

The development of intelligent ribozyme and RNA aptamer

whose activities switch on in response to K^+

via quadruplex formation

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Chapter 1

General introduction

1-1. Significance of Constructing Biopolymer Systems

The coming energy shortage is one of the most serious crises for the modern world. In recent years, the depletion of fossil fuel resources such as oil and gas is being serious and the new technologies for utilizing resources in the efficient manners are required. Nature has the exceptionally efficient biochemical reaction systems controlling by biopolymers. These systems work under mild condition such as at neutral pH, ordinary temperatures, ambient pressure and in aqueous solution. Therefore, efficient and low environmental impact systems can be constructed by utilizing such biopolymers. To utilize biopolymers, we need to understand the mechanisms how to control their functions and to construct artificial functional biopolymers.

1-2. Functional RNAs

RNAs tend to be recognized as functional molecules which catalyze biochemical reactions, recognize target molecules and control the gene expression. In 1981, as a new class of RNA, ribozyme which exerts enzymatic activity was discovered by Cech *et al.*^{1,2} In 1990, Szostak group and Gold group independently reported the technology known as Systematic Evolution of Ligands by EXponential enrichment (SELEX) for the selection of RNA aptamers which bind to the target molecule strongly and specifically.^{3,4} Moreover, recent researches reported many class of naturally occurring or artificial functional RNAs which regulate gene expressions as short-interfering RNAs,^{5,6} micro RNAs^{7,8} and riboswitches.⁹⁻¹² These functional RNAs relate to controlling the gene expression and also higher order life phenomena such as development and differentiation. Furthermore, the disorder of these functional RNAs may lead diseases. To understand and utilize the mechanism of structure and function of these RNAs leads the development of efficient molecular tools and new drugs.

The structure of the biopolymers such as functional RNAs and proteins play a significant role in their functions. When functional RNA and protein fold into a unique structure, they exert activities such as catalytic activity and specific molecular recognition. On the other hand, when they lose their unique structure, they rarely work. The structural information is useful and has been utilized to design functional biopolymers. Moreover to regulate a structural transition of a biopolymer in response to environmental change enables us to switch its function artificially.

1-3. Strategy of Construction of K⁺-responsive Functional RNAs

It is well known that guanosine (G)-rich DNAs and RNAs form a noncanonical structure called G-quadruplex organized in stacks of G:G:G:G tetrads, in which four guanosines are assembled in a planar arrangement by Hoogsteen base pairing. G-rich DNAs and RNAs form G-quadruplex structure in the presence of monovalent metal ions especially K⁺, but take on a single-stranded one in the absence of monovalent ions.¹³⁻¹⁷ The G-quadruplex is thought to play biologically significant roles such as DNA replication, transcription, translation, etc. and also relate to oncogene expression.

In the previous study, Mashima and coworkers have determined the quadruplex structure of RNA aptamer (r(GGAGGAGGAGGA)) which was named R12 in the presence of K⁺.¹⁸⁻²⁰ R12 folds into a compact parallel G-quadruplex structure (Figure 1-1-d), with G:G:G:G tetrad (Figure 1-1-a) and G:(A):G:G:(A):G hexad planes (Figure 1-1-b). The quadruplex of R12 forms a stable dimer through stacking interaction between the hexads (Figure 1-1-d). The water-entropy gain upon the dimerization of R12 is also an important driving force.²⁰ We also found that R12 takes on a single-stranded form in the absence of K⁺ (Figure 1-1-c). We also recognized R12 as a structural switch that can be turned 'ON' by using K⁺ as a switching stimulator. Thus, in this study, we attempted to make use of this conformational change in R12 to regulate a catalytic activity and target-binding activity of functional RNAs in response to K⁺.

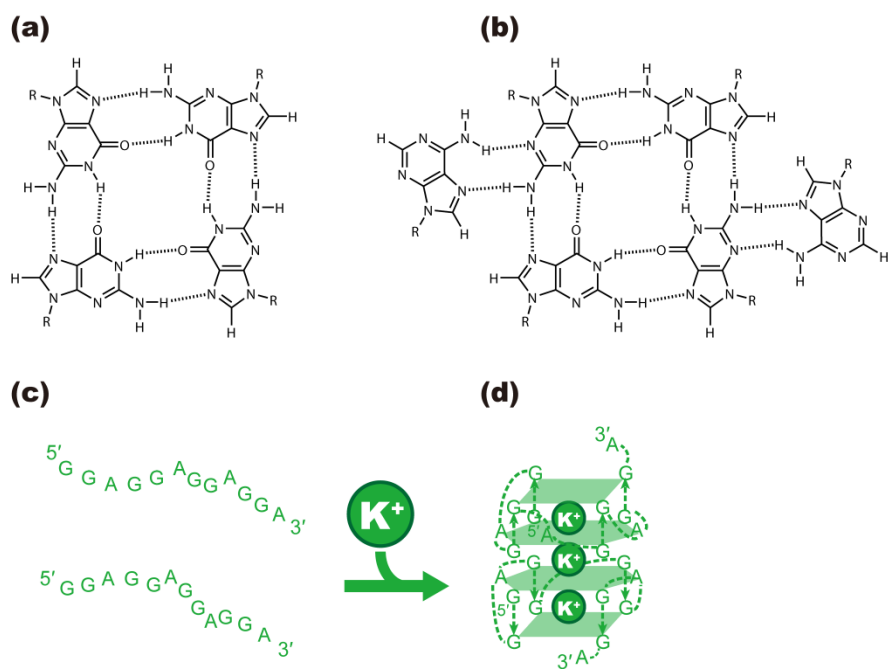


Figure 1-1. The G:C:G:G tetrad (a) and G(:A):G:G(:A):G hexad (b) planes. The hydrogen bonds are indicated by dotted lines. The sequence of R12. R12 takes on a single-stranded form in the absence of K^+ (c) and it forms G-quadruplex structure in the presence of K^+ (d).

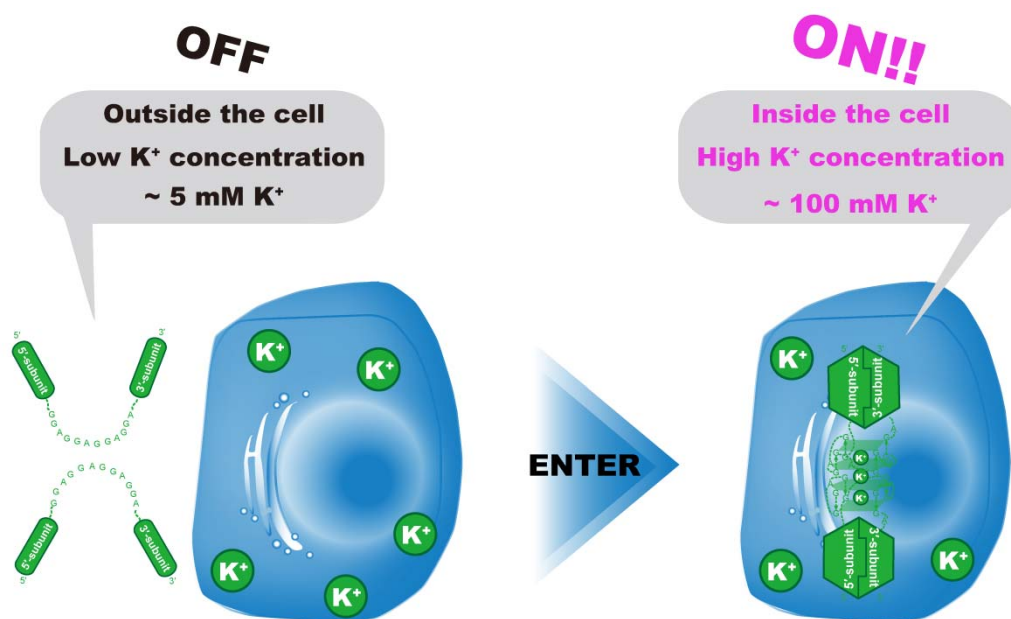


Figure 1-3. Schematic illustration of the activation of K^+ -responsive functional RNA which sense the differences between extra- and intracellular environments, especially K^+ concentrations.

1-5. Development of K^+ -responsive Ribozyme: Quadruplex Hammerhead Ribozyme (QHR)

Hammerhead ribozyme (HR) is one of the simplest and well examined ribozyme. It was discovered from a viroid and a satellite RNA which is a single-stranded RNA of plant viruses.²¹⁻²³ HR exhibits sequence-specific ribonuclease activity. Originally, HR is known as a cis-acting self-cleavage ribozyme. In 1987, Uhlenbeck reported a trans-acting HR.²⁴ He separated a HR to two strands which were composed of 19- and 24-nucleotides and these two strands assembled into a hammerhead conformation by the formation of three helices and exerted trans-cleavage activity.

In one of the designed trans-cleavage HR is composed of three components, a substrate and two enzymatic portions. The enzymatic portions of the HR are assembled each other by the stem II helix (Figure 1-4-a). The two closely located portions exhibit the ribozyme activity independently of the existence of K^+ .^{24,25} In the previous study,²⁶ Nagata and coworkers replaced the stem II by R12 or R11, which was deleted the 3'-terminal adenosine residue of R12 to develop the K^+ -responsive ribozymes. Two subunits of the catalytic core of a HR, the 5'-HR subunit (5'-GCGGUCUGAUGA-3') and the 3'-HR subunit (5'-GAAACAGG-3'), were connected to the 5'- and 3'-ends of R12 (or R11), respectively, with uracil residues as a linker. In these designed ribozymes, the 5'-HR and 3'-HR subunits are not brought into close proximity in the absence of K^+

because R12, or R11, takes on the single-stranded form (Figure 1-4-a), but they are brought into close proximity in the presence of K^+ due to the formation of the compact G-quadruplex of R12, or R11, by which designed ribozymes were expected to exert activities (Figure 1-4-b).

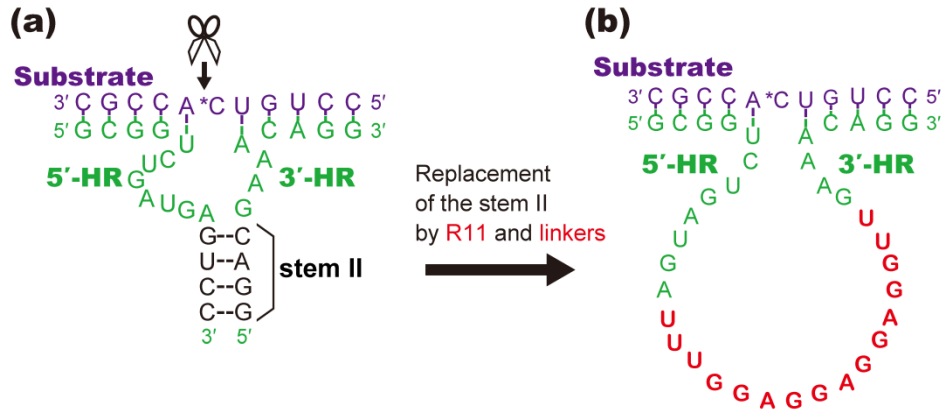


Figure 1-4. (a) The sequence of HR. The catalytic portion and stem portion of HR are shown in green and black characters, respectively. (b) The sequence of QHR. The catalytic portions are shown in green characters. R11 and linker sequences are also shown in red characters. The substrate RNA strands are indicated in purple.

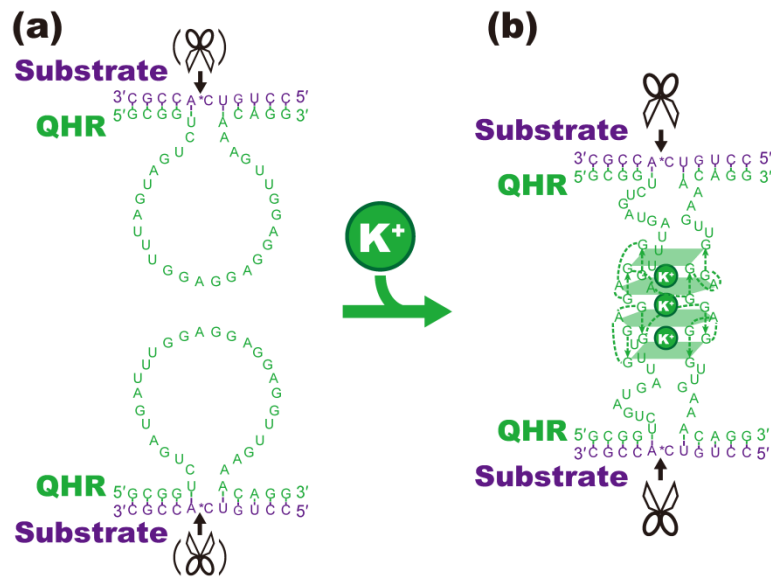


Figure 1-5. Schematic illustration of the activation of QHR. QHR exerts RNA cleavage activity via G-quadruplex formation in response to K^+ . In the absence of K^+ , R11 portion does not form the G-quadruplex and both subunits of HR are located far from each other (a).²⁶ In the presence of K^+ , R11 portion folds into the compact G-quadruplex and the two subunits are brought into close proximity and the active core structure is restored. Thus this QHR exerts the activity in response to K^+ (b).²⁶

The effects of K^+ on the ribozyme activities of the newly designed ribozymes which have various combination of a number of linker U residues were examined. One exhibited the expected character, *i.e.* the ribozyme activity was enhanced with increasing the concentration of K^+ . Others which have either no or small number of linker U residues exhibited the opposite character, *i.e.* repression of ribozyme activity in the presence of K^+ compared to the absence of K^+ rather than enhancement. The suppression of ribozyme activity in response to K^+ is suggested that a structural restriction that prevents the formation of ideal catalytic core was imposed on these ribozymes by formation of the G-quadruplex in response to K^+ . In contrast, the enhancement of the activity in response to K^+ suggests that the G-quadruplex and the proper number of linker U residues allow the location of the two subunits in an original close arrangement for formation of the ideal ribozyme core. Thus, the new ribozymes whose enzymatic activity is either suppressed or enhanced in response to K^+ have been produced. In particular, one of these constructed ribozymes whose activity switches on in response to K^+ was named Quadruplex Hammerhead Ribozyme (QHR, 5'-GCGGUCUGAUGAUUUGGAGGAGGAGGUUGAAACAGG-3'). QHR was consisted of 5'-HR subunit, three uracil residues, R11, two uracil residues, and 3'-HR subunit.

QHR exerts higher activity in the presence of K^+ than its absence as we have hypothesized. Unexpectedly, however, QHR showed unexpected ribozyme activity even in the absence of K^+ , namely residual activity. The residual activity resulted in low switching efficiency, the ratio of the activity between in the absence or presence of K^+ . It have been hypothesized that in the absence of K^+ , the R11 sequence of QHR is a flexible single-stranded form and that the unexpected active core of QHR is partially and/or transiently formed even in the absence of G-quadruplex.²⁶

The aim of this study was to develop the new QHR system which exhibits lower residual activity and higher switching efficiency and to develop newly designed K^+ -responsive RNA aptamer. First, we adopted three strategies to inhibit the formation of the partial and / or transient structure of the active core and repress the residual activity of QHR in the absence of K^+ . For the first strategy, we split QHR in the middle of the R11 sequence. For the second strategy, we applied partially complementary RNA strands to QHR. For the third strategy, we introduced G-rich complementary DNA strands into QHR. These new QHR systems not only repressed the residual activity in the absence of K^+ but also resulted in the improvement of the switching efficiency in response to K^+ . These QHR systems are prototypes to develop the novel K^+ -responsive functional RNAs which switch their activity between out/inside the cell.

Second, we applied the strategy that switch the activity in response to K^+ via G-quadruplex formation of R11 to develop K^+ -responsive RNA aptamer and expand the target functional RNA which is able to be applied this strategy. The RNA aptamer which tightly binds to Tat protein as a transcription factor of HIV was employed to develop the switching aptamer, quadruplex Tat-binding aptamer (QTAp). QTAp bound to arginine-rich peptide derived from Tat protein in the presence of

K⁺. On the other hand, it did not bind to the peptide in the absence of K⁺. We succeeded to show that our strategy using G-quadruplex of R11 can be applied to not only ribozyme but also RNA aptamer.

The strategy that switch the activity in response to K⁺ via G-quadruplex formation of R11 worked successfully in quadruplex hammerhead ribozyme and quadruplex Tat-binding aptamer as we expected. In principle, this strategy is able to be applied to all functional RNAs and also DNAs which require particular structures to exhibit their activities. These two systems can be prototypes for the development of such functional RNAs that sense differences in the concentration of K⁺ between intra/extracellular environment and regulate their activities.

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Chapter 2

Strategy for the Suppression of a Residual Activity: Development of split-Quadruplex Hammerhead Ribozyme

2-1. Introduction

For the development of the molecular tools which switch their activities, the repression of high residual activity which can be high background activity is important. Particularly, ribozymes catalyze biochemical reactions and small amount of active ribozyme should exhibit high residual activity. In the previous study, our designed ribozyme, quadruplex hammerhead ribozyme (QHR, 5'-GCGGUCUGAUGAUUUGGAGGAGGAGGUUGAAACAGG-3'), which switch on its activity in response to K^+ via quadruplex formation of R11 showed high residual activity even in the absence of K^+ .¹ In this chapter, we developed new K^+ -responsive QHR system which exhibits lower residual activity than original QHR.

2-2. Results

2-2-1. Design of split QHR

It was suggested that the residual activity of QHR was originated from the partial and/or transient formation of the active conformation.¹ In detail, due to covalent connection between the 5'- and 3'-subunits of hammerhead ribozyme (HR), named 5'-HR (5'-GCGGUCUGAUGA-3') and 3'-HR (5'-GAAACAGG-3') respectively, through R11 together with some uracil residues as linkers, the fluctuation of the structure of QHR in solution or binding of QHR to the substrate RNA may bring the two subunits, 5'-HR and 3'-HR, into close proximity, even without the G-quadruplex formation. This may lead to the residual activity. Along these lines, we adopt a strategy to suppress the partial and/or transient catalytic core formation and repress the residual activity of QHR in the absence of K^+ .

In this strategy, to keep the 5'-HR and 3'-HR subunits away from each other in the absence of K^+ , we split QHR in the middle of the R11 sequence (left of Figure 2-1). We named the resulting two subunits 5'-spQHR (5'-GCGGUCUGAUGAUUUGGAGGA-3') and 3'-spQHR (5'-GGAGGUUGAAACAGG-3'), respectively. In spQHR system, these 5'- and 3'-spQHRs are no longer covalently connected. We thought the partial and/or transient active core would rarely form and the suppression of residual activity is expected. In the presence of K^+ , however, we expected the

R11 sequences of the 5'- and 3'-spQHRs would assemble to each other, and form the same or a similar G-quadruplex structure via intermolecular Hoogsteen base pairings (right of Figure 2-1). This is likely since four d(GGAGGA) strands reportedly fold into a compact parallel quadruplex dimer composed of two G:G:G:G tetrad planes and two G:(A):G:G:(A):G hexad ones,² which resembles the structure of the dimeric quadruplex of R12.

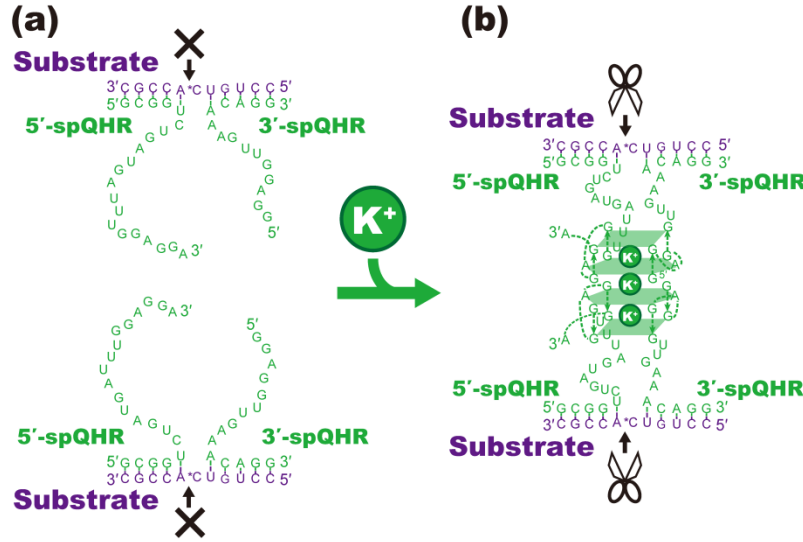


Figure 2-1. The sequences of 5'-spQHR and 3'-spQHR (a, green portions) and their expected structures in the presence of K⁺ (b).

2-2-2. Activity Assay of split QHR

The effects of K⁺ on the enzymatic activities of the mixture of the 5'- and 3'-spQHRs in the absence of K⁺ and presence of 100 mM K⁺ were analyzed by denatured gel electrophoresis, the resulting cleavage products being isolated and their band intensities being measured. The time course of the cleavage percentage is plotted for each case in Figure 2-2-a. The first-order rate constants of the mixture of the 5'- and 3'-spQHRs at 25 °C in the absence of K⁺ and presence of 100 mM K⁺ were $k_{\text{obs}}^{0\text{mM}} = 1.24 \times 10^{-3} \text{ min}^{-1}$ and $k_{\text{obs}}^{100\text{mM}} = 3.90 \times 10^{-3} \text{ min}^{-1}$, respectively (Figure 2-2-b). Therefore the switching efficiency by K⁺ ($k_{\text{obs}}^{100\text{mM}}/k_{\text{obs}}^{0\text{mM}}$) was 3.2 (Figure 2-2-c). The first-order rate constants of the original QHR were $k_{\text{obs}}^{0\text{mM}} = 0.04 \text{ min}^{-1}$ and $k_{\text{obs}}^{100\text{mM}} = 0.1 \text{ min}^{-1}$, respectively (Figure 2-2-b). These values indicate that in the absence of K⁺, the residual activity was reduced to one thirty-first, and the switching efficiency of the spQHR system was improved 1.4-fold as compared with in the case of the original QHR (Figure 2-2-c).

The first-order rate constants of the mixture of the 5'- and 3'-HRs were $k_{\text{obs}}^{0\text{mM}} = 1.41 \times 10^{-3} \text{ min}^{-1}$ and $k_{\text{obs}}^{100\text{mM}} = 1.19 \times 10^{-3} \text{ min}^{-1}$ (Figure 2-2-b). Both these values were comparable to $k_{\text{obs}}^{0\text{mM}}$ of the

spQHRs (Figure 2-2-b). It was clearly shown that there was no activity enhancement for the mixture of the 5'- and 3'-HRs upon the addition of K^+ (Figure 2-2-c), which is reasonable because, in the first place, the 5'- and 3'-HRs do not have a quadruplex-forming sequence.

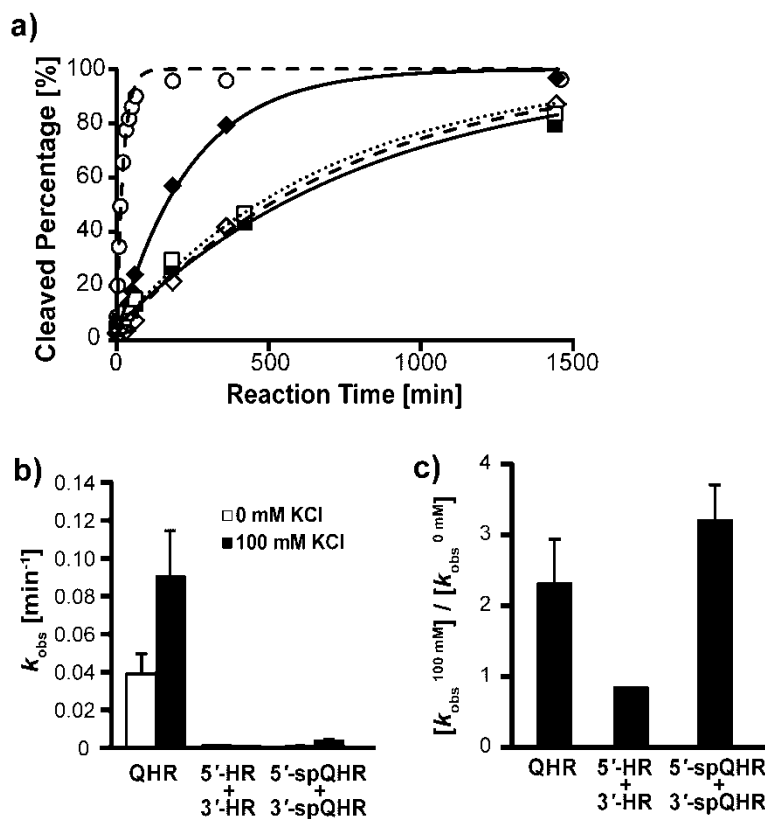


Figure 2-2. Activity of the split-Quadruplex Hammerhead Ribozyme (spQHR). (a) Time course of the increase in cleaved substrate with either the QHR in the absence of K^+ (open circles), the mixture of 5'- and 3'-spQHRs in the absence (open diamonds) and presence (filled diamonds) of 100 mM K^+ , or the mixture of 5'- and 3'-HRs in the absence (open squares) and presence (filled squares) of 100 mM K^+ . (b) The rate constants of the cleavages of the substrate with either the original QHR, the mixture of 5'- and 3'-HRs, or the mixture of 5'- and 3'-spQHRs, each in the absence ($k_{obs}^{0\text{mM}} / \text{min}^{-1}$, open boxes) and presence ($k_{obs}^{100\text{mM}} / \text{min}^{-1}$, filled boxes) of 100 mM K^+ . (c) The switching efficiency, $k_{obs}^{100\text{mM}} / k_{obs}^{0\text{mM}}$, is showed for each of the QHR, 5'-HR + 3'-HR, and 5'-spQHR + 3'-spQHR.

2-3 Discussion

Origin of the much lower Activity of spQHR than QHR

In the presence of 100 mM K^+ , the mixture of the 5'- and 3'-spQHRs exhibited a much lower ribozyme activity, k_{obs}^{100mM} , than the original QHR. We expected that this observation is partially due to the fact that other combinations of the 5'- and 3'-spQHRs are possible, although 5'-spQHR/3'-spQHR is the correct and active combination. Thus, in principle, if a stoichiometry of one-to-one pairing is assumed, the combinations of 5'-spQHR/5'-spQHR, 5'-spQHR/3'-spQHR, and 3'-spQHR/3'-spQHR are possible, all of which may form the G-quadruplex conformation. In theory, the population ratio should then be 5'-spQHR/5'-spQHR: 5'-spQHR/3'-spQHR: 3'-spQHR/3'-spQHR = 1:2:1. Since only 5'-spQHR/3'-spQHR should exhibit the ribozyme activity, *i.e.* the others should not be active, k_{obs}^{100mM} of the mixture of the spQHRs is expected to be half that of the original QHR. However, this was not the case and k_{obs}^{100mM} of the mixture of the spQHRs showed much lower activity than half of the activity of QHR. The reason for this may be the formation of an inactive G-quadruplex structure such as antiparallel forms which contain four RNA strands.

2-4. Conclusion

Here, we constructed the K^+ -responsive spQHR system which is consistent of 5'- and 3'-spQHRs, a mixture of which exhibited much lower residual activity in the absence of K^+ , and also improved the switching efficiency of the ribozyme activity by K^+ , both as compared with the original QHR. To our knowledge, this is the first study to utilize intermolecular G-quadruplex formation to control the activity switching of a ribozyme.

2-5. Experimental Procedures

Materials

All RNAs were synthesized, purified, and de-salted by FASMAC Co., Ltd. (Kanagawa, Japan).

Cleavage reaction

QHR (a final concentration is 10 μ M) or spQHR (final concentrations are 10 μ M 5'-spQHR and 10 μ M 3'-spQHR) was mixed in a solution comprising ca. 70 mM Tris-HCl buffer (pH 8.0) and either 0 or 100 mM KCl. The solution was heated at 95 $^{\circ}$ C for 5 min and then gradually cooled to 25 $^{\circ}$ C. $MgCl_2$ was then added to the final concentration of 50 mM. The cleavage reaction was started by

adding the FITC-labeled substrate RNA (a final concentration is 1 μ M). A small aliquot was taken from the reaction solution at various time points, and the reaction was stopped by adding EDTA and urea to the final concentration of 83 mM and 7.5 M, respectively. The temperature of the solution was kept at 25 °C through the reaction. These small aliquots were applied to and separated on a denaturing 20% polyacrylamide gel by electrophoresis. The amount of FITC-labeled substrate RNA was quantified by using Pharos FXTM Molecular Imager (BIO-RAD).

The cleaved percentage was defined as the amount of cleaved substrate divided by the total amount of the substrate, which is the addition of the un-cleaved and cleaved substrates. The first-order rate constant, k_{obs} , was determined by applying the time-course experiment data to the equation: $P(t) = P_{\text{max}} - (P_{\text{max}} - P_0) \exp(-k_{\text{obs}} t)$ where $P(t)$ is the cleavage percentage at time t , P_{max} is the cleavage percentage at infinite time, and P_0 is the extrapolated cleavage percentage at $t = 0$.^{3,4}

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Chapter 3

Strategy for the Suppression of a Residual Activity: Development of Complementary RNA Strand

3-1. Introduction

One of the important steps of the development of the molecular tools which switch their activities is the repression of high residual activity. In principle, the repression of residual activity leads to improvement of the switching efficiency which is defined as the activity ratio between in the presence/absence of 100 mM K^+ . In the case of spQHR system, however, we succeeded to repress the residual activity to lower than original QHR, the maximum ribozyme activity in the presence of K^+ was also repressed. To regulate the both repression of the activity in the absence and presence of K^+ , we developed the new QHR system based on Watson-Crick base pairing. In this chapter, we developed new K^+ -responsive QHR system which composes original QHR and the partially complementary RNA sequences against QHR.

3-2. Results

3-2-1. Design of Complementary RNA Strand (CS)

Here we designed several RNA sequences that are complementary to different regions of QHR, named them CSs, to restrict the formation of partial and/or transient catalytic core of QHR. We attempted to design a CS that can bind to QHR and form a duplex in the absence of K^+ (Figure 3-1-b), but in the presence of K^+ , dissociates from QHR through the formation of the G-quadruplex in the R11 sequence of QHR (Figure 3-1-c). In this way, we intended to keep the 5'-HR and 3'-HR subunits of the QHR apart completely from each other in the absence of K^+ and suppress the residual activity (Figure 3-1-b). On the other hand, when in the presence of K^+ , QHR is expected to restore full activity via the formation of a catalytic structure upon dissociation of the CS and G-quadruplex formation for R11.

Figure 3-2 shows the sequences of the CSs employed in this study. CS1 and CS2 are 5- and 11-nucleotides (nt) long, respectively, each targeting the R11 region of QHR. CS3, CS4, and CS5 are each 10-, 11-, and 12-nt long, respectively, all targeting sequences in the R11 region and in the 5'-HR subunit of QHR. Finally, CS6 is 12-nt long, and targets the same sequence as CS5 but has three base-pair mismatches as to QHR.

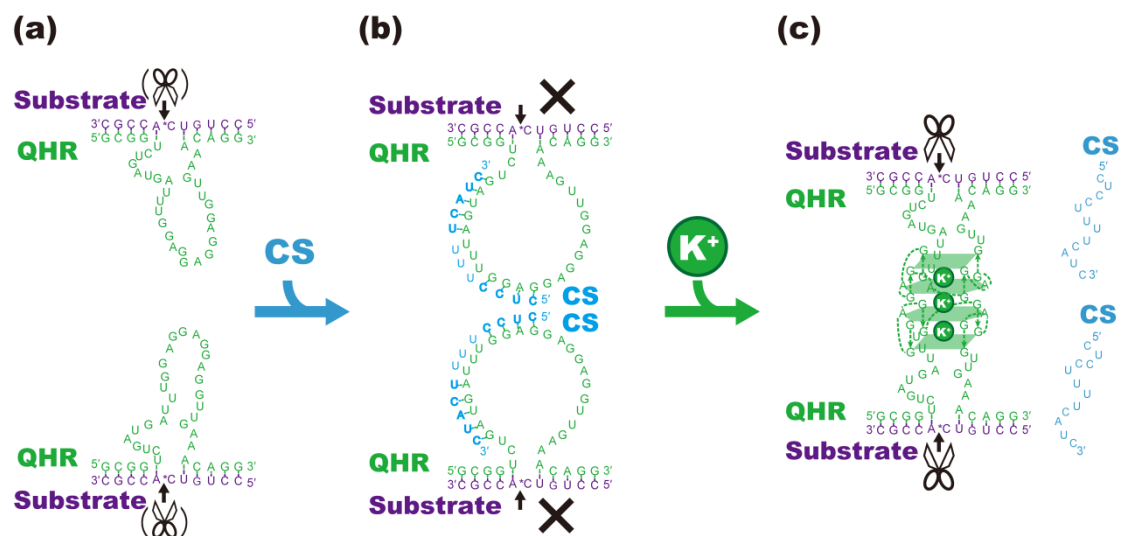


Figure 3-1. Schematic illustration showing that the QHR has the residual activity (a) and that the residual activity is repressed by CS (b). CS dissociates from QHR through the formation of the G-quadruplex in the R11 sequence of QHR (c).

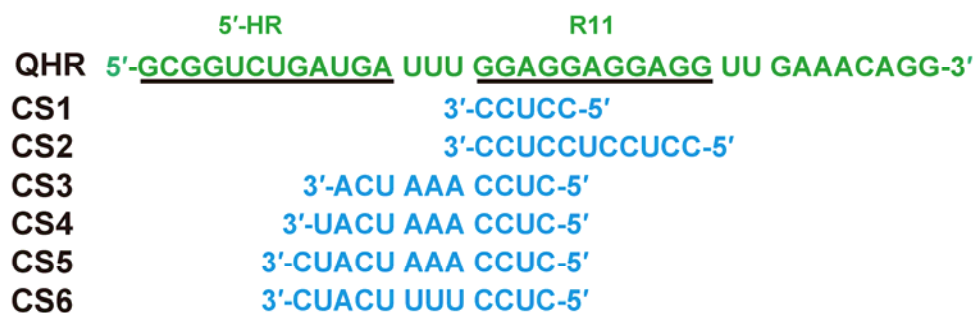


Figure 3-2. The sequences of the QHR (green) and CSs (blue). The 5'-HR subunit and R11 region are underlined.

3-2-2. Ribozyme Activity Assay of QHR in the Coexistence of CS

The ribozyme activities of the QHR with each CS (CS1 - CS6) in the absence and presence of 100 mM K^+ were analyzed by denatured gel electrophoresis (Figure 3-3-a). The first-order rate constants for all combinations of QHR with CSs were obtained by applying the results of electrophoresis (Figure 3-3-c). The ratios of the first-order rate constants ($k_{obs}^{100mM}/k_{obs}^{0mM}$), the switching efficiency, for all combinations of QHR with CSs were calculated and are shown in Figure 3-3-d. CS1 is 5-nt long and is complementary to a part of the sequence within R11 region of QHR. The residual activity of QHR in the coexistence of CS1, k_{obs}^{0mM} , was suppressed to one-third. Although this is rather mild suppression, the formation of the duplex within the R11 region was shown to be effective for the repression of the residual activity (Figure 3-3-c and Table 3-1). Additionally, the enhancement of the activity of QHR with CS1 by K^+ ($k_{obs}^{100mM}/k_{obs}^{0mM}$) was 5.9-fold. This is an improvement as compared with that of free QHR alone (2.3-fold), having a comparable k_{obs}^{100mM} value (just ca. 10% reduction) to QHR alone in the absence of any CS (Figure 3-3-c and Table 3-1).

Next, to achieve further repression of the residual activity, we designed CS2, which is fully complementary to the sequence of R11. The k_{obs}^{0mM} value of QHR in the presence of CS2, however, did not show further improvement as compared with that of QHR in the presence of CS1, exhibiting a similar residual activity (Figure 3-3-c and Table 3-1). This suggests that covering of only the sequence of R11 region and inhibition of its G-quadruplex formation are not sufficient to eliminate the residual activity, probably due to partial and/or transient formation of the catalytic core structure in the HR domains, even though there is no the G-quadruplex structure of R11. On the other hand, the $k_{obs}^{100mM}/k_{obs}^{0mM}$ value of QHR in the coexistence of CS2 turned out to be 0.92-fold, indicating that there was no enhancement of the activity by K^+ (Figure 3-3-d and Table 3-1). It is assumed that the duplex structure formed with CS2 is more stable than the G-quadruplex structure in the R11 region even in the presence of K^+ , and thereby activity switching by K^+ is restricted. Therefore, keeping an appropriate length for binding to the R11 region, while searching for an appropriate complementary sequence to other regions, such as the HR subunit, may lead to the finding of a suitable CS.

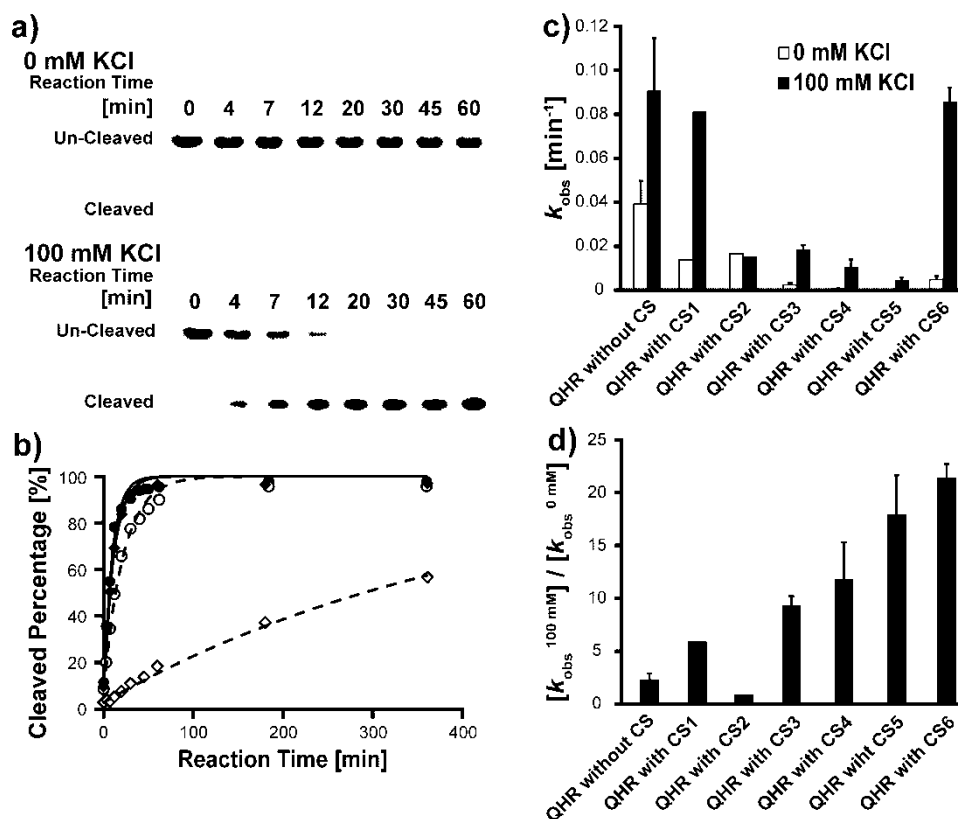


Figure 3-3. (a) Polyacrylamide gel electrophoresis of the products produced on cleavage of the substrate with the QHR in the presence of CS6. The top and bottom panels show cleavage in the absence of K^+ and in the presence of 100mM K^+ , respectively. (b) Time course of the increase in cleaved substrate with the QHR in the absence of K^+ (open circles, dashed line) or in the presence of 100mM K^+ (filled circles, solid line); and with the QHR and CS6 in the absence of K^+ (open diamonds, dashed line) or in the presence of 100 mM K^+ (filled diamonds, solid line). (c) Rate constants of the cleavage of the substrate with different combinations of the QHR and CSs, each in the absence of K^+ ($k_{obs}^{0\text{mM}}/\text{min}^{-1}$, open boxes) and in the presence of 100 mM K^+ ($k_{obs}^{100\text{mM}}/\text{min}^{-1}$, filled boxes). (d) The ratios of the rate constants, $k_{obs}^{100\text{mM}}/k_{obs}^{0\text{mM}}$, are indicated for different combinations of the QHR and CSs.

Table 3-1. The first-order rate constants and the switching efficiency of QHR either alone or in the presence of various CS.

	$k_{\text{obs}}^{0\text{ mM}} [\text{min}^{-1}]$	$k_{\text{obs}}^{100\text{ mM}} [\text{min}^{-1}]$	$[k_{\text{obs}}^{100\text{ mM}} / k_{\text{obs}}^{0\text{ mM}}]$
QHR without CS	3.91×10^{-2}	9.05×10^{-2}	2.3
QHR with CS1	1.38×10^{-2}	8.10×10^{-2}	5.9
QHR with CS2	1.66×10^{-2}	1.53×10^{-2}	0.9
QHR with CS3	2.46×10^{-3}	1.82×10^{-2}	9.3
QHR with CS4	9.52×10^{-4}	1.06×10^{-2}	11.8
QHR with CS5	3.39×10^{-4}	4.47×10^{-3}	17.9
QHR with CS6	4.77×10^{-3}	8.57×10^{-2}	21.4

In CS3, CS4, and CS5, the sequence that is partially complementary to the R11 region of QHR is short (4-nt long, which is one nucleotide shorter than CS1) and same, while the sequences that are complementary to 5'-HR subunit are varied (lengthened by one base in CS3, CS4, and CS5 in that order) (Figure 3-2). Expectedly, these three CSs repressed the residual activity of QHR significantly, the $k_{\text{obs}}^{0\text{mM}}$ values being in the range between one-fortieth and one two-hundredth of the value of QHR alone (Figure 3-3-c and Table 3-1). This indicates that connection of complementary sequences to parts of the 5'-HR subunit and that to the R11 region are highly effective for suppressing the residual activity of QHR. A probable explanation for this great suppression of the residual activity is that complementary sequences in CS3, CS4, and CS5 as to parts of the 5'-HR subunit of QHR have formed a duplex and thereby inhibiting the partial and/or transient formation of the active core structure in the HR subunits of QHR, which would otherwise exhibit residual activity even in the absence of the G-quadruplex structure. We then determined the switching efficiency values of QHR in the presence of CS3, CS4, and CS5, all of which improved in that order (9.3, 11.8, and 17.9), and were much larger than that of QHR in the absence of CS, 2.3-fold (Figure 3-3-c and Table 3-1). However, the $k_{\text{obs}}^{100\text{mM}}$ values of QHR with CS3, CS4, and CS5 decreased in that order, and more importantly, all had much smaller values than that of QHR without CS (Figure 3-3-c and Table 3-1). A probable reason for the latter point is that each of CS3, CS4, and CS5 forms a duplex with QHR through a rather long stretch (regions through a part of 5'-HR, linker uracil residues, and a part of the R11 region), thereby dissociation of CSs, formation of the quadruplex structure in the R11 region, and activity switching by addition of K^+ are strongly restricted. Although the further improvement of the suppression of the residual activity, $k_{\text{obs}}^{0\text{mM}}$, and the enhancement of the switching efficiency, $k_{\text{obs}}^{100\text{mM}}/k_{\text{obs}}^{0\text{mM}}$, for CS3, CS4, and CS5, as compared with CS1, is encouraging, $k_{\text{obs}}^{100\text{mM}}$ is required to be increased.

Finally, CS6 targets exactly the same region of QHR as CS5, except that CS6 has three base-pair mismatches as to the linker uracil residues of QHR (Figure 3-2). These base-pair mismatches were introduced to make an adjustment to obtain a preferred sequence that retains a similar level of residual activity suppression as CS5 but is capable of dissociating from QHR upon the G-quadruplex formation by R11 in the presence of K^+ . Although the k_{obs}^{0mM} of QHR with CS6 was not as low as that of QHR with CS5, QHR in the coexistence of CS6 exhibited effective suppression of the residual activity, the k_{obs}^{0mM} value being $4.77 \times 10^{-3} \text{ min}^{-1}$, which is one eighth of the value of QHR in the absence of any CS (Figure 3-3-c and Table 3-1). Importantly, the k_{obs}^{100mM} value was $8.57 \times 10^{-2} \text{ min}^{-1}$, which is just a minor decrease from the k_{obs}^{100mM} value of QHR alone (only ca. 5% reduction), and thereby the $k_{obs}^{100mM}/k_{obs}^{0mM}$ value turned out to be as high as 21.4-fold (Figure 3-3-d and Table 3-1). We suggest that in the case of CS6, the three base-pair mismatches against QHR were suitable to form a duplex with QHR in the absence of K^+ , and also for CS6 to dissociate from QHR upon formation of the G-quadruplex of R11, thereby the active structure of QHR being induced by addition of K^+ .

We attempted to design a CS that suppresses the residual activity of QHR in the absence of K^+ and also leads to QHR high switching efficiency ($k_{obs}^{100mM}/k_{obs}^{0mM}$ value) through G-quadruplex formation in response to K^+ . In total, we examined the effects of six CSs on the activity of QHR (Figure 3-2). Among them, CS6 exhibited good results in leaving QHR with the highly suppressed residual activity in the absence of K^+ , and causing high switching efficiency for QHR in response to K^+ . Thus, we have successfully developed a new QHR-CS system which switches its ribozyme activity with high contrast in response to K^+ .

3-3. Discussion

Relationship with Previous Study

Several ribozymes were reportedly conjoined with aptamers, by which the activities were altered in response to small compounds.¹⁻³ These ribozymes that are activated by small compounds are used as biosensors and gene regulators. Another activity switching ribozyme, that was reported, was composed of a ribozyme portion and a conjoined inhibitor portion, which is an antisense RNA against the ribozyme portion. This ribozyme-inhibitor system is activated upon the presence of a complementary RNA strand (named activator), by which the inhibitor portion is displaced. This system, which employs a cis-acting RNA duplex is somewhat similar to our QHR-CS system in the sense that it uses inhibitor of ribozyme.⁴ To our knowledge, only two groups have reported ribozymes connected with G-quadruplex forming nucleic acids instead of the aptamers. Hartig group replaced the stem of a hammerhead ribozyme with a G-rich RNA sequence,

r(GGGGUGGGUGGGUGGG), with which the activity was enhanced by the addition of meso-5,10,15,20-tetrakis-(N-methyl-4-pyridyl) porphine (TMPyP4), known as a G-quadruplex stabilizer.⁵ The activity switching efficiency of their designed ribozyme upon addition of TMPyP4 was about 8. Perreault group incorporated two G-rich RNA fragments into hepatitis delta virus (HDV) ribozyme.⁶ It is thought that one of the incorporated fragments forms a series of hydrogen bonds with part of the original HDV ribozyme in the absence of K^+ , resulting in a misfolded ribozyme that is inactive. In the presence of K^+ , however, the two fragments are suggested to fold into a G-quadruplex and thereby the active catalytic conformation is restored. In the latter two cases, the precise structures of the G-quadruplexes are not fully understood.

In our case, we have precise, at atomic resolution, structural knowledge of the G-quadruplex that we have used, which provides us a guide to obtain the ability of activity switching to other ribozymes and aptamers. Our strategy enabling induction of the activity of a ribozyme and aptamer in response to K^+ is rather straightforward. Firstly, the ribozyme or aptamer is divided into two portions, and each part is connected to 5'- and 3'-ends of R11 through some linker residues. At this step, we only need to focus on obtaining such a ribozyme or aptamer connected with R11 that exerts as high activity as possible in the presence of K^+ . This can be accomplished by finding an appropriate position to divide the ribozyme or aptamer, and adjusting the length of the linker residues. At the next step, we can use the strategy, which is fruit of this study, to suppress the residual activity in the absence of K^+ and to achieve highly efficient enhancement of the activity in response to K^+ ; introducing partially complementary RNA sequences. In summary, we have shown a method, which might be applicable to other ribozymes and aptamers, endowing them with activity switching capabilities that can be controlled.

3-4. Conclusion

Here, we constructed the new QHR system which is composed of original QHR and its complementary RNA strand, CS. The QHR-CS system, especially in the coexistence of CS6, exhibited much lower residual activity in the absence of K^+ . This suppression of the residual activity results in the significant improvement of the switching efficiency. When we develop the K^+ -responsive functional RNA, we might be able to apply the CS to suppress the residual activity in the absence of K^+ and to achieve highly contrasted activity switching in response to K^+ .

3-5. Experimental Procedures

Materials

All RNAs were synthesized, purified, and de-salted by FASMAC Co., Ltd. (Kanagawa, Japan).

Cleavage reaction

The ribozyme activity assay was performed using the described protocol in chapter 2, with slight modifications. QHR (a final concentration is 10 μ M) was mixed in a solution comprising ca. 70 mM Tris-HCl buffer (pH 8.0) and either 0 or 100 mM KCl. At cleavage reaction in the presence of CS, CS was also added to the final concentration of 30 μ M. The solution was heated at 95 °C for 5 min and then gradually cooled to 25 °C. MgCl₂ was then added to the final concentration of 50 mM. The cleavage reaction was started by adding the FITC-labeled substrate RNA (a final concentration is 1 μ M). A small aliquot was taken from the reaction solution at various time points, and the reaction was stopped by adding EDTA and urea to the final concentration of 83 mM and 7.5 M, respectively. The temperature of the solution was kept at 25 °C through the reaction. These small aliquots were applied to and separated on a denaturing 20% polyacrylamide gel by electrophoresis. The amount of FITC-labeled substrate RNA was quantified by using Pharos FXTM Molecular Imager (BIO-RAD).

The first-order rate constant of the ribozyme reaction was determined using the described protocol in chapter 2.

3-6. References

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Chapter 4

K⁺-responsive off-to-on Switching of Hammerhead Ribozyme through Dual G-quadruplex Formation Requiring no Heating and Cooling Treatment

4-1. Introduction

To suppress the residual activity of QHR in the absence of K⁺, we have introduced RNA, CS, which is partially complementary to QHR. The introduction of CS successfully repressed the residual activity of QHR in the absence of K⁺, while in the presence of K⁺ the activity is fully exerted and resulted in the switching efficiency of QHR drastically increased. In spite of these improvements, however, this system required an annealing treatment which is consist of heating and cooling processes for the K⁺-responsive conformational change of QHR from the inactive duplex form to the active G-quadruplex form (Figure 4-1-a, b, c). We found that the conformational change did not occur if the annealing treatment was not applied (Figure 4-1-b and d). In this chapter, in order to apply QHR to living cellular environments, we developed a new QHR system, in which the residual activity is repressed and transition to the active form is induced just by the addition of K⁺, especially without the annealing treatment. This newly constructed QHR system could be a prototype for the development of such switching-molecules that can be applied to living cellular environments.

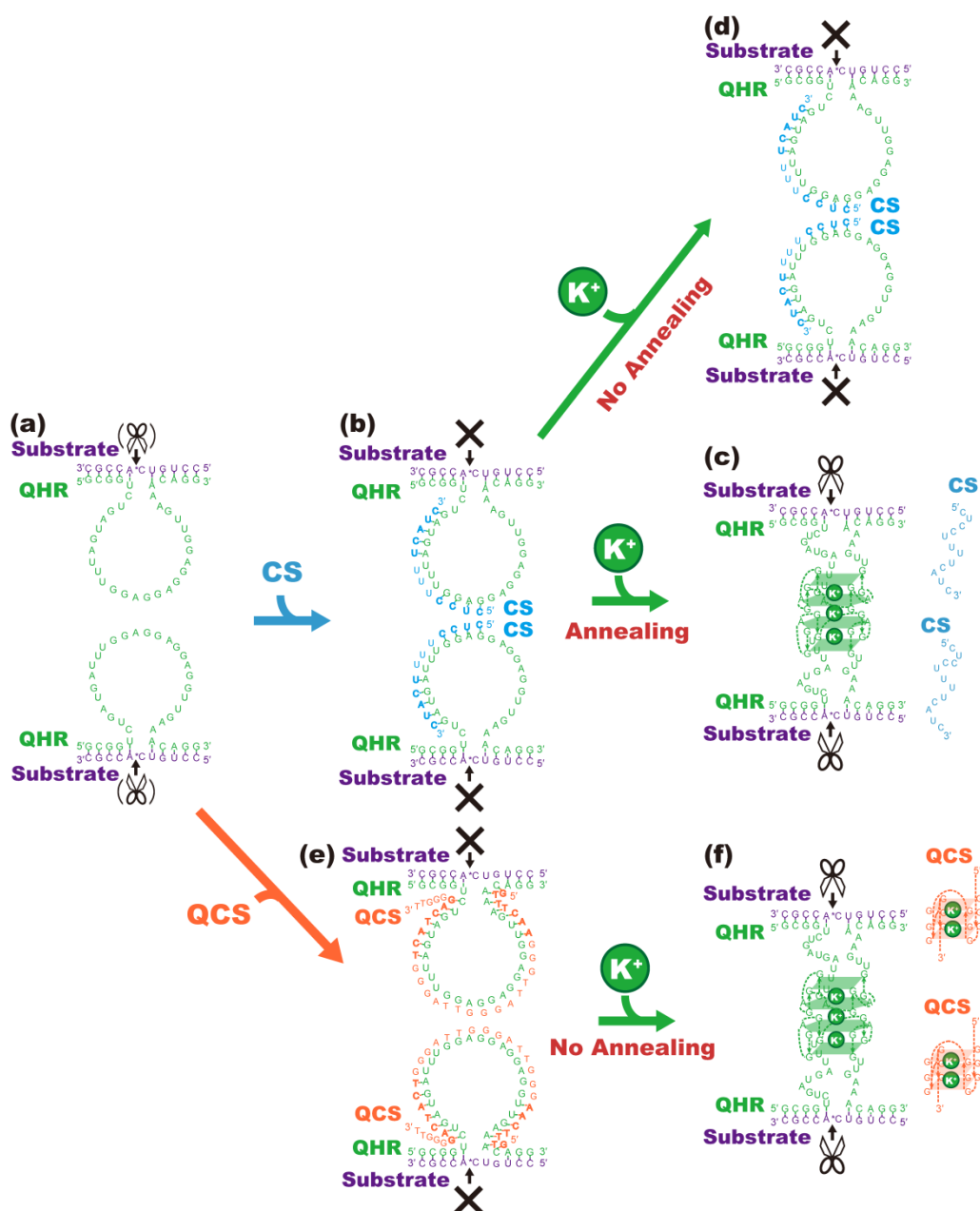


Figure 4-1. The schematic illustrations of architectures and activations of QHR-CS and QHR-QCS systems. The sequences of QHR (green), substrate RNA (purple), CS (blue), and QCS (orange) are shown. The residues of either CS or QCS that form base pairs with QHR are shown in bold. The cleavage site of the substrate RNA is indicated by a black arrow. QHR shows the residual activity even in the absence of K^+ (a). (a-d) QHR-CS system. The formation of the QHR-CS duplex suppresses the residual activity of QHR (b). Addition of K^+ followed by annealing induces activation of QHR (c). Addition of K^+ without annealing does not induce activation (d). (e, f) QHR-QCS system. The formation of the QHR-QCS duplex represses the residual activity of QHR (H). Addition of K^+ results in the formation of the G-quadruplex for both QHR and QCS, by which QHR exhibits its activity. Here, annealing is not required for the activation.

4-2. Results

4-2-1. Design of a G-Quadruplex-forming Complementary DNA Strand (QCS)

In the presence of CS, the conformational change of QHR from a duplex to a G-quadruplex occurs only when K^+ is added and at the same time the annealing treatment is performed (Figure 4-1-a, b, c). The duplex of QHR and CS dissociates during the heating process of the annealing treatment. Here, the G-quadruplex of QHR has a higher melting temperature than that of the QHR-CS duplex in the presence of K^+ . Therefore, the G-quadruplex of QHR is preferred over the QHR-CS duplex in and after the cooling process. Thus, in the coexistence of CS, QHR efficiently folds into G-quadruplex and exerts the activity in response to K^+ with the annealing treatment. However, in the presence of K^+ and without heating process, although it may be possible for the duplex of QHR and CS to transiently dissociate, reformation of the QHR-CS duplex is efficient and results in the inhibition of the conformational change of QHR to the G-quadruplex. Therefore, for activation that does not require the annealing, it seems necessary to repress the reformation of the QHR-CS duplex when K^+ is added. In this study, we attempted to trap CS in G-quadruplex structure upon dissociation from QHR in the presence of K^+ , which should prevent the reformation of the duplex between QHR and CS. We employed a human telomeric DNA sequence, (TTAGGG)₄TT, which was reported to form a G-quadruplex in the presence of K^+ ,¹⁻³ as a template of such a new class of CS. Two of the four TTA loop sequences of this DNA sequence were replaced with complementary sequences as to QHR, the resultant DNA being named the G-quadruplex-forming complementary DNA strand (QCS, 5'-d(GTTTCAAGGGTTAGGGTTAGGGTCATCAGGGGTT)-3', Table 4-1). In the absence of K^+ , the formation of the duplex between QHR and QCS is expected to repress the residual activity of QHR (Figure 4-1-a and e). On the other hand, QCS that transiently dissociates from QHR is expected to form a G-quadruplex in the presence of K^+ , losing its ability to re-form the duplex with QHR (Figure 4-1-f). Simultaneously, QHR is expected to fold into the G-quadruplex and exhibits its ribozyme activity just with the addition of K^+ alone, namely without the annealing treatment (Figure 4-1-f).

Table 4-1. The sequences of the QHR, CS, and QCS. The bases that form base pairs between QHR and CS are indicated by italic characters, and those between QHR and QCS are indicated by bold characters. The residues of R11 of QHR are indicated by underlines. The guanosine residues of QCS that form the tetrads of the G-quadruplex are indicated by underlines.

Name	Sequence
QHR	5'-r(GCGGUCUG <i>GAUG</i> AUUU <u>GGAGGAGGAGG</u> UUGAAACAGG)-3'
CS	3'-r(CUACUUU <u>UCCUC</u>)-5'
QCS	3'-d(TTGGGG <u>GACTACTGGG</u> ATTGGGATTGGGA <u>ACTTTG</u>)-5'

4-2-2. Ribozyme Activity Assay of QHR in the presence of QCS

QHR alone or with complementary nucleic acids (CS or QCS) either in the absence or presence of 100 mM K⁺ was incubated at 25 °C for 40 min. The RNA cleavage reaction of QHR was initiated by addition of 5'-end FITC-labeled substrate RNA to a final concentration of 1 μM. The ribozyme activities of the QHR in the absence and presence of 100 mM K⁺ were determined by polyacrylamide gel electrophoresis (Figure 4-2-a). First-order rate constants were obtained from these results (Figure 4-2-c). The switching efficiency, the ratio of the first-order rate constants under either 0 or 100 mM KCl conditions, $k_{\text{obs}}^{100\text{mM}} / k_{\text{obs}}^{0\text{mM}}$, were calculated and are shown in Figure 4-2-d. In the absence of neither CS nor QCS, the first-order rate constant of QHR in the absence of K⁺ ($k_{\text{obs}}^{0\text{mM}}$) was $2.8 \times 10^{-2} \text{ s}^{-1}$, while that at 100 mM K⁺ ($k_{\text{obs}}^{100\text{mM}}$) was $4.4 \times 10^{-2} \text{ s}^{-1}$ (Figure 4-2-c). In the case of QHR alone, the activity was indeed enhanced in response to K⁺, a switching efficiency of ca. 1.6 (Figure 4-2-d, “QHR”). However, in the absence of neither CS nor QCS, the QHR exhibited non-negligible residual activity even in the absence of K⁺ (Figure 4-2-c, “QHR”). On the other hand, in the presence of CS, $k_{\text{obs}}^{0\text{mM}} = 4.8 \times 10^{-3} \text{ s}^{-1}$ and $k_{\text{obs}}^{100\text{mM}} = 5.1 \times 10^{-3} \text{ s}^{-1}$ were obtained (Figure 4-2-c, “QHR+CS”). The residual activity of QHR with the coexistence of CS, was repressed to approximately 17 % of the level for QHR alone (compare “QHR” and “QHR+CS” in Figure 4-2-c). However, addition of KCl alone failed to induce the activity of QHR with CS (“QHR+QCS” in Figure 4-2-c and d). In the case of QHR in the presence of CS, the annealing treatment is needed to switch “ON” the activity along with the addition of KCl. In fact, when annealing is performed after the addition of K⁺, the switching efficiency of 21.4 was achieved for QHR in the presence of CS (Figure 3-3 in Chapter 3).

In the case of the QHR system where QCS coexists, $k_{\text{obs}}^{0\text{mM}} = 2.6 \times 10^{-3} \text{ s}^{-1}$ and $k_{\text{obs}}^{100\text{mM}} = 3.1 \times 10^{-2} \text{ s}^{-1}$ were obtained (Figure 4-2-c, “QHR+QCS”). The introduction of QCS significantly repressed the residual activity, which was 9 % of the level for QHR alone. Importantly, the activity

enhancement occurred in response to just K^+ , the annealing not being required (Figure 4-2-c and d). Thus, the introduction of QCS resulted in repression of the residual activity and the K^+ -responsive activity enhancement without annealing treatment at the same time. As a result of the successful suppression of residual activity, the switching efficiency increased to 11.6 (Figure 4-2-d). The switching efficiency of QHR alone was 1.6 and almost no enhancement (the value is 1.1) was observed for the QHR with CS when annealing was not performed (Figure 4-2-d). Thus, the great improvement of the switching efficiency of QHR was achieved as a result of the introduction of QCS even without the annealing treatment.

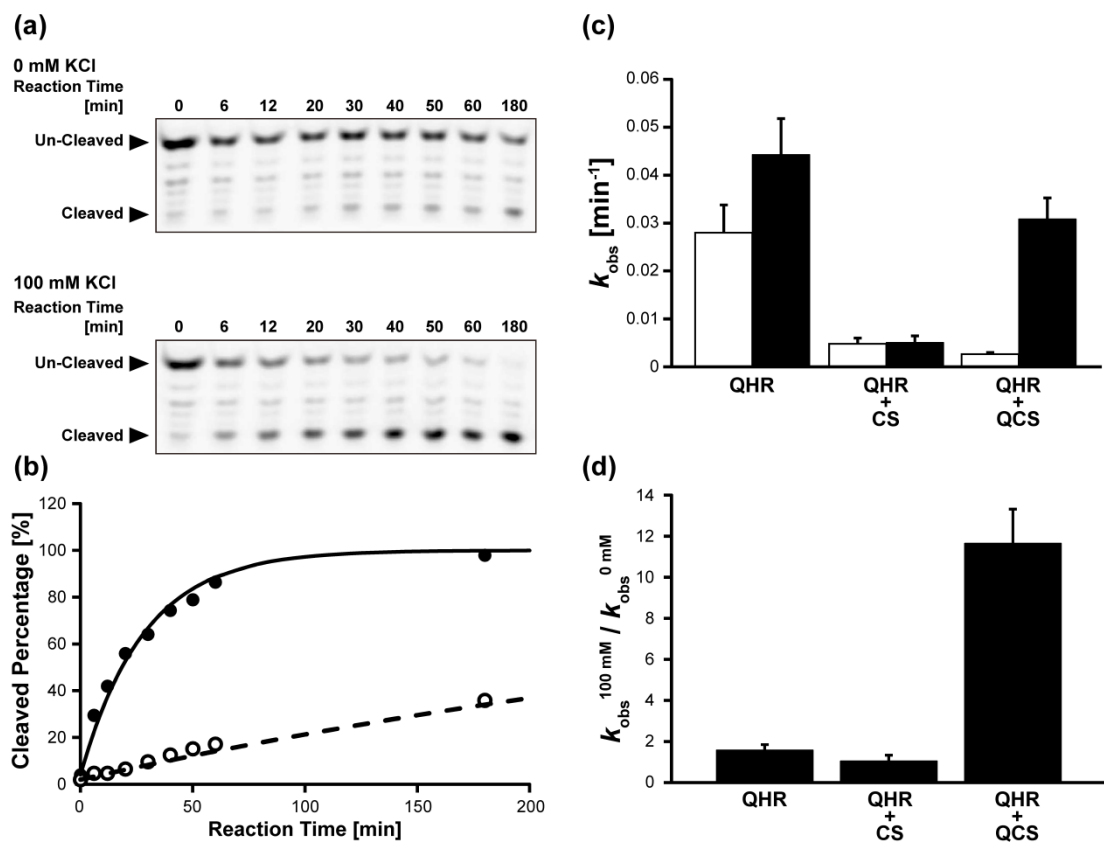


Figure 4-2. Activity assays of each QHR systems. (a) Polyacrylamide gel electrophoresis of the resultant products by the cleavage of the FITC-labeled substrate RNA with the mixture of QHR and QCS. The reactions were performed either in the presence of 0 (top) or 100 mM KCl (bottom). (b) Time course of increasing cleavage product with the mixture of QHR and QCS either in the presence of 0 mM KCl (open circles, dashed line) or 100 mM KCl (filled circles, solid line). (c) The first-order rate constants (k_{obs}) obtained by curve fitting of the time-course experiment results for the cleavage reaction. Bar graphs present k_{obs} for reactions performed either in the presence of 0 mM K^+ ($k_{obs}^{0\text{mM}}$, open boxes) or 100 mM K^+ ($k_{obs}^{100\text{mM}}$, filled boxes). (d) Gray boxes present the ratios of the rate constants, $k_{obs}^{100\text{mM}} / k_{obs}^{0\text{mM}}$.

4-2-3. K^+ -responsive Conformational Change of QHR and QCS from a Duplex to a G-quadruplex Examined by CD and NMR

CD spectra of the mixture of QHR and QCS were measured in buffer containing either 0 mM or 100 mM K^+ (dashed and solid lines of Figure 4-3-a, respectively). Addition of 100 mM K^+ resulted in an increase in the intensity of the positive band at around 260 nm (Figure 4-3-a), which suggests the formation of a parallel type G-quadruplex.^{4,5} This is consistent with our design scheme in which the R11 portion of QHR is expected to form a parallel G-quadruplex upon elevation of the K^+ concentration (Figure 4-1-f).^{6,7} Additionally, a positive band appeared at around 295 nm when 100 mM K^+ was added (Figure 4-3-a). This suggests the formation of a hybrid-type mixed parallel/antiparallel-stranded G-quadruplex,^{3,8,9} which is also consistent with the design scheme in which QCS involving the sequences of human telomeric DNA is expected to form a hybrid-type mixed parallel/antiparallel-stranded G-quadruplex in the presence of K^+ ¹⁻³ (Figure 4-1-f).

The duplex to G-quadruplex structural transition of QHR and QCS was examined by NMR. In the absence of K^+ , the observation of imino proton resonances at 11.6 - 12.6 ppm and 13.0 - 14.4 ppm (top of Figure 4-3-b) indicates the formation of G:C and A:T (A:U) base pairs in the duplex, respectively, which is consistent with our design scheme (Figure 4-1-e). In the presence of K^+ , the intensity of these resonances dramatically decreased. On the other hand, new resonances appeared at 10.8 - 12.0 ppm (bottom of Figure 4-3-b), which indicates the formation of tetrads of the G-quadruplexes. These findings confirm the duplex to G-quadruplex transition, which is consistent with the design scheme (Figure 4-1-f).

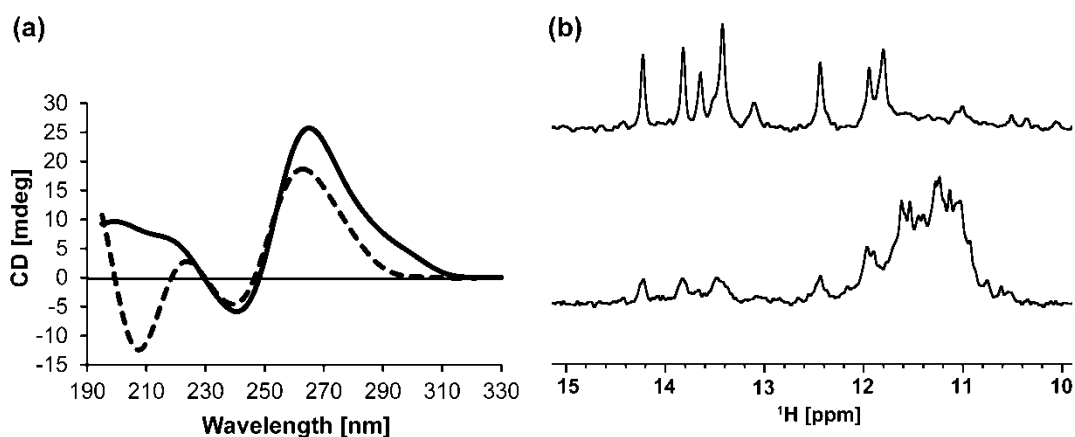


Figure 4-3. (a) CD spectra of the mixture of QHR (14 μ M) and QCS (14 μ M) dissolved in 5 mM sodium phosphate buffer (pH 6.5) containing 5 mM $MgCl_2$, and either 0 mM (dashed line) or 100 mM (solid line) KCl at 5 $^{\circ}C$. (b) NMR spectra of the mixture of QHR (70 μ M) and QCS (70 μ M) dissolved in 5 mM sodium phosphate buffer (pH 6.5) containing 5 mM $MgCl_2$, and either 0 mM (top) or 100 mM (bottom) KCl at 25 $^{\circ}C$.

4-2-4. K^+ and Na^+ Dependence of the Ribozyme Activity of the QHR-QCS System

The activity of the QHR-QCS system was examined at each K^+ concentration without the annealing treatment. With increasing the K^+ concentration, the activity of QHR increased (Figure 4-4). The activity almost reached a plateau in the presence of 300 mM KCl. Therefore, the fraction of the active form of QHR (f_{active}) was estimated using equation (4-1) on the assumption that QHR was fully activated at 300 mM KCl. The half maximal effective concentration of KCl (EC_{50}) was determined to be 23.0 mM.

Moreover, the activity of the QHR-QCS system turned out to be very low in the presence of 100 mM Na^+ , the value of $k_{obs}^{100mM NaCl}$ being just 15 % of the first-order rate constant at 100 mM K^+ ($k_{obs}^{100mM KCl} = 3.1 \times 10^{-2} s^{-1}$) (Figure 4-5).

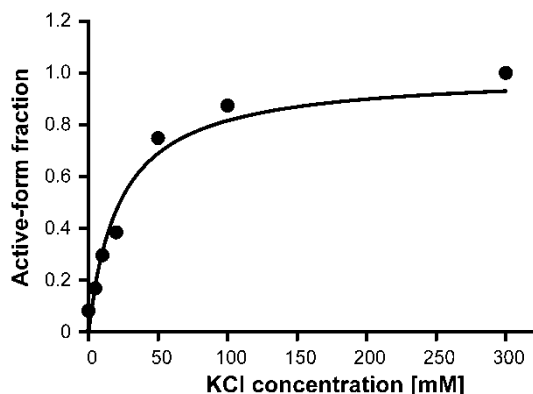


Figure 4-4. K^+ -concentration dependence of the ribozyme activity of the QHR-QCS system. The fraction of the active form of QHR at each KCl concentration was deduced from the rate constants (k_{obs}) for the cleavage reaction. The solid line was drawn by the curve fitting with equation (4-2).

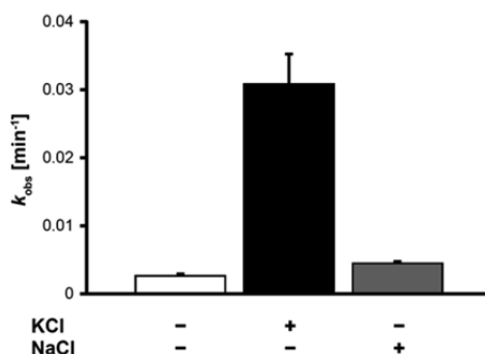


Figure 4-5. The effect of Na^+ on the activity of the QHR-QCS system. The first-order rate constants of the cleavage reaction with the mixture of QHR and QCS, were obtained for the reaction performed either in the absence of K^+ and Na^+ (open box), in the presence of 100 mM K^+ (black box), or in the presence of 100 mM Na^+ (gray box).

4-3. Discussion

Relationship with Previous Studies and the Possibility of Application

Switching of the ribozyme and DNAzyme activity through a duplex-quadruplex structural transition with K^+ , Na^+ , ATP, oligo DNA, and proteins has been developed by some groups including ours.^{5,8-13} To the best of our knowledge, however, our QHR-QCS system is the first one that involves switching of the ribozyme activity through the duplex - G-quadruplex transition in response to K^+ without the annealing treatment. This system should be appropriate for cellular application when the annealing treatment cannot be performed. Furthermore the activity was induced by K^+ with an EC_{50} value of 23 mM, but not by Na^+ . Since the K^+ concentration is ca. 100 mM inside a cell, while the K^+ concentration is ca. 5 mM outside a cell, the QHR-QCS system should be inactive outside a cell, but once it enters the cell, its activity is expected to be exhibited autonomously. When a drug delivery system (DDS) is considered, QHR-QCS system may work as a way to release functional RNAs that target intracellular molecules from carriers. Thus the system may be useful to develop a new efficient DDS.

Our method can be applied to other functional nucleic acids such as an RNA aptamer, DNA aptamer, and DNAzyme. There could be situations in which an RNA aptamer that targets either a certain protein or a small molecule should be silent outside the cell to avoid side effects and should work only inside the cell. Our method involving the dual G-quadruplex formation has potential to afford an RNA aptamer with these required properties.

4-4. Conclusion

We developed the new generation QHR system which is composed of QHR and QCS. The residual activity of QHR-QCS system is effectively suppressed by the formation of the QHR-QCS duplex in the absence of K^+ . It is remarkable that the R11 portion of QHR forms the G-quadruplex just by the elevation of the K^+ concentration, by which QHR exhibits the activity without requiring the annealing treatment. This is in contrast to the previous QHR-CS system which requires the annealing treatment for its activation. In the QHR-QCS system, QCS that transiently dissociates from QHR forms G-quadruplex and is trapped in this conformation in the presence of K^+ , which prevents the reformation of the QHR-QCS duplex. Accordingly, the R11 portion of QHR is not restricted to form the G-quadruplex, any more, which results in the restoration of the active core structure of the hammerhead ribozyme. This is how the QHR-QCS system switches on its activity in response to K^+ without the annealing treatment. Switching of the activity of the QHR-QCS system is induced by K^+ , not by Na^+ . EC_{50} of K^+ for the activity switching is 23.0 mM, which is within the range of

extra-/intracellular K^+ concentrations (ca. 5 mM and 100 mM, respectively). Thus, the QHR-QCS system involving dual G-quadruplex formation may have a potential for the development of cellular applications.

4-5. Experimental Procedures

Oligonucleotides

The quadruplex hammerhead ribozyme (QHR) (5'-r(GCGGUCUGAUGAUUUGGAGGAGGAGGUUGAAACAGG)-3'), complementary RNA strand (CS) (5'-r(CUCCUUUUCAUC)-3'), G-quadruplex-forming complementary DNA strand (QCS) (5'-d(GTTTCAAGGGTTAGGGTTAGGGTCATCAGGGGTT)-3'), and 5'-end fluorescein-5-isothiocyanate (FITC)-labelled substrate RNA (5'-CCUGUCACCGC-3') were synthesized, purified, and desalted by FASMAC Co., Ltd. (Kanagawa, Japan).

Cleavage reactions and kinetic measurements

The ribozyme activity assay was performed using the described protocol in chapter 3, with slight modifications. QHR was dissolved either alone or with a complementary nucleic acid (CS or QCS) in ca. 70 mM Tris-HCl buffer (pH 8.0), and then incubated at 95 °C for 5 min. It was then cooled to 30 °C in 60 min, followed by incubation at 25 °C for 30 min. QHR (10 μ M) either alone or with a complementary nucleic acid (CS or QCS, 30 μ M) dissolved in 50 mM Tris-HCl buffer (pH 8.0), 50 mM $MgCl_2$, and either 0 or 100 mM NaCl with various concentrations of KCl was incubated at 25 °C for 40 min. The cleavage reaction was initiated by addition of the 5'-end FITC-labeled substrate RNA (1 μ M). The other experimental conditions and instruments using the ribozyme activity assay are same as previous study reported in chapter 2 and 3.

The first-order rate constant of the ribozyme reaction was determined using the described protocol in chapter 2 and 3.

K^+ -concentration dependence of the ribozyme activity of the QHR-QCS system

Various amounts of KCl were added to samples containing the pre-formed QHR-QCS duplex. Then the activity of QHR was examined at each K^+ concentration without the annealing treatment. The fraction of the active form of QHR (f_{active}) was deduced using equation (4-1) on the assumption that QHR was fully activated at 300 mM KCl.

$$f_{\text{active}} = \frac{k_{\text{obs}}^{[K^+]}}{k_{\text{obs}}^{[300 \text{ mM}]}} \quad (4-1)$$

The half maximal effective concentration of KCl (EC₅₀) for activation of QHR was obtained with equation (4-2).

$$f_{\text{active}} = \frac{[\text{K}^+]}{\text{EC}_{50} + [\text{K}^+]} \quad (4-2)$$

Circular dichroism (CD) measurements

QHR (14 μM) and QCS (14 μM) were dissolved in 5 mM sodium phosphate buffer (pH 6.5) and 5 mM MgCl₂, and then incubated at 95 °C for 5 min. The mixture was then cooled to 30 °C in 60 min, followed by incubation at 25 °C for 30 min using a thermal cycler. Subsequently, KCl was added to the sample solution to the final concentration of either 0 or 100 mM. CD spectra were recorded at 5 °C with a cell of 1 mm path length using a J-720 spectropolarimeter (JASCO, Tokyo, Japan).

Nuclear magnetic resonance (NMR) measurements

QHR and QCS were dissolved at the final concentration of 70 μM in 5 mM sodium phosphate buffer (pH 6.5), containing 5 mM MgCl₂. Each sample solution was incubated at 95 °C for 5 min and cooled to 30 °C in 60 min, and then incubated at 25 °C for 30 min using a thermal cycler. Subsequently, KCl was added to the sample solution to the final concentration of either 0 or 100 mM. NMR spectra were recorded at 25 °C using a Bruker BioSpin DRX600 and AVANCE III HD 600 spectrometer equipped with a cryogenic probe with a Z-gradient. 2,2-dimethylsilapentane-5-sulfonic acid (DSS) was used as an internal chemical shift reference.

4-6. References

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Chapter 5

Development of Quadruplex Tat-binding RNA Aptamer

5-1. Introduction

5-1-1. Development of K⁺-responsive RNA aptamer

In the proof of concept study, we have demonstrated to switch the activity of hammerhead ribozyme in response to K⁺ by the development of QHR systems. We expect that our method can be applied to other functional nucleic acids such as an RNA aptamer, DNA aptamer, and DNAzyme. As a next step, in this chapter, we also attempted to alter the target-binding activity of RNA aptamer in response to K⁺. There could be situations in which an RNA aptamer that targets either a certain protein or a small molecule should be silent outside the cell to avoid side effects and should work only inside the cell. Our method may have potential to endow an RNA aptamer with these preferred properties. In this study, we employed a Tat-binding RNA Aptamer (TAp)¹ whose 3D structure have been determined by Matsugami *et al.* by using NMR.² We hypothesized that a modified TAp in which a part of sequence is replaced by R11 with linker residues can alter its activity in response to K⁺ via G-quadruplex formation in R11 in the same way as QHR.

5-1-2. HIV Tat and Tat-binding RNAs

Tat is one of the accessory protein of Human Immunodeficiency Virus (HIV). Tat protein is well known as a transcriptional factor to promote the transcription of HIV genome and also as an important pharmacological target. Tat protein enhances the efficiency of viral transcription through binding to the trans-activating region (TAR, Figure 5-1-a) located at the 5'-end of all pre-messenger RNA transcripts. HIV Tat protein is composed of 86 residues and contains a highly basic region.³ The arginine-rich basic region is essential for specific binding to TAR.^{4,5} An arginine-rich short peptide derived from the Tat binds to TAR RNA with specificity and affinity similar to that of the full length of Tat protein.^{4,6}

Previously, a Tat-binding RNA Aptamer (TAp, Figure 5-1-c) that binds Tat 100 times more strongly than the TAR was obtained by means of the *in vitro* selection method. Moreover, TAp inhibited the Tat dependent trans-activation of transcription both *in vitro* and in cultured cell.^{1,2,7} TAp has a similar structural motif of TAR RNA containing unpaired bulge bases (Figure 5-1-c). The bulge residues of TAR and TAp are necessary for specific binding to Tat.¹ In fact, double bulges

5-2. Results

5-2-1. Design of Quadruplex Tat-binding RNA aptamer (QTAp)

TAp has a hairpin structure containing two bulges (Figure 5-1-c) and these bulges are essential for TAp binding to Tat protein. In the present study, we replaced the GAAA sequence which is known to form a stable tetranucleotide loop structure⁸ and a GC base pair located in next to GAAA loop by R11 and uracil residues to design Quadruplex Tat-binding RNA aptamer (QTAp, Figure 5-1-e).

We employed three and two uracil residues as a linker located 5'- and 3'-end of R11, respectively because this combination successfully worked to activate ribozyme in response to K^+ , in the case of QHR.⁹ We expected that QTAp is inactive in the absence of K^+ because the G-quadruplex of R11 is rarely formed and 5'-/3'-subunits of TAp are located away from each other (Figure 5-2-b). On the other hand, in the presence of K^+ , R11 sequence in QTAp folds into the compact G-quadruplex and 5'-/3'-TAp subunits of QTAp are brought into close proximity. Thus 5'-/3'-TAp subunits of QTAp restore the active conformation (Figure 5-2-c) in the same way as the activation of QHR and exhibit Tat-binding activity (Figure 5-2-d).

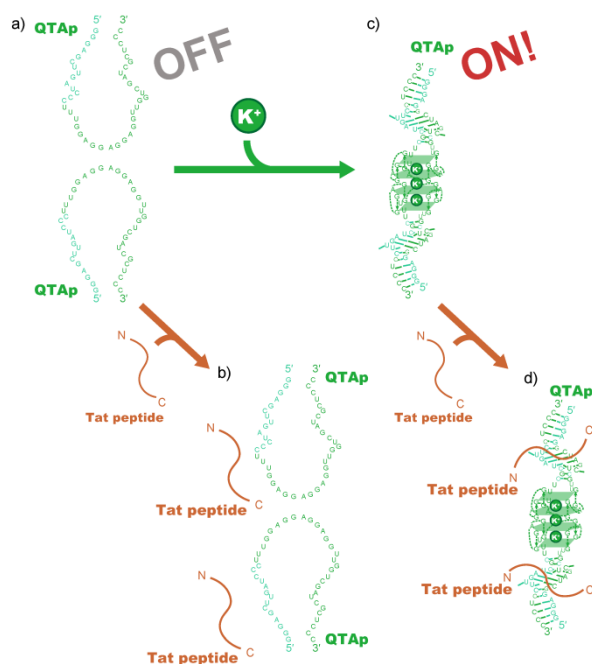


Figure 5-2. The architecture and activation scheme of QTAp. QTAp (green) composed of 5' and 3' TAp sequences connected by R11 sequences with uracil linkers are shown. The Tat peptides are also shown in orange. (a, b) In the absence of K^+ , QTAp does not form the quadruplex structure and not exert Tat-binding activity. (a, c) Addition of K^+ results in activation of QTAp through G-quadruplex formation. (d) QTAp exerts Tat-binding activity via G-quadruplex formation in response to K^+ .

5-2-2. QTAp-binding Assay on Tat using Fluorescence Resonance Energy Transfer

An arginine-rich short peptide derived from the Tat (Tat peptide, aa 49-57, RKKRRQRRR) bound with enhanced specificity to TAR RNA, as shown by Churcher *et al.*¹⁰ In addition, 13-mer peptide containing RKKRRQRRR strongly bound to TAp.² We performed fluorescence resonance energy transfer (FRET) based QTAp-binding assay in the same way as reported by Matsumoto *et al.*¹¹ The FRET based binding assay was performed with non-labeled RNAs and Tat49-57 containing 16-mer peptide (FAMtatTMR, FAM-AAARKKRRQRRRAAAC-TMR) labeled by Carboxyfluorescein (FAM) at its N-terminus and tetramethylrhodamine (TMR) at its C-terminus with alanine and cysteine linkers. The intramolecular dimerization between the fluorescein and rhodamine in aqueous solution leads to a contact quenching of fluorescence.¹¹⁻¹⁴ Also, FAM (donor) and TMR (acceptor) is one of the well characterized and commonly used as a FRET pair. When these dyes keep an appropriate distance (ca. 1-10 nm) from each other, FRET is observed. Due to the flexibility of the Tat peptide, it is possible to assemble into an intramolecular dimer of the dyes, FAM and TMR, and this intramolecular dimerization inactivates FRET¹⁵ (left of Figure 5-3). As the result of intramolecular dimerization of the dyes, the fluorescence emission of free FAMtatTMR was significantly quenched in the absence of RNA ligands. Upon binding with RNA ligands such as TAp, the intramolecular dimer of FAM and TMR dissociates, placing distance between the two dyes and enabling TMR to exert the fluorescence of FRET.^{11,14} In the absence of any RNA ligand, the fluorescence of FAM and TMR of FAMtatTMR was strongly quenched at either 100 mM NaCl (Data not shown) or KCl (left of Figure 5-4, black line). This result is consistent with the previous study by Matsumoto *et al.*¹¹

Next, we measured the fluorescence spectra of the mixture of FAMtatTMR and QTAp either in the presence of K^+ or Na^+ . We used TAp as a positive control that already has been reported to bind to Tat peptide, and del-TAp that already has been reported to show low binding affinity for Tat peptide as a negative control. We also used del-QTAp as a control to examine the influence of G-quadruplex structure itself for Tat-binding activity. The del-TAp and del-QTAp do not have bulge sequences which are essential for Tat-binding activity. In the presence of TAp, fluorescence of FRET acceptor, TMR, at around 575 nm was observed at either 100 mM NaCl (left of Figure 5-4, blue line) or KCl (left of Figure 5-4, red line). On the other hand, in the presence of del-TAp (Figure 5-1-d), any fluorescence was not observed even at 100 mM KCl (left of Figure 5-4, orange line). These results indicate that TAp bound to FAMtatTMR with no ion selectivity and the binding of TAp induced a dissociation of the intramolecular dimer of FAM and TAMRA through the conformational change of FAMtatTMR even in the presence of either 100 mM NaCl or KCl (Figure 5-5-a and b). These results also showed that del-TAp did not bind to FAMtatTMR even in the presence of either 100 mM NaCl or KCl.

In the presence of 100 mM KCl, the addition of QTAp induced strong fluorescence of FRET acceptor (TMR) at around 580 nm (right of Figure 5-4, red line). On the other hand, in the absence of KCl (under 100 mM NaCl condition), the addition of QTAp did not induced fluorescence of FAMtatTMR (right of Figure 5-4, blue line). In the presence of K^+ , the existence of del-QTAp resulted in a little fluorescence enhancement (right of Figure 5-4, orange line). These results indicated that QTAp bound to FAMtatTMR only in the presence of KCl but did not bind to it in the absence of KCl even in the presence of NaCl (Figure 5-5-c and d). At 100 mM KCl, the observation of a little fluorescence even in the presence of del-QTAp which has no bulge sequences of TAP portions suggests that the G-quadruplex structure of R11 slightly bound to FAMtatTMR directly. Both FAM and TMR may interact to G-quadruplex by stacking interaction. The population of FAMtatTMR bound to G-quadruplex structure is ca. 20 %, which indicates that the FAMtatTMR bound to TAP subunits of QTAp is dominant. This is consistent with the design scheme for QTAp in which the R11 portion of QTAp is expected to fold into a compact G-quadruplex upon increasing the K^+ concentration and binds to Tat peptide (Figure 5-2-a, c and d).

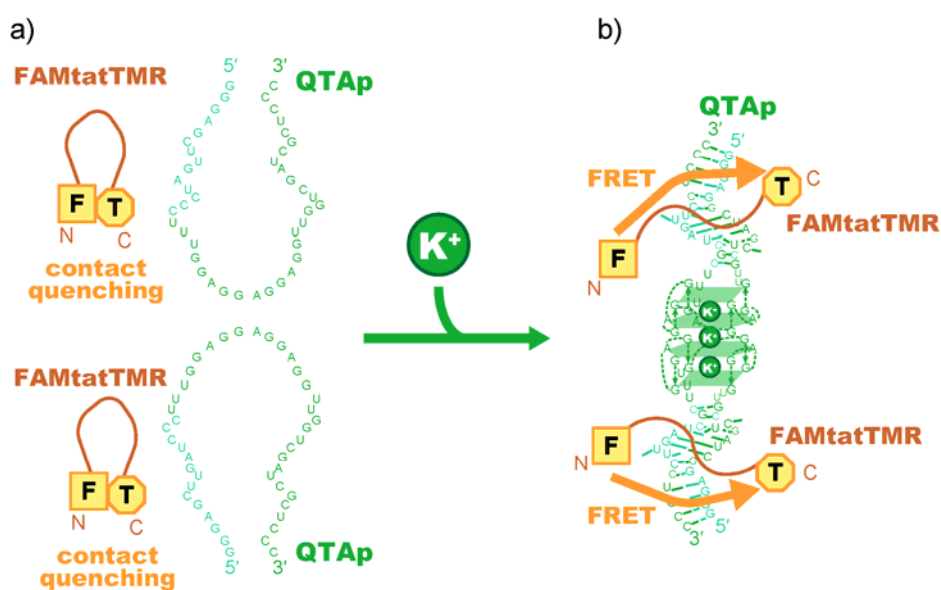


Figure 5-3. Schematic illustration of the principle of FRET based QTAp-binding assay on FAMtatTMR. The FAMtatTMR (orange line) and QTAp (green character) are shown.

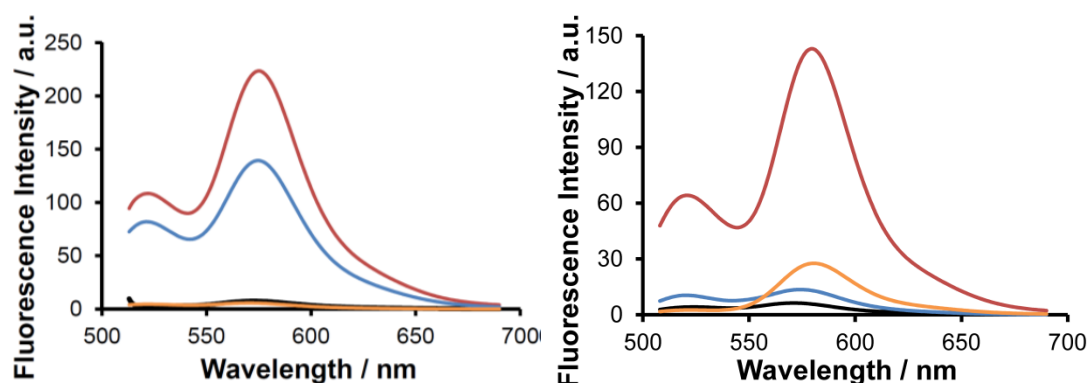


Figure 5-4. (A) Fluorescence spectra of FAMtatTMR in the absence of TAp at 100 mM KCl (black) and in the presence of TAp at 100 mM KCl (red) and 100 mM NaCl (blue). Fluorescence spectrum of FAMtatTMR in the presence of del-TAp at 100 mM KCl (orange). (B) Fluorescence spectra of FAMtatTMR in the absence of QTAp at 100 mM KCl (black) and in the presence of QTAp at 100 mM KCl (red) and 100 mM NaCl (blue). Fluorescence spectrum of FAMtatTMR in the presence of del-QTAp at 100 mM KCl (orange).

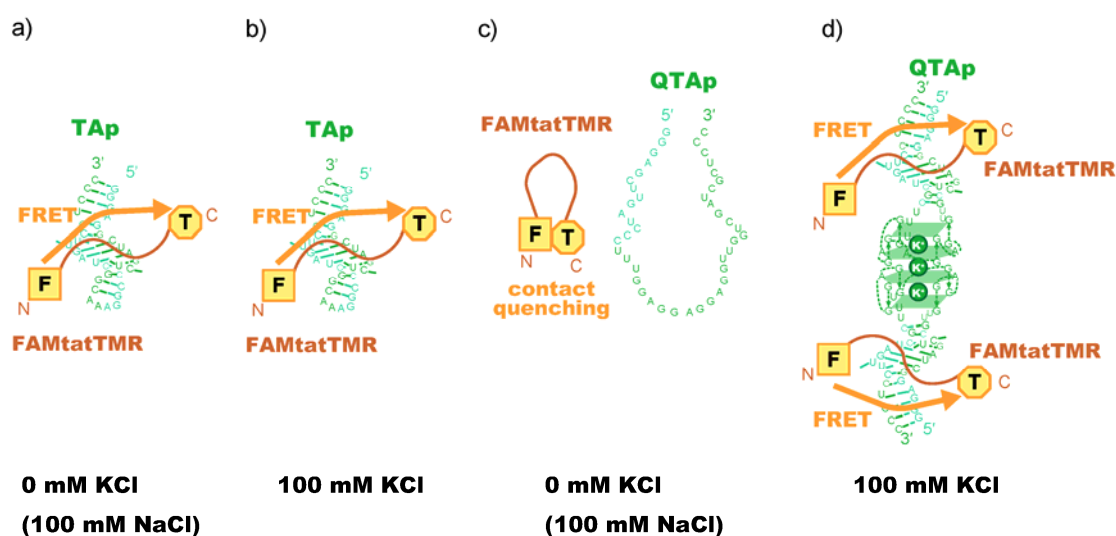


Figure 5-5. Schematic illustration of FRET based QTAp-binding assay on FAMtatTMR. The FAMtatTMR (orange line), TAp subunits of QTAp (purple line), uracil linker residues (red characters) and R11 region of QTAp (green character) are shown.

To evaluate the dissociation constant, K_d , of the binding of QTAp to Tat peptide, we performed the titration experiment. In this study, we titrated the non-fluorescence labeled QTAp to FAMtatTMR and measured the fluorescence intensity at various concentration of QTAp either in the presence of KCl (Figure 5-6-a, filled circle) or NaCl (Figure 5-6-a, open box). We also performed the titration with TAp and FAMtatTMR in the presence of KCl (Figure 5-6-b).

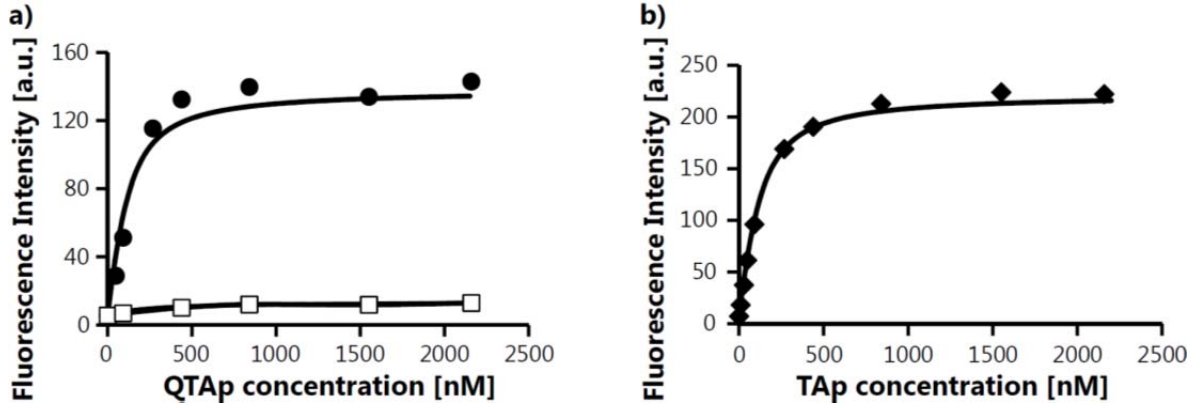


Figure 5-6. The fluorescence intensity derived from TMR of FAMtatTMR in the course of the titration with QTAp (a) and TAp (b) either at 100 mM KCl (filled circle and filled diamond) or 100 mM NaCl (open box).

With the increase in the QTAp concentration, the fluorescence intensity of FAMtatTMR increased (Figure 5-6-a). The increasing fluorescence almost reached the plateau at 800 nM QTAp (in the presence of 10 times excess amount of QTAp compared with FAMtatTMR). In the previous study, TAp binds to Tat peptide with 1:1 stoichiometry.² The K_d value was obtained by the curve fitting using the equation (5-1)¹¹ on the assumption that the binding stoichiometry of QTAp and FAMtatTMR is 1:1.

$$F = F_0 + \Delta F \left(\frac{[\text{RNA}]_0 + [\text{Tat}]_0 + K_d}{\left(([\text{RNA}]_0 + [\text{Tat}]_0 + K_d)^2 - 4[\text{RNA}]_0[\text{Tat}]_0 \right)^{1/2}} - 1 \right) \quad (5-1)$$

where F and F_0 are the fluorescence intensities of the TMR in the presence and the absence of the ligand RNA (QTAp or TAp), respectively. ΔF is the difference between the fluorescence intensity of FAMtatTMR in the presence of an infinite concentration of the ligand RNA (QTAp or TAp) and in the absence of one. $[\text{RNA}]_0$ and $[\text{Tat}]_0$ are the initial concentrations of the ligand RNA (QTAp or TAp) and FAMtatTMR, respectively. As a result of the curve fitting (Figure 5-6-a, filled circle), K_d

value of the binding between QTAp and FAMtatTMR in the presence of KCl was determined to be 6.0×10^{-8} M (Table 5-1). On the other hand, K_d value of the binding between TAp and FAMtatTMR in the presence of KCl was determined to be 6.3×10^{-8} M (Figure 5-6-b, filled diamond and Table 5-1). These results indicate that 5'- and 3'-TAp subunits of QTAp fully restore the binding activity against Tat peptide compared with original TAp.

Table 5-1. Dissociation constants, K_d , of QTAp and TAp at 37 °C, under 100 mM KCl condition.

	K_d (M)
QTAp	6.0×10^{-8}
TAp	6.3×10^{-8}

5-2-3. Structural Analysis of QTAp

The G-quadruplex formation of QTAp in response to K^+ was examined by NMR. Figure 5-7 shows the 1D 1H -NMR spectra of QTAp in the presence of KCl (Figure 5-7-a) or NaCl (Figure 5-7-b).

In the presence of K^+ , the observation of imino proton resonances at 11.2 - 12.0 ppm (Figure 5-7-a) indicates the formation of the tetrad planes of the G-quadruplexes. Moreover, at 12.0 -14.4 ppm, some imino proton resonances were also observed (Figure 5-7-a). This indicates the formation of G:C and A:U base pairs in the stem region of the TAp portion, respectively. These results indicate that in the presence of K^+ , R11 sequence of QTAp folds into G-quadruplex and 5'-/3'-subunits of TAp in QTAp also form stem helices, as we have expected. On the other hand, in the absence of K^+ , there is almost no imino proton resonance at 11.0 - 12.0 ppm (Figure 5-7-b). This result indicates that in the absence of K^+ , QTAp does not form the G-quadruplex structure even in the presence of Na^+ . Unexpectedly, even in the absence of G-quadruplex, there are some imino proton resonances at 12.0 -14.4 ppm (Figure 5-7-b), which suggests the duplex formation of QTAp in the absence of K^+ . We discuss about this structural formation in the absence of K^+ in section 5-3-1. Nevertheless, the formation of G-quadruplex was observed only in the presence of K^+ but Na^+ .

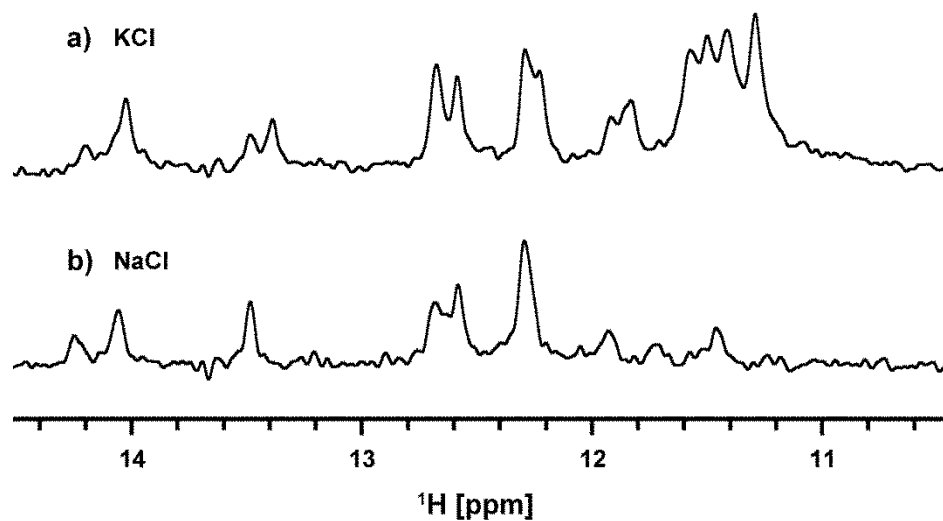


Figure 5-7. Imino proton spectra of QTAP either in the presence of 100 mM KCl or NaCl.

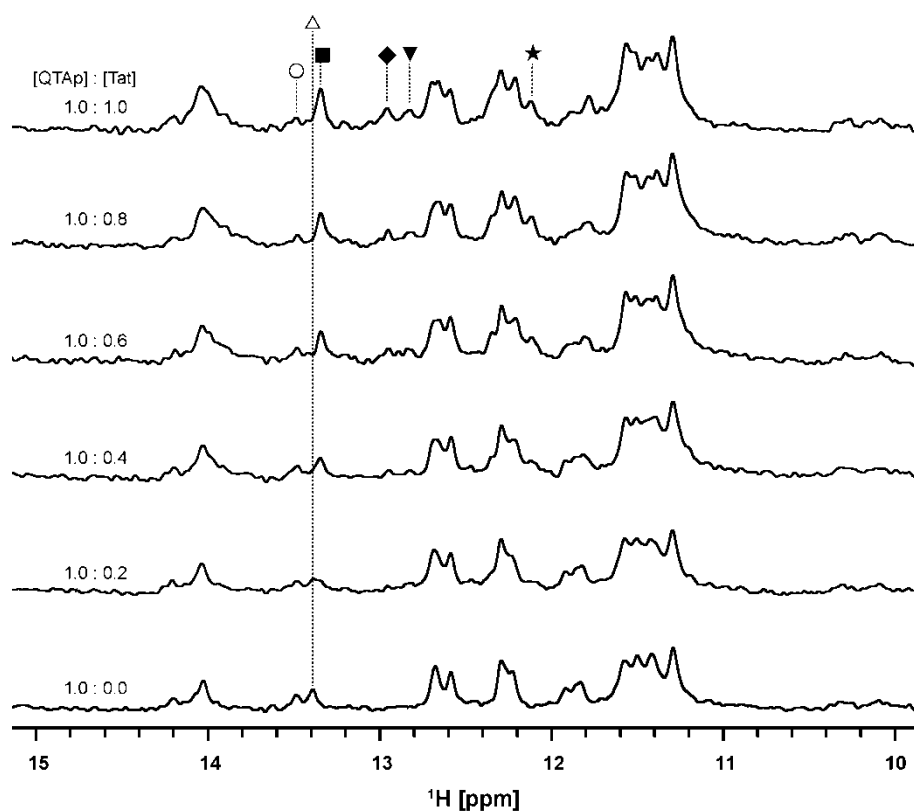


Figure 5-8. The imino proton spectra of QTAP in the course of the titration with Tat peptide at 37 °C in the presence of 100 mM KCl. The molar ratio is indicated at the left.

Next, the titration experiment was performed and each spectrum measured in the presence of various amount of non-labeled Tat peptide (RKKRRQRRR) was shown in Figure 5-8. With increasing the ratio of Tat peptide, intensities of resonances marked $\circ$ and Δ decreased (Figure 5-8) and intensities of a resonances marked $\blacksquare$, $\blacklozenge$, $\blacktriangledown$ and $\star$ increased (Figure 5-8). In the previous study, resonances observed around 13.4 ppm was assigned² to an imino proton of G5 (Figure 5-1-c) and the upfield shift of the resonance upon binding of Tat peptide was reported.¹⁶ In the case of QTAp, the decreasing of the intensities of $\circ$ and Δ resonances ($\circ$ and Δ , Figure 5-8) and the increasing of intensity of $\blacksquare$ resonance ($\blacksquare$, Figure 5-8) seemed to occur coordinately. This result suggests that the resonance marked $\circ$ and Δ originated from free-form of G5 (Figure 5-1-e) and the resonance marked $\blacksquare$ originated from bound-form. The observation of the resonances originated from both free- and bound-forms indicated that free- and bound-forms of QTAp were in slow exchange on the NMR time scale and this is consistent of the previous study.^{1,2, 16}

We also performed the course of titration experiment at 100 mM NaCl with non-labeled Tat peptide (Figure 5-9). With increasing the ratio of Tat peptide, the course of spectra changed. Especially, the resonances of imino proton of G5 (Figure 5-1-e) were changed ($\circ$ and $\blacksquare$, Figure 5-9). This result suggests that QTAp bound to Tat peptide even in the absence of K^+ . We discuss about this result in section 5-3-1.

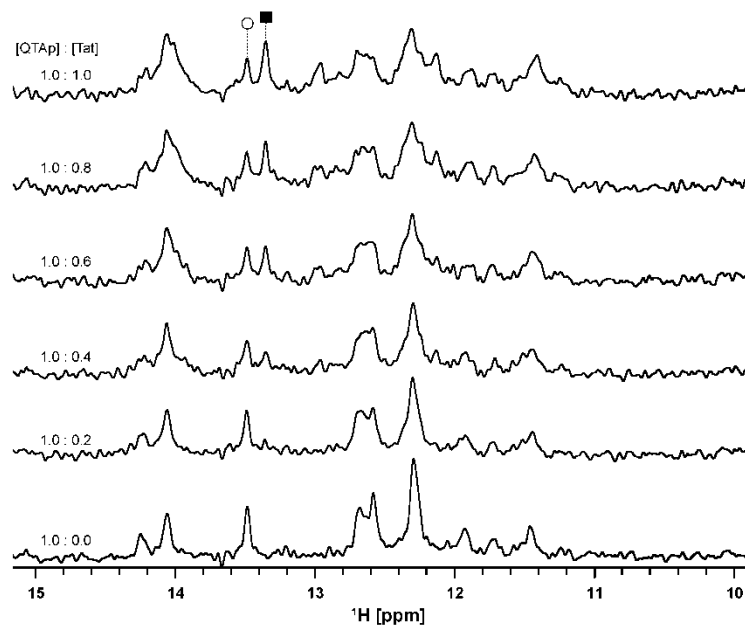


Figure 5-9. The imino proton spectra of QTAp in the course of the titration with Tat peptide at 37 °C in the presence of 100 mM NaCl. The molar ratio is indicated at the left.

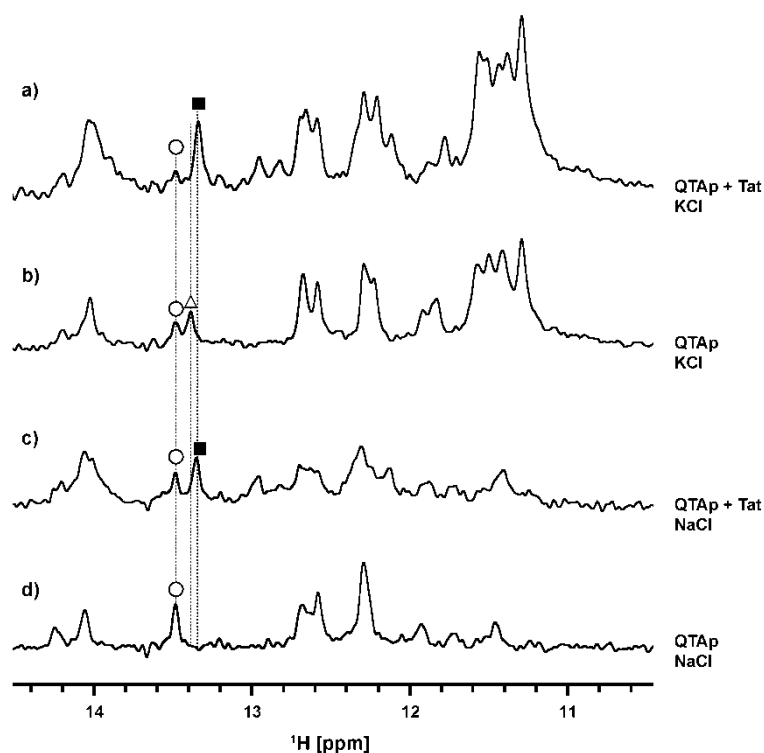


Figure 5-10. Imino proton spectra of QTAp either with Tat peptide (a) or without Tat peptide (b) in the presence of 100 mM KCl. Imino proton spectra of QTAp either with Tat peptide (c) or without Tat peptide (d) in the presence of 100 mM NaCl.

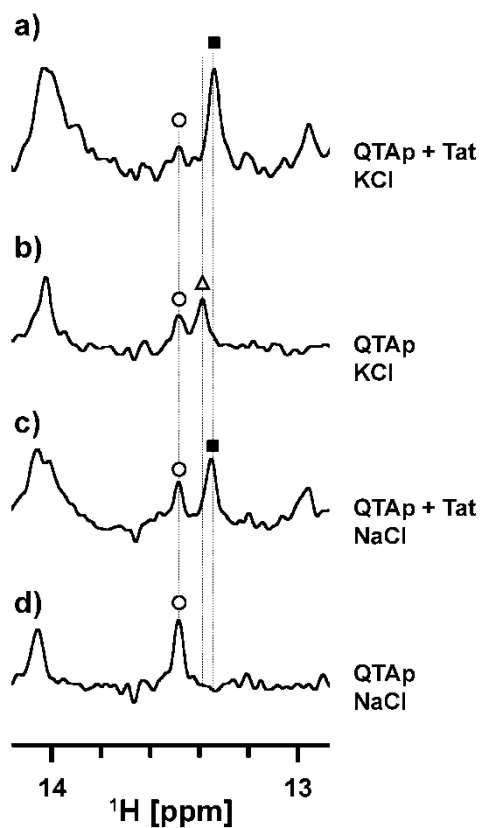


Figure 5-11. Expansions of the 14.0-13.0 ppm region of the imino proton spectra are shown.

5-3. Discussion

5-3-1. Origin of the High and Low Binding Activity of QTAp in the Presence and Absence of K^+ , Respectively, in Structural Aspects

In the absence of K^+ , the imino proton resonances originated from G-quadruplex were not observed at 11.0 - 12.0 ppm (Figure 5-10-c and d), which indicates that in the absence of K^+ , the G-quadruplex structure of QTAp was not formed. Even though there is no G-quadruplex, the imino proton resonances were observed at 12.0 -14.4 ppm, which indicates QTAp formed a particular structure being composed of Watson-Crick base pairs, G:C and A:U (Figure 5-10-c and d). There are some differences, especially, about the imino proton resonance of G5 between in the presence and absence of K^+ (Figure 5-10 and 5-11). In the absence of Tat peptide, two ($\circ$ and Δ , Figure 5-11-b) and one ($\circ$, Figure 5-11-d) resonances of G5 were observed at 100 mM and 0 mM KCl, respectively. They formed similar but partially different structure each other. We expect that the resonances indicated by $\circ$ and Δ originated from a low and high affinity structures, respectively. Actually, in the presence of K^+ , with increasing the ratio of [Tat]:[QTAp] from 0:1 to 1:1, the intensity of the resonance marked Δ disappeared (compared with Figure 5-11-a and b) and the intensity of the resonance marked $\blacksquare$ appeared. This result indicates that the high affinity structure of QTAp which gives the resonance Δ completely bound to Tat peptide and forms QTAp-Tat complex which gives the resonance $\blacksquare$ at the ratio of [Tat]:[QTAp] = 1:1. On the other hand, approximately 50 % of the intensity of resonance indicated by $\circ$ was remaining even at the ratio of [Tat]:[QTAp] = 1:1 (compared with Figure 5-11-a and b). This result indicates that a half of low affinity structure of QTAp which gives the resonance $\circ$ bound to Tat peptide and forms the QTAp-Tat complex which gives the resonance $\blacksquare$, while the half of low affinity one was still alone at the ratio of [Tat]:[QTAp] = 1:1. In the absence of K^+ , a half of intensity of resonance $\circ$ was also remaining (compared with Figure 5-11-c and d). This result also indicates that the resonances $\circ$ originated from a low affinity structure which was remaining even at the ratio of [Tat]:[QTAp] = 1:1. The resonance $\circ$ originated from the low affinity structure was observed both in the presence and absence of K^+ (Figure 5-11-d), while the one Δ originated from the low affinity structure was only observed in the presence of K^+ (Figure 5-11-b). Thus, we expect that the high population of the high affinity structure is the origin of the high Tat-binding activity of QTAp in the presence of K^+ .

In the previous study, it is proposed that TAP forms two U:A:U base triples when TAP binds to the peptide originated from Tat.^{1,2} In the case of TAR^{15,17,18} and TAP,^{1,2} the base triples and the phosphate groups which are adjacent to the base triples were reported to be important for the recognition of Tat. The double base triple motifs of the high affinity structure may be smoothly induced by Tat peptide but not from the low affinity one. We expect that in the case of high affinity

structure, the 5'- and 3'-TAp subunits of QTAp fully hybridized each other, except the bulge sequences (U7, U8, U33 and C34, Figure 5-1-e), due to the stabilization by the G-quadruplex of R11. On the other hand, in the case of the low affinity structure, it may not fully form the base pairs, especially C13:G32 and C14:G31 (Figure 5-1-e), without the stabilization by G-quadruplex formation.

FRET based binding assay showed that QTAp did not bind to Tat peptide in the absence of K^+ . Although, the titration experiment using NMR indicated that QTAp bound to Tat peptide both in the presence and absence of K^+ . In the absence of K^+ , QTA formed the particular structure which has low (but not no) binding activity as to the Tat peptide. In the case of NMR measurements, a binding between high concentration Tat peptide ($\sim 50 \mu M$) and QTAp ($\sim 50 \mu M$) in the low affinity structure can occur.

Nevertheless, both the formation of high affinity structure which is stabilized by G-quadruplex formation and the resultant high Tat-binding activity were observed only in the presence of K^+ but not Na^+ .

5-3-2. Relationship with Previous Studies and Possibility of Application

In the present study, we have developed a K^+ -responsive RNA aptamer making use of the G-quadruplex formation of RNA. In the previous study, including our group, G-quadruplexes are reportedly used for the engineering of molecular probes and functional nucleic acids, and in each case the G-quadruplex formation plays important roles in the regulation of functions, such as FRET efficiency,^{19,20} ribozyme activity,^{9,21} DNAzyme activity,²²⁻²⁴ and electrochemical property.^{25,26} To the best of our knowledge, however, there is no report of K^+ -responsive RNA aptamer and our QTAp is the first one that switches the target-binding activity through the G-quadruplex formation in response to K^+ .

In the present study, we demonstrated that the binding activity of QTAp was induced by K^+ , but not by Na^+ . Considering the development of new RNA aptamer which sense the difference of cellular environment, this ion selectivity is essential because of the large amount of existence of Na^+ outside the cell. There could be situations in which an RNA aptamer that targets either a certain protein or a small molecule should be silent outside the cell to avoid side effects and should function only inside the cell. Our method has potential to afford an RNA aptamer with these required properties.

QTAp was developed using a Tat-binding RNA aptamer, but this principle can be applied to other functional nucleic acids such as a DNA aptamer, DNAzyme, and also artificially modified oligo nucleotides. In particular, the artificially modified oligo nucleotides such as 2'-OMe RNA, LNA,

PNA, etc., have high RNase resistance and this property is useful for development of cellular application.

5-4. Conclusion

Here, we constructed the novel K^+ -responsive RNA aptamer QTAp using Tat-binding RNA aptamer and R11. In the fluorescence experiment, QTAp only exhibit its binding activity in the presence of K^+ but not in the presence of Na^+ . The results of NMR experiments indicate that the additional structural analysis is needed to evaluate the design scheme. Nevertheless, both the formation of high affinity structure which is stabilized by G-quadruplex formation and the resultant high Tat-binding activity were observed only in the presence of K^+ but not Na^+ . It can be a prototype of the development of K^+ -responsive RNA aptamer which is activated by K^+ only when it enters inside the cell.

5-5. Experimental Procedures

Materials

All RNAs were synthesized, purified, and de-salted by FASMAC Co., Ltd. (Kanagawa, Japan). All peptide, including fluorescence labeled one, were synthesized, purified by Toray Research Center, Inc. (Tokyo, Japan).

Fluorescence Resonance Energy Transfer (FRET) based QTAp-binding assay

FAMtatTMR (FAM-AAARKKRRQRRRAAAC-TMR) was dissolved at the final concentration of 100 nM in 50 mM Tris-HCl buffer (pH 8.0), containing 100 mM KCl (or NaCl). Fluorescence spectra were recorded at 37 °C using a FP-8500 Spectrofluorometer (JASCO, Japan).

In the titration experiments, QTAp was dissolved at the final concentration of 10 or 100 μ M in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl (or NaCl). Each sample solution was incubated at 95 °C for 5 min and cooled to 37 °C in 10 min using a thermal cycler.

Nuclear magnetic resonance (NMR) measurements

QTAp was dissolved at the final concentration of 70 μ M in 5 mM sodium phosphate buffer (pH 6.5). Each sample solution was incubated at 95 °C for 5 min and cooled to 37 °C in 10 min using a thermal cycler. Subsequently, KCl (or NaCl) was added to the sample solution to the final concentration of 100 mM. NMR spectra were recorded at 37 °C using a Bruker BioSpin DRX600

and AVANCE III HD 600 spectrometer equipped with a cryogenic probe with a Z-gradient. 2,2-dimethylsilapentane-5-sulfonic acid (DSS) was used as an internal chemical shift reference.

In the titration experiments, non-fluorescence labeled Tat peptide (RKKRRQRRR) was added step-by-step to QTAp in a solution comprising 5 mM sodium phosphate buffer (pH 6.5) and 100 mM of KCl (or NaCl) at 37 °C

5-6. References

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Chapter 6

General Conclusion

6-1. General Conclusion

In this study, we developed the novel functional RNA that switched its activity in response to K^+ via G-quadruplex formation of the GGA repeat sequence, R11. In proof of concept study, the hammerhead ribozyme and Tat-binding RNA aptamer were employed to develop the prototypes of K^+ -responsive functional RNAs, named quadruplex hammerhead ribozyme (QHR) and quadruplex Tat-binding RNA aptamer (QTAp). Moreover, we proposed methods to repress the residual activity in the absence of K^+ especially for QHR system. As a result of the repression of the residual activity, the switching efficiency which is defined as the ratio of the activity between in the presence and absence of K^+ increased significantly.

In chapter 2, we develop the split QHR (spQHR) to repress the residual activity in the absence of K^+ . The origin of the residual activity was expected that the active core structure is formed partially and/or transiently even in the absence of G-quadruplex formation. We hypothesized that the covalent connection of 5'- and 3'-HR subunits through R11 can contribute to form the active core partially and/or transiently. To keep the 5'- and 3'-HR subunits away from each other, we split QHR in the middle of R11 region and named them spQHRs. The residual activity of spQHRs was significantly reduced to one thirty-first as compared with in the case of the original QHR in the absence of K^+ . On the other hand, in the presence of K^+ , the activity of spQHRs was enhanced to 3.1-fold. Although this strategy succeeded to suppress the residual activity, the maximum activity in the presence of K^+ was also repressed. As a next step, we tried to achieve both a high maximum activity and low residual activity.

In chapter 3, we also proposed and utilized the method to repress the residual activity by inhibition of the partial and/or transient active core structure. To achieve this, we introduced a complementary strand RNA (CS) against QHR. The CS inhibits to form an active conformation by the formation of duplex with QHR. The CS which is complement to both R11 and 5'-HR sequence of QHR and have three base mismatches most successfully worked. The introduction of the CS effectively repressed the residual activity. Importantly, in the presence of K^+ , the activity of QHR was almost fully restored in QHR-CS system. QHR in CS system retained ca. 90 % activity of free QHR, while spQHR retained only 4 % activity of original QHR. The introduction of the CS enabled us to achieve both a high maximum activity and low residual activity. The high and low activity in the presence and absence of K^+ , respectively, result in the activity switching with high contrast.

In chapter 4, we developed a new class of complementary strand DNA which does not require an

annealing treatment. QHR-CS system has a weak point, which requires both K^+ and an annealing treatment to switch the activity of QHR. The new complementary DNA, containing some G-stretches, can form G-quadruplex in the presence of K^+ . The quadruplex-forming complementary DNA strand (QCS) forms the duplex with QHR in the absence of K^+ . On the other hand, it forms G-quadruplex in the presence of K^+ . The quadruplex formation of QCS inhibited the reformation of the duplex with QHR in the presence of K^+ and enable QHR to fold into active quadruplex structure. Thus QHR-QCS system can switch its activity without annealing treatment. This is the desired property for the development of cellular application which senses the differences between intra- and extracellular environments.

In chapter 5, we developed the prototype of K^+ -responsive RNA aptamer. We used Tat-binding RNA aptamer to design K^+ -responsive RNA aptamer and named it quadruplex Tat-binding RNA aptamer (QTAp). QTAp only bound to Tat peptide in the presence of K^+ . We demonstrated that the strategy of the development of K^+ -responsive functional RNA using R11 is applicable to not only ribozymes but also RAN aptamers.

In this study, we succeeded to switch the activity of the ribozyme and aptamer in response to K^+ . Furthermore we proposed and utilized the concept of spQHR, CS, and QCS to repress the residual activity of QHR. Especially, introduction of CS or QCS dramatically improved the switching efficiency of QHR. These concepts should be applicable to any K^+ -responsive functional RNAs. The progresses in this thesis may lead to the development new class of functional RNA which can sense the difference between intra- and extracellular environments and then switch its function. This new class of functional RNAs may be useful to develop a new efficient DDS, biosensors, and low side effect drugs.

To internalize our designed ribozyme and aptamer into cells, we plan to employ a methodology for DDS using RNA aptamer. In the previous study, McNamara and coworkers reported cell-type specific DDS using the conjugate of siRNA (drug) and RNA aptamer (carrier) which binds to the target membrane protein.^{1,2} Upon the aptamer binding to the membrane protein, aptamer-siRNA chimera is taken up by the target cells through the endocytosis. While the triple complex of QHR, siRNA and RNA aptamer which binds to a membrane protein is silent outside the cell, this complex sense the K^+ and activate itself only after the internalization into cells through the aptamer binding to the membrane protein. The activated QHR can cleave itself and separate the drugs from the carrier aptamer which can hinder a proper delivery of the drug inside the cell. The system can be a more efficient DDS which can release the drugs when it internalizes into target cells.

6-2. References

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List of Publications

Publications that constitute this thesis

1.

Boosting of activity enhancement of K^+ -responsive quadruplex hammerhead ribozyme

Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata, and Masato Katahira

Chemical Communications, (2015) **51**, 5898-5901.

(Chapter 2, 3)

2.

K^+ -responsive off-to-on switching of hammerhead ribozyme through dual G-quadruplex formation requiring no heating and cooling treatment

Yudai Yamaoki, Takashi Nagata, Tsukasa Mashima, and Masato Katahira

Biochemical and Biophysical Research Communications, (2015) **468**, 27-31.

(Chapter 4)

3.

The development of Quadruplex Tat-binding RNA aptamer whose activity switches on in response to K^+

Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata and Masato Katahira

In preparation

(Chapter 5)

Other Publication

1.

An FT-IR Study on Packing Defects in Mixed β -Aggregates of Poly(L-glutamic acid) and Poly(D-glutamic acid): A High-Pressure Rescue from a Kinetic Trap

Yudai Yamaoki, Hiroshi Imamura, Aleksandra Fulara, Sławomir Wójcik, Łukasz Bożycki, Minoru Kato, Timothy A. Keiderling, and Wojciech Dzwolak

The Journal of Physical Chemistry B (2012) **116**, 5172-5178.

List of Presentations

International Conferences

1.

Pressure effects on the secondary structure of a β -hairpin model peptide: Trpzip4

○Yudai Yamaoki, Keisuke Fujimura, Hiroshi Imamura, Eri Chatani, Minoru Kato

2010 International Chemical Congress of Pacific Basin Societies (Pacifichem 2010), December 15-20, 2010, Honolulu, Hawaii, USA

2.

The relationship between the abilities of amyloid fibril formation and turn structure formation: Study of amyloid- β peptide fragment A β_{19-34} and its mutants with high turn propensity

○Yudai Yamaoki, Eri Chatani, Hiroshi Imamura, Eisuke Takao, Minoru Kato

6 th International Meeting on Biomolecules under Pressure (IMBP 2011), December 12-16, 2011, Shiga, Japan

3.

Development of intelligent ribozyme whose activity switches on in response to K^+ via quadruplex formation

○Yudai Yamaoki, Tsukasa Mashima, Yu Sakurai, Yukari Hara, Takashi Nagata, Masato Katahira

The 39th International Symposium on Nucleic Acids Chemistry (ISNAC 2012), November 15-17, 2012, Nagoya, Japan

4.

Dual-quadruplex forming ribozyme distinctly switches on its activity in response to K^+

○Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata, Masato Katahira

The 40th International Symposium on Nucleic Acids Chemistry, November 13-15, 2013, Kanagawa, Japan

5.

Creation of novel Tat-binding aptamer and ribozyme whose activities switch on in response to K^+ via quadruplex formation

○Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata, Masato Katahira

The 41th International Symposium on Nucleic Acids Chemistry, November 5-7, 2014, Fukuoka, Japan

Domestic Conferences

1.

β ヘアピンモデルペプチド Trpzip4 の二次構造に及ぼす圧力効果

藤村 契介, ○山置 佑大, 今村 比呂志, 加藤 稔

第 11 回日本蛋白質科学会年会, 2011 年 6 月 7-9 日, 大阪

2.

GB1 ペプチドアミノ酸残基置換体のコンフォメーションに及ぼす圧力効果

○藤村 契介, 山置 佑大, 今村 比呂志, 加藤 稔

第 52 回 高圧討論会, 2011 年 11 月 9-11 日, 沖縄

3.

天然状態の α -lactalbumin と Thioflavin T の結合

○今村 比呂志, 山置 佑大, 加藤 稔

神戸大学先端融合科学シンポジウム「タンパク質アセンブリ ー会合、超分子化、凝集ー」, 2012 年 1 月 31 日-2 月 1 日, 神戸

4.

アミロイド β_{19-34} 断片と変異体モノマーのターン構造形成能とアミロイド線維形成能

○山置 佑大, 茶谷 絵理, 今村 比呂志, 高尾 英佑, 加藤 稔

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5.

天然状態の α -lactalbumin と Thioflavin T の複合体形成

○今村 比呂志, 山置 佑大, 加藤 稔

第 12 回 日本蛋白質科学会年会, 2012 年 6 月 20-22 日, 愛知

6.

カリウムイオン依存的四重鎖形成によってリボザイム活性をオンにする機能性核酸の創製

○山置 佑大, 真嶋 司, 櫻井 佑, 原 ゆかり, 永田 崇, 片平 正人

第 30 回 タンパク質・核酸構造理論勉強会, 2012 年 7 月 6-7 日, 京都

7.

カリウムイオン濃度を感知して活性がオンになるインテリジェントリボザイムの創製

○山置 佑大, 真嶋 司, 櫻井 佑, 原 ゆかり, 永田 崇, 片平 正人
第 14 回 日本 RNA 学会年会, 2012 年 7 月 18-20 日, 宮城

8.

Potential Assessment of Establishing Photovoltaic System in Kyoto University Main Campus

Mohamed Omer, Emi Yamakawa, Yu Qin, Mohd Radzi Abu Mansor, ○Kengoh Kuroda, Ha Yoo Sung, Yudai Yamaoki, Hao Min Sun, Le Hoang Long, Takeo Kotake and Nuki Agya Utama
Seminar on GCOE Group Research, August 3, 2012, Kyoto

9.

Development of intelligent ribozyme whose activity switches on in response to K^+ via quadruplex formation

○Yudai Yamaoki, Tsukasa Mashima, Yu Sakurai, Yukari Hara, Takashi Nagata, Masato Katahira
第 50 回 日本生物物理学会年会, 2012 年 9 月 22-24 日, 愛知

10.

Creation of novel ribozyme whose activity switches on in response to K^+ via quadruplex formation

○Yudai Yamaoki, Tsukasa Mashima, Yu Sakurai, Yukari Hara, Tsutomu Kodaki, Takashi Nagata, Masato Katahira

第 35 回 日本分子生物学会年会, 2012 年 12 月 11-14 日, 福岡

11.

カリウムイオン濃度依存的四重鎖形成によって自律的に活性をスイッチングする新規リボザイムの開発

○山置 佑大

京都大学グローバル COE プログラム 地球温暖化時代のエネルギー科学拠点 平成 24 年度
年次報告会, 2013 年 1 月 16 日, 京都

12.

Estimation of LCA for Gas-Cooled Fast Reactor

Ha Yoo Sung, Takeo Kotake, Yudai Yamaoki, Kengoh Kuroda, Yu Qin, Hao Min Sun, Le Hoang Long, Feng Wu, Mohamed Omer, ○Emi Yamakawa, Mohd Radzi Abu Mansor and Agya Utama
京都大学グローバル COE プログラム 地球温暖化時代のエネルギー科学拠点 平成 24 年度
年次報告会, 2013 年 1 月 16 日, 京都

13.

Development and improvement of quadruplex ribozyme which switches its activity in response to K^+

○Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata, Masato Katahira

第 15 回日本 RNA 学会年会, 2013 年 7 月 24-26 日, 愛媛

14.

カリウムイオン依存的に活性の ON/OFF を制御する四重鎖リボザイムの創製：四重鎖形成相補鎖によるスイッチング能の向上

○山置 佑大, 真嶋 司, 永田 崇, 片平 正人

第 53 回生命科学夏の学校, 2013 年 8 月 30 日-9 月 1 日, 静岡

15.

カリウムイオン依存的に活性の ON/OFF を制御する四重鎖リボザイムの創製：四重鎖形成相補鎖によるスイッチング能の向上

○山置 佑大, 真嶋 司, 永田 崇, 片平 正人

RNA Frontier Meeting 2013, 2013 年 9 月 3-5 日, 静岡

16.

Aptamer that exerts anti-prion activity and intelligent ribozyme whose activity switches in response to K^+

○Masato Katahira, Tsukasa Mashima, Yudai Yamaoki, Takashi Nagata, Fumiko Nishikawa, Satoshi Nishikawa, Yuji Kamatari, Kazuo Kuwata

The 51st Annual Meeting of the Biophysical Society of Japan, September 25-27, 2013 Kyoto

17.

Pressure and Temperature variable FTIR study on the structural stability of β -hairpin model peptides of mutants of GB1(41-56)

○Keita Tsuchiya, Yudai Yamaoki, Minoru Kato

The 51st Annual Meeting of the Biophysical Society of Japan, September 25-27, 2013 Kyoto

18.

Enhancement of a Quadruplex-ribozyme activity in response to K^+ : a quadruplex-forming complementary DNA enables accurate switching

○Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata, Masato Katahira

The 51st Annual Meeting of the Biophysical Society of Japan, September 25-27, 2013, Kyoto

19.

Development of quadruplex hammerhead ribozyme which switches on its ribozyme activity: introduction of a quadruplex-forming complementary DNA to repress a basal activity

○Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata, Masato Katahira

The 36th Annual Meeting of the Molecular Biology Society of Japan, December 3-6, 2013, Hyogo

20.

K⁺依存的に活性の ON/OFF を制御する人工機能性核酸の創製

○山置 佑大, 真嶋 司, 永田 崇, 片平 正人

超異分野若手シンポジウム 2014, 2014 年 3 月 22 日, 大阪

21.

Development of Tat binding aptamer and ribozyme which switch their activities in response to K⁺

○山置 佑大, 真嶋 司, 永田 崇, 片平 正人

第 15 回若手 NMR 研究会, 2014 年 7 月 11-13 日, 千葉

22.

カリウムイオンを感知して自らの活性をスイッチングする Tat 捕捉アプタマーおよびリボザイムの創製

○山置 佑大, 真嶋 司, 永田 崇, 片平 正人

第 16 回日本 RNA 学会年会, 2014 年 7 月 23-25 日, 愛知

23.

Development of Tat binding aptamer and ribozyme which switch their activities in response to K⁺

○Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata, Masato Katahira

The 52st Annual Meeting of the Biophysical Society of Japan, September 25-27, 2014, Hokkaido

24.

CCND1 転写抑制とテロメア短縮をもたらす TLS/FUS による非コード RNA と DNA 認識機構の NMR 法による解明

○近藤 敬子, 真嶋 司, 山置 佑大, 大吉 崇文, 黒川 理樹, 永田 崇, 片平 正人

第 37 回日本分子生物学会年会, 2014 年 11 月 25-27 日, 神奈川

25.

Invention of Tat-binding aptamer and hammerhead ribozyme which switch their functions in

response to K^+

○山置 佑大, 真嶋 司, 永田 崇, 片平 正人

第9回バイオ関連化学シンポジウム, 2015年09月10-12日, 熊本

List of Honors

1.

JSPS Research Fellowship (DC2)

Japan Society for the Promotion of Science, April 1, 2013 – March 31, 2014

2.

第 15 回若手 NMR 研究会 優秀ポスター賞

第 15 回若手 NMR 研究会, 2014 年 7 月 13 日

3.

第 16 回日本 RNA 学会年会 優秀賞（口頭発表）

日本 RNA 学会, 2014 年 8 月 1 日

4.

平成 26 年度エネルギー理工学研究所表彰 学生賞

京都大学エネルギー理工学研究所, 2015 年 3 月 6 日

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