo*-cGPS isomer (open circles) or complexed with 3′GMP (closed circles) is shown. Rex values express the motions on the micro-to-millisecond time scale, reflecting the catalytic action. The number of residues with Rex values for RNase T1 complexed with *exo*-cGPS isomer was significantly increased compared with that for RNase T1 complexed with 3′GMP.

Table 1. **Summary of the spectral density function models used for optimization of the model-free motional parameters.**

Model	Data used in fitting	Optimized parameters
1	*T*1, *T*2, NOE	S^2
2	*T*1, *T*2, NOE	S^2, *R*ex
3	*T*1, *T*2, NOE	S^2, *te*
4	*T*1, *T*2, NOE	Sf^2, Ss^2, *ts*
5	*T*1, *T*2, NOE	S^2, *te*, *R*ex

Most of the RNase T1 molecules formed a complex with both substrates under the measurement conditions based on previous reports (*4, 17*) regarding the affinity of RNase T1 with *exo*-cGPS or 3′GMP. Moreover, slow-exchanging peaks were observed in the ^{1}H-^{15}N HSQC spectra of RNase T1 complexed with *exo*-cGPS or 3′GMP (data not shown). These results indicate that our interpretation of Rex values for RNase T1 complexed with exo-cGPS isomer and 3′GMP may be reasonable.

The above results suggest that the internal motions on the micro-to-millisecond time scale in RNase T1 complexed with *exo*-cGPS isomer were different from those in that complexed with 3′GMP.

CONCLUSION

^{15}N relaxation measurements have allowed detailed characterization of the backbone dynamics of RNase T1 in the presence of *exo*-cGPS isomer and in that of 3′GMP. Our results indicate that the internal motions on the pico-to-nanosecond time scale of RNase T1 complexed with a productive substrate differed from those of complexed with a nonproductive substrate, even in regions other than the active site in RNase T1. Moreover, the internal motions on the micro-to-millisecond time scale of RNase T1 complexed productive substrate differed from that of that complexed with a nonproductive substrate.

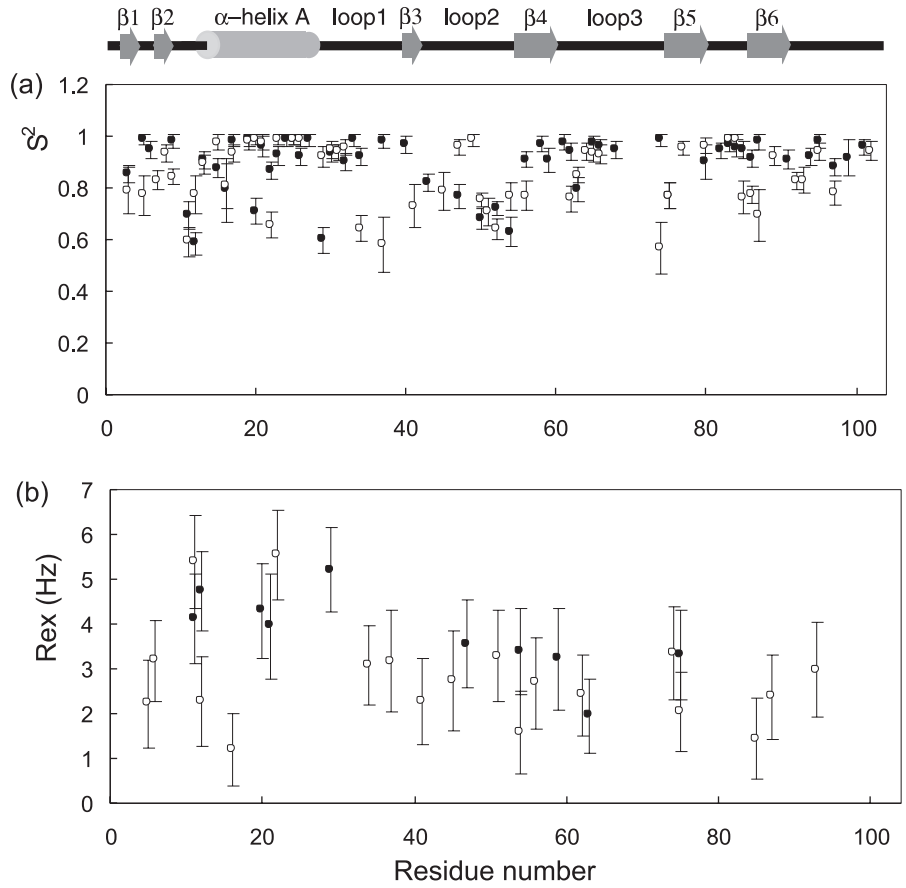

Fig. 4. **Comparison of the order parameters and Rex.** (a) Order parameters for RNaseT1 complexed with *exo*-cGPS isomer (open circles) or 3′GMP (closed circles). (b) Rex for RNaseT1 complexed with *exo*-cGPS isomer (open circles) or 3′GMP (closed circles).

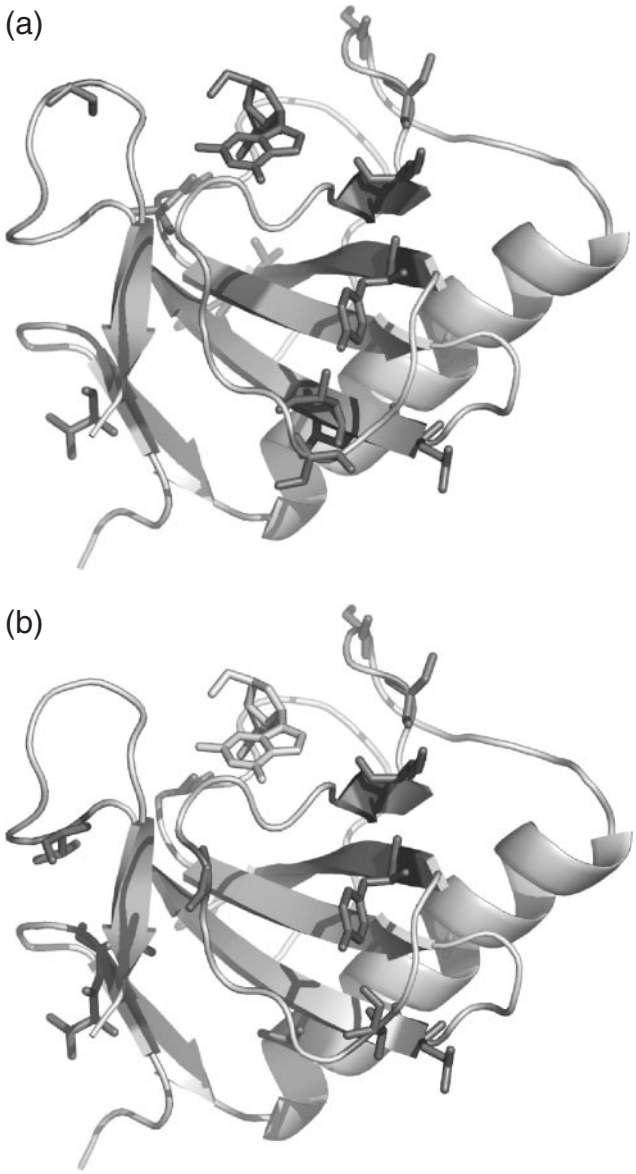

Fig. 5. **Crystal structure of RNase T1 complexed with *exo*-cGPS isomer, cited from Zegers *et al.*** (1998). For comparison of *R*ex values between RNase T1 complexed with *exo*-cGPS isomer and that complexed with 3′GMP, residues of lower order parameters (below 0.8) (a), and Rex values (b) observed only for RNase T1 complexed with *exo*-cGPS isomer are shown as a stick model.

From these results, we conclude that the results of analysis of the conformational dynamics of a enzyme using a non-productive substrate do not always reflect the dynamics during enzymatic catalysis. The present observations provide new insight into the internal motions of an enzyme in the productive substrate-binding state.

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