FEBS Letters 473 (2000) 106–112 FEBS 23633

Significant change in the structure of a ribozyme upon introduction of a phosphorothioate linkage at P9: NMR reveals a conformational fluctuation in the core region of a hammerhead ribozyme

Ken-ichi Suzumura[a], Masaki Warashina[b,c], Koichi Yoshinari[b,d], Yoshiyuki Tanaka[b], Tomoko Kuwabara[b,c], Masaya Orita[a], Kazunari Taira[b,e,*]

[a] *Yamanouchi Pharmaceutical Co. Ltd., 21 Miyukigaoka, Tsukuba Science City 305-8585, Japan*
[b] *National Institute for Advanced Interdisciplinary Research, Tsukuba Science City 305-8562, Japan*
[c] *Institute of Applied Biochemistry, University of Tsukuba, Tennoudai 1-1-1, Tsukuba Science City 305-8572, Japan*
[d] *National Institute of Bioscience and Human Technology, Tsukuba Science City 305-8566, Japan*
[e] *Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Tokyo 113-8656, Japan*

Received 10 March 2000; received in revised form 7 April 2000

Edited by Takashi Gojobori

Abstract A modified hammerhead ribozyme (R32S) with a phosphorothioate linkage between G_8 and A_9, a site that is considered to play a crucial role in catalysis, was examined by high-resolution ^1H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Signals due to imino protons that corresponded to stems were observed, but the anticipated signals due to imino protons adjacent to the phosphorothioate linkage were not detected and the ^{31}P signal due to the phosphorothioate linkage was also absent irrespective of the presence or absence of the substrate. ^{31}P NMR is known to reflect backbone mobility, and thus the absence of signals indicated that the introduction of sulfur at P9 had increased the mobility of the backbone near the phosphorothioate linkage. The addition of metal ions did not regenerate the signals that had disappeared, a result that implied that the structure of the core region of the hammerhead ribozyme had fluctuated even in the presence of metal ions. Furthermore, kinetic analysis suggested that most of the R32S–substrate complexes generated in the absence of Mg^{2+} ions were still in an inactive form and that Mg^{2+} ions induced a further conformational change that converted such complexes to an activated state. Finally, according to available NMR studies, signals due to the imino protons of the central core region that includes the P9 metal binding site were broadened or not observed, suggesting that this catalytically important region might be intrinsically flexible. Our present analysis revealed a significant change in the structure of the ribozyme upon the introduction of the single phosphorothioate linkage at P9 that is in general considered to be a conservative modification.

Key words: Hammerhead ribozyme; Nuclear magnetic resonance; Conformational change; Phosphorothioate; Kinetics

1. Introduction

The hammerhead motif is one of the motifs of self-cleaving RNA found in small RNA plant pathogens and it catalyzes the sequence-specific cleavage of RNA [1–4]. Divalent metal ions are fundamental participants in the catalytic cleavage of the phosphodiester bond in an RNA to a 2′,3′-cyclic phosphate by hammerhead ribozymes. Thus, these ribozymes are metalloenzymes [5–21]. However, under certain conditions (in the presence of 1–4 M monovalent cations, such as Li^+, Na^+, and NH_4^+), hammerhead ribozymes do not require divalent metal ions for catalytic activity [22]. Most studies of crystal structures of hammerheads have identified a metal-binding site between the *pro-R*p oxygen of the phosphate of A_9 (P9 oxygen) and the N7 atom of $G_{10.1}$ [23–25]. Even though this metal binding site (P9 site) within the crystal structure is located approximately 20 Å from the scissile phosphodiester bond, this metal binding site is thought to play a crucial role in achieving maximal cleavage activity for the following reasons. The substitution of the *pro-R*p-phosphoryl P9 oxygen by a sulfur results in a decrease in Mg^{2+}-dependent catalytic activity [26]. Furthermore, replacement of $G_{10.1}$ by a pyrimidine also results in a substantial decrease in the ribozyme's activity [27,28]. Moreover, Uhlenbeck's group and Herschlag's group reported that their kinetic studies of ribozymes with a phosphorothioate modification at the P9 phosphate indicated that an *R*p-phosphorothioate linkage reduced the cleavage rate by a factor of 10^3. However, the rate returned to the control value after the addition of a low concentration of Cd^{2+} ions, which are thiophilic [29]. Thus, it appears that the P9 oxygen is one of the metal binding sites that is required for efficient catalysis [18–21].

The kinetics of the reaction catalyzed by R32, a 32-mer hammerhead ribozyme (Fig. 1a), have been studied in considerable detail [16,30]. Although we intended initially to design this ribozyme such that it would not adopt any inactive conformations under physiological conditions, the existence of an inactive form of R32 (Fig. 1b) in the absence of Mg^{2+} ions was revealed by nuclear magnetic resonance (NMR) spectroscopy [31]. However, we were able to monitor the Mg^{2+} ion-induced changes in the structure of R32 that are favorable for recognition of the RNA substrate by NMR spectroscopy. No conformational change in R32 occurred unless Mg^{2+} ions were present [31]. Moreover, monovalent metal ions, such as Na^+ ions, were unable to substitute for Mg^{2+} ions. This observation implies that divalent metal ions play a direct role not only in the chemical cleavage of the phosphodiester bond but also in the formation of the correctly folded active conformation [31–34]. However, the metal binding site was not clearly revealed in the R32–substrate complex because signals

*Corresponding author. Fax: (81)-298-54 3019.
E-mail: taira@chembio.t.u-tokyo.ac.jp

PII: S0014-5793(00)01499-X

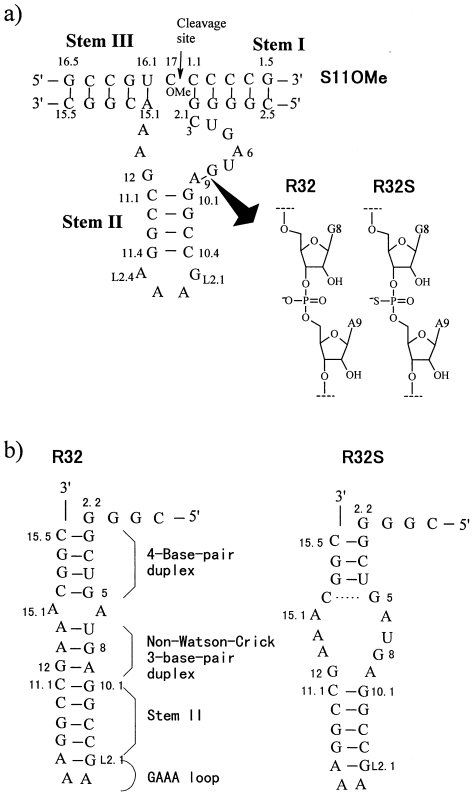

Fig. 1. a: Sequences and proposed secondary structures of the 32-mer ribozymes (R32 and R32S) and the uncleavable substrate (S11OMe). b: Proposed secondary structures of R32 and R32S in the absence of substrate in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.0).

due to imino protons in the central core region (U_7-G_8-A_9: G_{12}-A_{13}-A_{14}) were broadened.

In the present study, in order to characterize the P9 site, which appears to be important not only structurally but also catalytically [18,19], we examined the physicochemical properties of R32S (see Fig. 1a), which contains a phosphorothioate moiety between G_8 and A_9 (at position P9), by NMR spectroscopy. We hoped initially that this replacement of a phosphate by a phosphorothioate linkage would enable us to monitor directly, by NMR, the binding of a metal ion at the P9 phosphate. Since the Cd^{2+} ion is a thiophilic metal ion, we postulated that binding of Cd^{2+} to the sulfur atom would induce a change in the chemical shift of this specific thiophosphate at P9. Unfortunately, because of the broadening of the signal due to the P9 thiophosphate in R32S, we could not detect this ^{31}P signal at low temperatures. Nevertheless, our analysis revealed a significant change in the structure of the ribozyme upon the introduction of the single phosphorothioate linkage at P9. Moreover, this change enhanced the conformational fluctuation of the core region of the hammerhead ribozyme. It has been reported that the formation of ribozyme–substrate complex depends on the sequence of recognition arms. However, two ribozymes analyzed in this study had an identical sequence except for the introduction in one case of a phosphorothioate linkage at P9. Therefore, effects of a sulfur atom on the structure of a ribozyme could be investigated. Moreover, identification of the conformational changes that lead to the active hammerhead ribozyme–substrate complex should be important not only for our understanding of the mechanism of action of the wild-type hammerhead ribozyme but also for our understanding of that of maxizymes, which are capable of forming dimeric structures that are very active [35–39]. We demonstrate here the flexibility in the vicinity of P9 that might be important for catalysis [18,19].

2. Materials and methods

2.1. Preparation of RNA molecules, namely the modified ribozyme R32S and the uncleavable pseudosubstrate S11OMe

Synthesis and purification of the wild-type ribozyme R32, its substrate (S11) and uncleavable pseudosubstrate (SdC11) were described previously [31]. Crude samples of the modified ribozyme R32S and an uncleavable pseudosubstrate (S11OMe), in which the nucleophilic 2′-OH at the cleavage site (C_{17}) of the normal substrate (S11) was replaced by 2′-OMe, were purchased from Genset Corporation (France). Two kinds of pseudosubstrates (SdC11 and S11OMe) were used in this study, but the difference between SdC11 and S11OMe was not important in the present analyses. RNAs were purified by reversed-phase HPLC on a Capcellpak AG120 column (Shiseido, Tokyo, Japan), with elution with a linear gradient of CH_3CN (5–20%, v/v) in 0.1 M triethylammonium acetate buffer (pH 7.0). The preparations of purified oligonucleotides were desalted on a column of Sephadex G-25 (Fast Desalting column; Pharmacia Biotech Inc.). After evaporation of the solvent, all counterions were replaced with sodium by successive treatments on small columns of Dowex 50W-X2 (pyridine form), Dowex 50W-X2 (sodium form), and Chelex-100 (sodium form) resins. The appropriate fractions were collected and evaporated to dryness by lyophilization.

The presence and the position of the phosphorothioate linkage in the modified ribozyme (R32S) were confirmed by treating an aliquot of the solution of 5′-^{32}P-labeled R32S (after NMR spectra had been recorded; see below), at 37°C for 8 h, with an equal volume of methyl iodide. Enhanced cleavage of R32S specifically at P9 was confirmed by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel. Since we recently realized that the speed of migration of hydrolysis reaction products decreased in the following order: 2′ or 3′ acyclic (thio)phosphate > cyclic phosphate > cyclic thiophosphate [19], we also confirmed the proper introduction of the phosphorothioate linkage at the P9 site by analyzing the speed of migration of the hydrolysis reaction products of R32 and R32S. In order to facilitate the analysis, R32 and R32S were first cleaved by a DNA enzyme (5′-CGG CCT CAG GCT AGC TAC AAC GAC AGC CCC G-3′) designed [40,41] to cleave off the nine nucleotides from the 5′-side of R32 and R32S, to generate a 23-mer 3′-fragment (5′-$UG_{p(s)}$A GGC CGA AAG GCC GAA ACG GC-3′) that was subsequently hydrolyzed and the speed of migration of hydrolysis reaction products was analyzed (see Fig. 6 of Yoshinari and Taira [19] for more details). The slower migration of the hydrolyzed dinucleotide (cyclic thiophosphate 5′-UG_{ps}) originating from the 23-mer 3′-fragment of R32S confirmed unambiguously that a phosphorothioate linkage was introduced specifically at the P9 position (data not shown).

2.2. Kinetic measurements

Kinetic measurements were made basically as described previously [13,42]. The 5′-terminus of the substrate was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara, Japan). Reaction rates were measured in 25 mM $MgCl_2$ and 50 mM Tris–HCl (pH 8.0, adjusted at 5°C) under ribozyme-saturating (single-turnover) conditions (1 μM ribozyme and less than 2 nM substrate) at 5°C. After preincubation of the reaction mixture, reactions were initiated by adding a solution that contained either $MgCl_2$ or the cleavable substrate S11. Reactions were stopped by the removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equal volume of a solution of 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue. The substrate and 5′-cleaved product were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and the extent of cleavage was determined from the radioactivity of the substrate and product with a Bio-Image Analyzer (Storm830, Molecular Dynamics, USA).

2.3. NMR spectroscopy

Samples were prepared by dissolving purified oligomers in 0.12 ml of a solution, prepared in 90% H_2O and 10% D_2O, of 0.1 M NaCl and 10 mM phosphate buffer (pH 7.0). All spectra were recorded on a spectrometer (Alpha-500; Jeol, Tokyo) operated at 500 MHz for ^1H spectra and 202 MHz for ^{31}P spectra at 5°C. The chemical shifts of protons were determined relative to the signal from the internal standard, 2-methyl-2-propanol (1.23 ppm). The ^{31}P chemical shifts were referenced to an external trimethyl phosphate (10% solution in ethanol). To suppress signals due to water, a 1–1 solvent suppression sequence [43], incorporating pulsed-field gradients, was employed in the one-dimensional proton spectra. Each sample was heated to 90°C and then cooled slowly over the course of 30 min. Concentrated solutions of $MgCl_2$ and $CdCl_2$ were added directly to the sample tube. However, in the presence of metal ions, the oligomers were not heated to 90°C because Mg^{2+} ions at high concentrations can destroy RNA oligomers at high temperature.

3. Results

3.1. ^{31}P NMR studies

Fig. 2 shows the ^{31}P NMR spectra of the purified R32S sample at 5°C (a) and 60°C (b). The ^{31}P signals due to normal phosphodiester bonds around 0 ppm (expansion of −6 to −1 ppm at 5°C) were well defined but the signal derived from the phosphorothioate linkage, which usually gives ^{31}P signals at approximately 55 ppm [44–46], was absent from the ^{31}P NMR spectrum taken at 5°C (a). Since the chemical analysis of the R32S sample confirmed the existence of a phosphorothioate linkage (see Section 2) and since the similarly prepared S11S substrate that contained a phosphorothioate linkage at the cleavage site showed the expected ^{31}P signals around 52–55 ppm (a mixture of *R*p and *S*p isomers; data not shown), we postulated that this result might have been due to broadening of the signal because of medium-range exchanges in conformations, occurring on the NMR time scale, around the P9 phosphorothioate linkage of R32S. In order to confirm that the medium-range exchanges caused the disappearance of the phosphorothioate signal, the ^{31}P NMR was then recorded at a high temperature (Fig. 2b). In the ^{31}P NMR spectrum taken at 60°C, a signal due to the P9 phosphorothioate was detected at 52 ppm (Fig. 2b, arrow). The high temperature enhanced the hydrolysis of RNA (see the new signal at 17 ppm of the hydrolysis products, cyclic phosphates). Thus, at least in the case of this ribozyme, the sequence around A_9 appeared to gain flexibility upon the introduction of the phosphorothioate linkage. This possibility was supported by results of ^1H NMR experiments in which the signals due to imino protons adjacent to the phosphorothioate linkage, which had been observed in the NMR spectrum of the parental R32 ribozyme (Fig. 3b), disappeared upon introduction of the P9 phosphorothioate linkage (Fig. 3a).

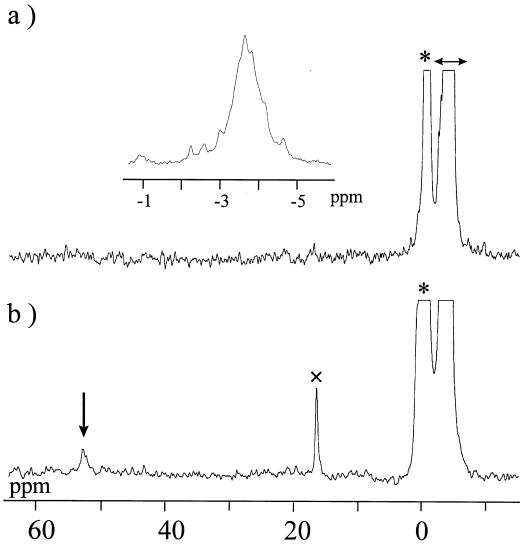

Fig. 2. ^{31}P NMR spectra of R32S in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.0). The spectra were acquired on a Jeol Alpha-500 MHz spectrometer using proton decoupling during the 0.33-s acquisition time and with a sweep width of 25 000 Hz, a 1.5-s recycle delay, and 150 000 scans, at 5°C (a) and at 60°C (b). Arrow: Signal due to phosphorothioate at P9; ×: signal due to hydrolysis products; *: signal due to phosphate buffer. See text for details.

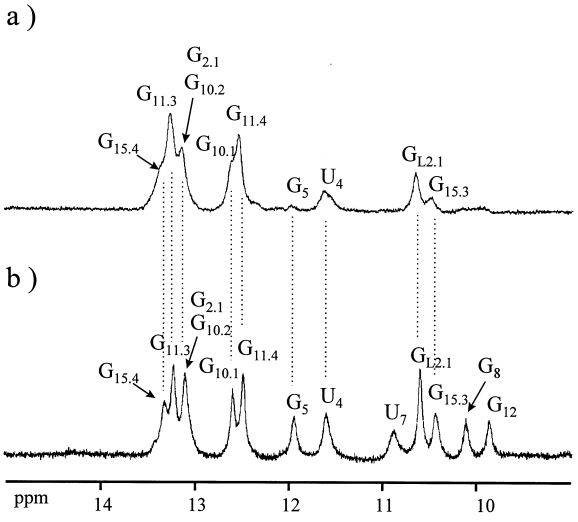

Fig. 3. Signals due to imino protons of the phosphorothioate-modified ribozyme R32S (a) and the unmodified ribozyme R32 (b) in the absence of pseudosubstrate and divalent metal ions. The spectra were recorded in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.0) at 5°C with the 1.17-s acquisition time and with a sweep width of 14 005 Hz, a 1.0-s recycle delay, and 35 000 scans. See text for further details.

No signal due to phosphorothioate was observed at 5°C after the addition of the substrate and metal ions, which are known to induce formation of a stable conformation in the case of the parental R32. Since we did not attempt to separate the diastereomeric *R*p- and *S*p-phosphorothioated forms of R32S, we had initially expected two ^{31}P NMR signals, with one originating from each isomer (as we observed in the ^{31}P NMR spectra of S11S substrate that contained a phosphorothioate linkage at the cleavage site; data not shown). However, as described above, such signals were not observed in the ^{31}P spectrum at low temperatures (at which RNA hydrolysis can be avoided; Fig. 2) and, thus, we could not monitor the binding of metal ions to the sulfur atom at P9 directly. Therefore, we decided to investigate conformational exchanges by monitoring imino protons, as follows.

3.2. Assignments of imino proton resonances of R32S

The imino proton resonances (Fig. 3a) of R32S, in the absence of the pseudosubstrate and divalent metal ions, were assigned by reference to the assignments of imino proton resonances of the parental R32 (Fig. 3b and Table 1 [31]). The previously determined secondary structure of R32, as deter-

mined from NOE studies performed in the absence of metal ions, is shown in Fig. 1b. The sequence from $C_{15.2}$ to $C_{15.5}$, corresponding to the recognition arm of stem III, forms base pairs with the sequence from G_5 to $G_{2.1}$ (4-bp duplex). The recognition arm of stem III is, thus, closed and R32 forms a large duplex, composed of stem II, a non-Watson–Crick 3-bp duplex and the 4-bp duplex. In the case of R32S, we observed signals due to the imino protons of the GAAA loop ($G_{L2.1}$, 10.66 ppm), stem II ($G_{11.4}$, 12.54 ppm; $G_{11.3}$, 13.27 ppm; $G_{10.2}$, 13.14 ppm; $G_{10.1}$, 12.62 ppm) and 4-bp duplex (G_5, 11.98 ppm; U_4, 11.62 ppm; $G_{15.3}$, 10.49 ppm; $G_{15.4}$, 13.36 ppm; $G_{2.1}$, 13.14 ppm). However, the signals due to the imino protons that corresponded to the non-Watson–Crick 3-bp duplex (U_7, G_8, G_{12}) were not detectable (or were only very weakly detectable) in the absence of the substrate and metal ions (Fig. 3a). In other words, no signals due to imino protons in the central core, which had been deduced to be the Mg^{2+} binding site, were observed even though the signals due to the duplex imino protons of both edges of the long helix were observed in the absence of the substrate and metal ions (see the proposed secondary structure of R32S in Fig. 1b). It seems likely that a medium-range exchange in conformations occurred, upon introduction of sulfur at the P9 position, in the central core region (U_7-G_8-A_9: G_{12}-A_{13}-A_{14}) that included the phosphorothioate linkage, and that this change was responsible for the disappearance of the signal. These results and the disappearance of the ^{31}P NMR signal that corresponded to the phosphorothioate linkage support each other.

3.3. Effects of addition of an uncleavable pseudosubstrate to a solution of R32S

Since the chemical shifts of signals due to imino protons reflect the arrangement of base pairs extremely well, they can provide information about interactions between a ribozyme and its substrate upon formation of a complex. In the case of R32, we observed signals due to imino protons of the non-Watson–Crick 3-bp duplex in the presence of a pseudosubstrate, in which the sugar of the residue on the 5′-side of the cleavage site in the natural substrate (S11) had been changed from ribose to deoxyribose [31], without metal ions. This result implies that no ribozyme–pseudosubstrate complex was formed in the absence of Mg^{2+} ions. Upon addition of Mg^{2+} ions, R32 was able to open its substrate recognition arm and form a complex with the uncleavable pseudosubstrate.

Table 1
Chemical shifts (ppm) of signals due to imino protons of R32 and R32S at 5°C in the absence of substrate and metal ions

Imino proton	R32 (chemical shift[a])	R32S (chemical shift[a])
Stem I		
$G_{2.1}$	13.10	13.14
Stem II		
$G_{10.1}$	12.60	12.62
$G_{10.2}$	13.10	13.14
$G_{11.3}$	13.22	13.27
$G_{11.4}$	12.49	12.54
Stem III		
$G_{15.4}$	13.32	13.36
$G_{15.3}$	10.45	10.49
GAAA loop		
$G_{L2.1}$	10.61	10.66
Others		
U_4	11.64	11.62
G_5	11.94	11.98
U_7	10.88	n.d.[b]
G_8	10.13	n.d.
G_{12}	9.88	n.d.

[a]Relative to 2-methyl-2-propanol (1.23 ppm).
[b]No signal detected.

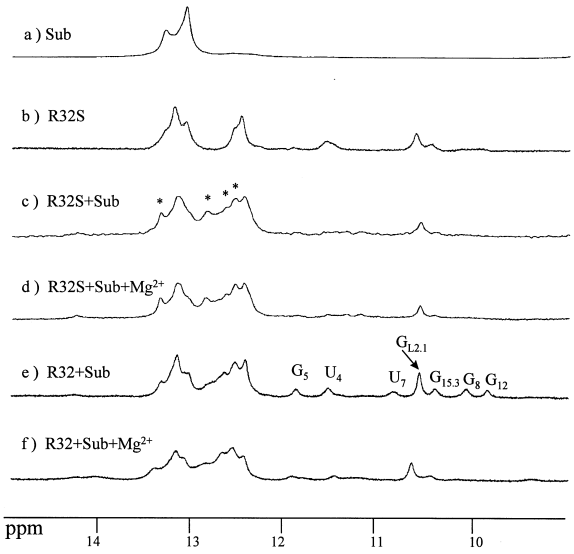

Fig. 4. Signals due to imino protons of R32S and R32. The spectra were recorded in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.0) at 5°C with the 1.17-s acquisition time and with a sweep width of 14 005 Hz, a 1.0-s recycle delay, and 35 000 scans. (a) Sub (S11OMe; a pseudosubstrate); (b) R32S; (c) R32S plus Sub (S11OMe); (d) R32S plus Sub (S11OMe) plus Mg^{2+} (1 mM); (e) R32 plus Sub (SdC11; a pseudosubstrate); (f) R32 plus Sub (SdC11) plus Mg^{2+} (5 mM). *: New signals (12.60, 12.71, 12.92 and 13.41 ppm)

In the case of R32S (Fig. 4b), the addition of the uncleavable pseudosubstrate (Fig. 4a) resulted in new signals due to imino protons at 12.60 ppm, 12.71 ppm, 12.92 ppm and 13.41 ppm, as indicated by asterisks in Fig. 4c, while signals due to imino protons of U_4 and G_5 disappeared, even in the absence of metal ions (Fig. 4c). It is noteworthy that the ^1H NMR spectrum of R32S plus the pseudosubstrate in the absence of metal ions (Fig. 4c) and the spectrum of R32 plus the pseudosubstrate, but only in the presence of Mg^{2+} ions (Fig. 4f), were almost identical. This correspondence indicates that R32S could form a ribozyme–substrate complex without divalent metal ions while R32 needed divalent metal ions for formation of the ribozyme–substrate complex. Apparently, replacement by a sulfur atom of the P9 oxygen significantly destabilized the stem structure of R32S as depicted in Fig. 1b and, as a result, the formation of a complex between R32S and its substrate became possible even in the absence of divalent metal ions.

The addition of Mg^{2+} ions to the solution of R32 plus the pseudosubstrate induced the disappearance of the signals due to imino protons (U_4, G_5, U_7, G_8, G_{12}; Fig. 4e,f), but the addition of $MgCl_2$ (1 mM) to R32S plus the pseudosubstrate did not result in any change in the ^1H NMR spectrum (Fig. 4d). Furthermore, even the addition of Cd^{2+} (150 μM) ions to the solution of R32S plus the pseudosubstrate did not affect the ^1H NMR spectrum (data not shown). All our data indicated that the conformation of the R32S–substrate complex, with or without Mg^{2+} ions, was close to that of parental R32–substrate complex in the presence of Mg^{2+} ions. No ^{31}P NMR

signals due to the phosphorothioate linkage of R32S were observed, even in the presence of the pseudosubstrate, irrespective of the presence or absence of metal ions, implying that a medium-range exchange in conformations on the NMR time scale must have occurred around the non-Watson–Crick 3-bp duplex. Accordingly, we can deduce that the central core of R32S does not form a stable conformation, even in a complex with a pseudosubstrate, regardless of the presence or absence of metal ions.

3.4. *Effects of the order of addition of substrate and Mg^{2+} ions to the reaction mixture on the rates of ribozyme-catalyzed reactions*

Our NMR studies suggested that R32S could form an R32S–substrate complex in the absence of Mg^{2+} ions while, by contrast, the formation of the parental R32–substrate complex requires Mg^{2+} ions [31]. In previous kinetic studies [31], it was apparent that the order of addition of substrate and Mg^{2+} ions to the reaction mixture affected the rate of R32-mediated cleavage at the relatively low temperature of 5°C (on ice). When reactions were initiated by the addition of Mg^{2+} ions to a preincubated solution of R32 and its substrate ('Mg^{2+} start'; open squares in Fig. 5), the rate of the reaction was lower than that of the reaction initiated by the addition of substrate to a preincubated solution of R32 and Mg^{2+} ions ('substrate start'; closed squares). These observations suggest that in the absence of Mg^{2+} ions, R32 was trapped, at least to some extent, in an inactive conformation without formation of a complex with the substrate.

R32S was able to form a complex with the pseudosubstrate in the absence of Mg^{2+} ions. Therefore, we wondered whether such a complex, in the absence of Mg^{2+} ions, might be an almost active complex that can cleave the bound substrate immediately upon capture of the necessary catalytic Mg^{2+} ions. If this were the case, we anticipated that, in contrast to the results for the parental R32–S11 system, the rate of the reaction initiated by the addition of Mg^{2+} ions to the R32S–S11 complex ('Mg^{2+} start'; open circles in Fig. 5) would be as high as (or even higher than) the rate of the reaction initiated by the addition of substrate to the R32S-Mg^{2+} complex ('substrate start'; closed circles). As shown in Fig. 5, the initial rate of the 'Mg^{2+} start' reaction catalyzed by R32S was much lower than that of the 'substrate start' reaction catalyzed by R32S and also than that of the reaction catalyzed by the parental R32. It should be noted that, in the case of R32S-catalyzed reactions, we monitored cleavage of the substrate by the diastereomeric *S*p-phosphorothioated R32S exclusively since the activity of the *R*p isomer is negligible under our present conditions [18,19,29]. This aspect of the study explains, at least in part, the fact that the activity of the R32S-catalyzed reactions was lower than that of the R32-catalyzed reactions. The lower rate of the 'Mg^{2+} start' reaction of R32S implies that the conformation of the R32S–S11 complex, which was examined in the NMR study, was still an inactive ground state and that, for formation of the transition-state structure of the R32S–S11 complex, further conformational changes that generated an activated ground state were needed. However, we cannot completely exclude the possibility that the lower concentrations of ribozyme and substrate used in the kinetic analysis, as compared to those in the NMR study, might have disrupted the putative formation of an active R32S–substrate complex in the absence of Mg^{2+} ions.

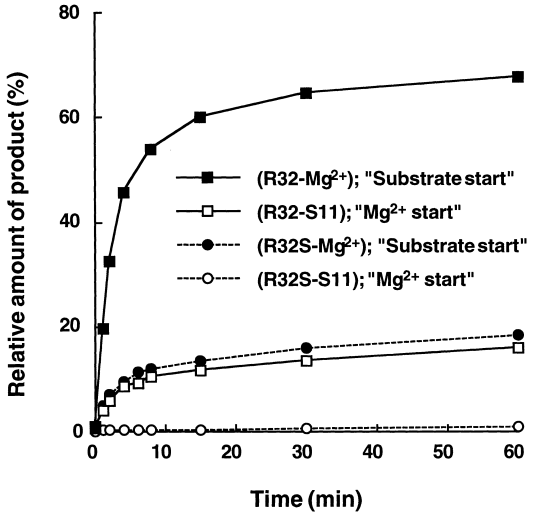

Fig. 5. Single-turnover reactions in the R32S/S11/Mg^{2+} and R32/S11/Mg^{2+} systems. Reactions were initiated by adding either the substrate S11 or Mg^{2+} ions. Solid squares, S11-initiated reaction mediated by R32 ('substrate start'); open squares, Mg^{2+}-initiated reaction mediated by R32 ('Mg^{2+} start'); solid circles, S11-initiated reaction mediated by R32S ('substrate start'); open circles, Mg^{2+}-initiated reaction mediated by R32S ('Mg^{2+} start'). Reactions were carried out with 1 μM ribozyme and less than 2 nM substrate, S11, at 5°C in the presence of 25 mM $MgCl_2$. Relative amounts of product were determined after electrophoresis of reaction mixtures.

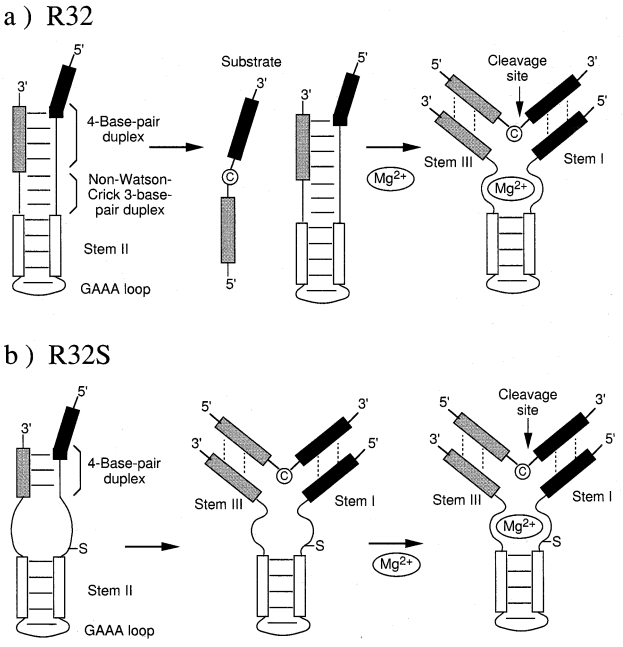

Fig. 6. Schematic representation of the conversion of inactive ribozymes to active forms. Formation of the active R32–substrate complex is possible only in the presence of Mg^{2+} ions. The R32S–substrate complex is formed only upon addition of substrate in the absence of Mg^{2+} ions. © represents the cleavage site in the substrate.

4. Discussion

To our knowledge, this is the first report of a study by ^1H

and ^{31}P NMR of the structure and reaction kinetics of a hammerhead ribozyme that include a phosphorothioate linkage at the P9 position. Fig. 6 shows a schematic representation of the secondary structure of R32S and of the proposed structural changes caused by the pseudosubstrate S11OMe and Mg^{2+} ions in R32S (Fig. 6b), as compared with the parental ribozyme R32 (Fig. 6a).

In the case of the unmodified ribozyme R32, we observed the signals due to imino protons of the GAAA loop, stem II, the non-Watson–Crick 3-bp duplex and the 4-bp duplex (Fig. 3b). The modified ribozyme R32S yielded imino proton resonances from the GAAA loop, stem II and the 4-bp duplex but no signals corresponding to imino protons in the non-Watson–Crick 3-bp duplex, even in the absence of substrate and divalent metal ions (Fig. 3a). Moreover, the signal due to the phosphorothioate linkage was absent in the ^{31}P NMR spectrum at 5°C (Fig. 2). This absence implies that a medium-range conformational exchange occurred in the core region (U_7-G_8-A_9: G_{12}-A_{13}-A_{14}) on the NMR time scale. The overall shape of R32 is presumed to be that of a long duplex composed of the GAAA loop, stem II, the non-Watson–Crick 3-bp duplex and the 4-bp duplex. By contrast, the global structure of R32S is composed of one loop (the GAAA loop), two stable stems (stem II and the 4-bp duplex) and a labile bulge (three non-Watson–Crick base pairs) that is sandwiched between the two stable stems (Fig. 6b).

Upon addition of the pseudosubstrate, the signals due to imino protons of U_4 and G_5 disappeared (Fig. 4c) and new base pairs formed in R32S in the absence of metal ions, implying that the recognition arms of R32S were opened, with resultant formation of stems I and III. By contrast, signals due to the imino protons of the 4-bp duplex of R32 remained apparent (Fig. 4e) upon the addition of the pseudosubstrate only, and R32 did not form a complex with the pseudosubstrate in the absence of divalent metal ions (Fig. 6a). Thus, the introduction at P9 of a single phosphorothioate linkage, which is generally considered to be a conservative change, increases the fluctuations of the backbone in R32S that destabilize a stem structure. As a result, R32S and the pseudosubstrate are able to form a complex more efficiently.

The addition of Mg^{2+} ions to a solution of R32 induces a change in the structure of the recognition arms and formation of the R32–substrate complex occurs (Fig. 4f). In combination with R32, Mg^{2+} ions not only have a catalytic role, but they also induce structural changes that are favorable for recognition of the substrate. In R32, the 4-bp duplex is stabilized by the adjacent non-Watson–Crick 3-bp duplex. Therefore, the substrate cannot split the 4-bp duplex of R32 and no ribozyme–substrate complex can form upon addition of the substrate only. Thus, in the case of the hammerhead ribozyme that has a stable base-pair duplex in the region of the uridine turn, the inactive monomeric hairpin structure must be disrupted for formation of the ribozyme–substrate complex. Mg^{2+} ions might affect the stability of the non-Watson–Crick 3-bp duplex and might induce a split in the 4-bp duplex. As a result, the Mg^{2+} ions support the formation of the ribozyme–substrate complex.

The recognition arms of R32S can be opened easily and R32S can form a complex with the substrate in the absence of metal ions. Addition of divalent metal ions did not change the NMR spectrum of the R32S–substrate complex (Fig. 4d), moreover, this spectrum was very similar to that of the R32–substrate complex formed in the presence of Mg^{2+} ions (Fig. 4f). Thus, the conformations of these two complexes appear to be very similar. In addition, the absence of signals due to imino protons of R32S and of ^{31}P signals from R32S, as well as the signals due to imino protons of R32 around A_9, suggests that this region is flexible and does not form a stable stem.

In previous NMR studies, signals due to the imino protons of this central core region were broadened or not observed [47–51]. These results and ours suggest that this catalytically important region might be intrinsically flexible. Moreover, a single replacement of the phosphate at P9 by phosphorothioate had a dramatic effect on structure. Although replacement of oxygen by sulfur is considered, in general, to be a very conservative modification, several cases are known in which such a substitution abolished catalytic function [52,53], directed cleavage to the next unmodified phosphodiester bond [54], or displaced essential catalytic metal ions from the active site as a consequence of the size of the bulky sulfur atom [55]. Accordingly, in our case, a single substitution of the phosphate at the catalytically important P9 position significantly altered the properties of a hammerhead ribozyme as depicted in Fig. 6. Our chemical probing data (Nakamatsu et al., unpublished) also support the conclusion that, in contrast to what one might expect from X-ray studies that indicate a pseudo-A-form helix at the P9 position [23–25], this region does not always form a stable helix in solution. The flexibility at P9 might be important for catalysis in hammerhead ribozyme-catalyzed reactions [18,19].

References

[1] Forster, A.C. and Symons, R.H. (1987) Cell 49, 211–220.
[2] Forster, A.C. and Symons, R.H. (1987) Cell 50, 9–16.
[3] Haseloff, J.P. and Gerlach, W.L. (1988) Nature 334, 585–591.
[4] Symons, R.H. (1989) Trends Biochem. Sci. 14, 445–450.
[5] Piccirilli, J.A., Vyle, J.S., Caruthers, M.H. and Cech, T.R. (1993) Nature 361, 85–88.
[6] Steitz, T.A. and Steiz, J.A. (1993) Proc. Natl. Acad. Sci. USA 90, 6498–6502.
[7] Pyle, A.M. (1993) Science 261, 709–714.
[8] Yarus, M. (1993) FASEB J. 7, 31–39.
[9] Dahm, S.C., Derrick, W.B. and Uhlenbech, O.C. (1993) Biochemistry 32, 13040–13045.
[10] Uebayashi, M., Uchimaru, T., Koguma, T., Sawata, S., Shimayama, T. and Taira, K. (1994) J. Org. Chem. 59, 7414–7420.
[11] Sawata, S., Komiyama, M. and Taira, K. (1995) J. Am. Chem. Soc. 117, 2357–2358.
[12] Pontius, B.W., Lott, W.B. and von Hippel, P.H. (1997) Proc. Natl. Acad. Sci. USA 94, 2290–2294.
[13] Zhou, D.-M., Zhang, L.-H. and Taira, K. (1997) Proc. Natl. Acad. Sci. USA 94, 14343–14348.
[14] Birikh, K.R., Heaton, P.A. and Eckstein, F. (1997) Eur. J. Biochem. 245, 1–16.
[15] Lott, W.B., Pontius, B.W. and von Hippel, P.H. (1998) Proc. Natl. Acad. Sci. USA 95, 542–547.
[16] Zhou, D.-M. and Taira, K. (1998) Chem. Rev. 98, 991–1026.
[17] Lilley, D.M.J. (1999) Curr. Opin. Struct. Biol. 9, 330–338.
[18] Wang, S., Karbstein, K., Peracchi, A., Beigelman, L. and Herschlag, D. (1999) Biochemistry 38, 14363–14378.
[19] Yoshinari, K. and Taira, K. (2000) Nucleic Acids Res. 28, 1730–1742.
[20] Nakamatsu, Y., Warashina, M., Kuwabara, T., Tanaka, Y., Yoshinari, K. and Taira, K. (2000) submitted.
[21] Warashina, M., Takagi, Y., Stec, W.J. and Taira, K. (2000) submitted.
[22] Murray, J.B., Seyhan, A.A., Walter, N.G., Burk, J.M. and Scott, W.G. (1998) Chem. Biol. 5, 587–595.

[23] Pley, H.W., Flaherty, K.M. and McKay, D.B. (1994) Nature 372, 68–74.
[24] Scott, W.G., Finch, J.T. and Klug, A. (1995) Cell 81, 991–1002.
[25] Murray, J.B., Terwey, D.P., Maloney, L., Karpeisky, A., Usman, N., Beigelman, L. and Scott, W.G. (1998) Cell 92, 665.
[26] Ruffner, D.E. and Uhlenbeck, O.C. (1990) Nucleic Acids Res. 18, 6025–6029.
[27] Ruffner, D.E., Stormo, G.D. and Uhlenbeck, O.C. (1990) Biochemistry 29, 10695–10702.
[28] Tuschl, T. and Eckstein, F. (1993) Proc. Natl. Acad. Sci. USA 90, 6991–6994.
[29] Peracci, A., Beigelman, L., Scott, E.C., Uhlenbeck, O.C. and Herschlag, D. (1997) J. Biol. Chem. 272, 26822–26826.
[30] Zhou, D.-M., Usman, N., Wincott, F.E., Matulic-Adamic, J., Orita, M., Zhang, L.-H., Komiyama, M., Kumar, P.K.R. and Taira, K. (1996) J. Am. Chem. Soc. 118, 5862–5866.
[31] Orita, M., Vinayak, R., Warashina, M., Chiba, A., Kaniwa, H., Nishikawa, F. and Nishikawa, S. (1996) J. Biol. Chem. 271, 9447–9454.
[32] Bassi, G.S., Mollegaard, N.E., Murchie, A.I.H., von Kitzing, E. and Lilley, D.M.J. (1995) Nature Struct. Biol. 2, 45–55.
[33] Ojha, R.P., Dhingra, M.M., Sarma, M.H., Myer, Y.P., Setlik, R.F., Shibata, M., Kazim, A.L., Ornstein, R.L., Rein, R., Turner, C.J. and Sarma, R.H. (1997) J. Biomol. Struct. Dyn. 15, 185–215.
[34] Hansen, M.R., Simorre, J.-P., Hanson, P., Mokler, V., Bellon, L., Beigelman, L. and Pardi, A. (1999) RNA 5, 1099–1104.
[35] Amontov, S.V. and Taira, K. (1996) J. Am. Chem. Soc. 118, 1624–1628.
[36] Kuwabara, T., Warashina, M., Orita, M., Koseki, S., Ohkawa, J. and Taira, K. (1998) Nature Biotechnol. 16, 961–965.
[37] Kuwabara, T., Warashina, M., Tanabe, T., Tani, K., Asano, S. and Taira, K. (1998) Mol. Cell 2, 617–627.
[38] Kuwabara, T., Warashina, M., Nakayama, A., Ohkawa, J. and Taira, K. (1999) Proc. Natl. Acad. Sci. USA 90, 6991–6994.
[39] Tanabe, T., Takata, I., Kuwabara, T., Warashina, M., Kawasaki, H., Tani, K., Ohta, S., Asano, S. and Taira, K. (2000) Biomacromolecules 1, 108–117.
[40] Santoro, S.W. and Joyce, G.F. (1997) Proc. Natl. Acad. Sci. USA 94, 4262–4266.
[41] Warashina, M., Kuwabara, T., Nakamatsu, Y. and Taira, K. (1999) Chem. Biol. 6, 237–250.
[42] Shimayama, T., Nishikawa, F., Nishikawa, S. and Taira, K. (1993) Nucleic Acids Res. 21, 2605–2611.
[43] Sklenar, V. and Bax, A. (1987) J. Magn. Reson. 74, 469–479.
[44] Stec, W.J., Zon, G., Egan, W. and Stec, B. (1984) J. Am. Chem. Soc. 106, 6077–6079.
[45] LaPlanche, L.A., James, T.L., Powell, C., Wilson, W.D., Uznanski, B., Stec, W.J., Summers, M.F. and Zon, G. (1986) Nucleic Acids Res. 14, 9081–9093.
[46] Jaroszewski, J.W., Clausen, V., Cohen, J.S. and Dahl, O. (1996) Nucleic Acids Res. 24, 829–834.
[47] Heus, H.A. and Pardi, A. (1991) J. Mol. Biol. 217, 113–124.
[48] Heus, H.A., Uhlenbeck, O.C. and Pardi, A. (1990) Nucleic Acids Res. 18, 1103–1108.
[49] Odai, O., Kodama, H., Hiroaki, H., Sakata, T., Tanaka, T. and Uesugi, S. (1990) Nucleic Acids Res. 18, 5955–5960.
[50] Pease, A.C. and Wemmer, D.E. (1990) Biochemistry 29, 9039–9046.
[51] Simorre, J.-P., Legault, P., Hangar, A.B., Michiels, P. and Pardi, A. (1997) Biochemistry 36, 518–525.
[52] Warnecke, J.M., Fürste, J.P., Hardt, W.-D., Erdmann, V.A. and Hartmann, R.K. (1996) Proc. Natl. Acad. Sci. USA 93, 8924–8928.
[53] Warnecke, J.M., Green, C.J. and Hartmann, R.K. (1997) Nucleosides Nucleotides 16, 721–725.
[54] Warnecke, J.M., Sontheimer, E.J., Piccirilli, J.A. and Hartmann, R.K. (2000) Nucleic Acids Res. 28, 720–727.
[55] Brautigam, C.A. and Steitz, T.A. (1998) J. Mol. Biol. 277, 363–377.