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전반사 형광 현미경법을 이용한 10–23 디옥시라이보자임의 효소적 메커니즘과 동역학에 대한 단분자 연구

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Abstract


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Total internal reflection fluorescence (TIRF) microscopy, with the aid of the fluorescence resonance energy transfer (FRET) phenomenon, enables the real-time observation of nanometer-scale structural changes, which are often hidden in ensemble measurements, from a fluorescently labeled single biomolecule. Using TIRF microscopy, we investigated the stepwise enzymatic reaction mechanism of 10–23 deoxyribozyme, an RNA-cleaving DNA enzyme which has been widely studied in various fields of applied science.
First, we analyzed the kinetics of the four normal reaction sub-steps of 10-23 deoxyribozyme of enzyme-substrate binding, substrate cleavage, and dissociations of each of the two products by varying the molecular environments specifically the temperature, the pH, and the residue of the cleavage site. Kinetic parameters and rates depending on the environmental changes revealed significant features with regard to the molecular mechanisms of each reaction.

Secondly, we identified two additional individual reaction sub-steps which involve the half-binding of a new substrate before the second product dissociation that we termed “shortcut binding” and the subsequent strand displacement of the remaining product by the half-bound substrate. We determined that shortcut binding and strand displacement can occur and conducted a kinetic analysis of each reaction sub-step while varying the substrate concentration. Shortcut binding occurs more frequently at a higher substrate concentration and the rate of shortcut binding and the second dissociation are dependent on the substrate concentration, as the two reactions compete with each other. We calculated the rate equation, noting that shortcut binding accelerates the enzymatic turnover rate.

To sum up, by adapting TIRF, we revealed the characteristics of 10-23 deoxyribozyme. These may be essential in the study of the chemical mechanisms and for explorations of the wider use of catalytic nucleic acids.
Keywords: 10–23 Deoxyribozyme, Kinetics, Enzymatic Mechanism, Single-Molecule, Total Internal Reflection Fluorescence Microscopy (TIRFM), Fluorescence Resonance Energy Transfer (FRET)

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# Table of Contents

**Abstract**

1. Introduction...........................................................................................................1

2. Basic Principles
   2.1 Fluorescence Resonance Energy Transfer (FRET).......4
   2.2 Total Internal Reflection Fluorescence Microscopy (TIRFM)
      2.2.1 Total internal reflection and evanescent wave.......8
      2.2.2 Prism-type TIRFM setup..............................................11
      2.2.3 Quartz surface preparation for sample immobilization........................................14
   2.3 10-23 Deoxyribozyme...............................................................................15
   2.4 References ......................................................................................................18

3. The Effects of the Temperature, pH, and Unpaired RNA Residue on the Kinetics of the Four Reaction Sub-Steps of 10-23 Deoxyribozyme
   3.1 Introduction...................................................................................................21
3.2 Experimental

3.2.1 Sample preparation: oligonucleotides..............................24

3.2.2 Imaging buffer....................................................................26

3.2.3 Data acquisition and analysis..............................................29

3.3 Results and Discussion

3.3.1 Reaction sub-steps: binding, cleavage, dissociation1, and dissociation2..........................32

3.3.2 The transition states for binding, cleavage, and sequential dissociation reactions..............36

3.3.3 The effects of the pH and unpaired RNA residue on reaction rates....................................43

3.4 Conclusion.............................................................................49

3.5 References.............................................................................50


4.1 Introduction............................................................................53

4.2 Experimental..........................................................................56

4.3 Results and Discussion
4.3.1 TIRF measurements of reaction pathways including shortcut binding and strand displacement..............59

4.3.2 Verifying shortcut binding and strand displacement.........................................................64

4.3.3 Dependence of rates for each reaction sub-step on the substrate concentration.................................70

4.3.4 The rate equations: implications on the catalytic turnover of 10–23 deoxyribozyme..............................77

4.4 Conclusion..........................................................................................................................87

4.5 References..........................................................................................................................88

5. Appendix: The Effects of Glycerol on the Kinetics of the Four Reaction Sub-steps of 10–23 Deoxyribozyme

5.1 Introduction.......................................................................................................................90

5.2 Experimental......................................................................................................................92

5.3 Results and Discussion.......................................................................................................96

5.4 Conclusion........................................................................................................................102

5.5 References........................................................................................................................103

국문초록

Acknowledgement(감사의 글)
1. Introduction

Single-molecule fluorescence resonance energy transfer (smFRET) methods have assisted with the remarkable progress made in understanding the structures, reaction mechanisms and molecular interactions of many biomolecules, such as proteins, lipids, and nucleic acids, which are difficult to understand with typical ensemble measurements. Total internal reflection fluorescence (TIRF) microscopy, first established by Daniel Axelrod in the early 1980s, is one of the most popular and widely used single-molecule techniques. It utilizes an optical effect, in this case an evanescent wave, to study molecular and cellular phenomena at liquid/solid interfaces. This technique enables the real-time tracking of a single molecule to provide valuable information on folding dynamics, interactions, and kinetics. Many interactions between biomolecules and enzymatic reactions have been successfully studied by the TIRF-FRET method.

Among the biological macromolecules, DNA can exhibit enzymatic activity analogous to RNA enzymes, i.e., ribozymes. These types of catalytic DNA are referred to as deoxyribozymes. Various types of deoxyribozymes that catalyze, for instance, RNA/DNA cleavage, ligation, and self-phosphorylation reactions have been identified by in vitro selection. Owing to their high stability and
modest cost, deoxyribozymes have been widely studied in various fields of science.

10–23 Deoxyribozyme is one of the most actively studied DNA enzymes. It catalyzes the cleavage of RNA by transesterification at the phosphorus located between an unpaired purine and paired pyrimidine residue. This single-stranded DNA recognizes its single-stranded RNA substrate by binding via Watson–Crick base pairing. A conserved sequence of the catalytic core between the two binding arms is known to play an essential role in the cleavage reaction. Many ensemble experiments have been conducted in an effort to identify its chemical and biological properties, but there are limits to these efforts, such as ensemble averaging and insufficient structural information with regard to the reaction pathway.

Using the TIRF–FRET method, observations of each of the main sequential reaction steps on the mechanism of 10–23 deoxyribozyme is straightforward via a distinct sequence of the FRET value indicating structural changes. The main reaction steps are enzyme–substrate binding, substrate cleavage, and the sequential dissociation of the two products. Here, taking advantage of the ability to identify each reaction sub-step, the effects of three variables, the temperature, the pH, and the residue of the cleavage site, were investigated, in terms of how they affect each reaction step in a different manner.
In addition to the four main sub-reactions, two other important reaction steps were distinguished: shortcut binding and subsequent strand displacement. A new substrate can bind before the full dissociation of the last product in a process which we termed shortcut binding, and the half-bound substrate rapidly replaces the remaining bound product. We demonstrated that this pathway is possible and investigated the dependence of substrate concentration on the occurrence of shortcut binding and the rates of each sub-step. We calculated the rate equation for the mechanism of shortcut binding and ran comparisons with the Michaelis–Menten equation. From our results, shortcut binding occurs more frequently at higher substrate concentration and it competes with the second product-dissociation reaction making the enzymatic turnover rate faster.

Overall, we dissected the reaction pathway and revealed kinetic factors hidden at the ensemble level gaining insight into the chemical mechanisms of 10–23 deoxyribozyme.
2. Basic Principles

2.1 Fluorescence Resonance Energy Transfer (FRET)

Fig. 2.1. A schematic diagram of FRET. D is the donor and A is the acceptor fluorophore. $S_0$ is the ground state and $S_1$ is the first excited state of fluorophores. Curved lines indicate vibrational relaxations.
Fluorescence resonance energy transfer (FRET), also called as Förster resonance energy transfer named after the German scientist Theodor Förster\(^1\), is a nonradiative energy transfer phenomenon which becomes effective when the distance between two fluorophores draw near (Fig. 2.1). The fluorophore that donates the energy is called a “donor”, and that accepts the energy is called an “acceptor”. When the donor is excited and the distance between the donor and acceptor is under 10 nm, FRET occurs well, resulting in the excitation and fluorescence of the acceptor. The FRET efficiency depends on many physical parameters. The FRET pair should have the spectral overlap of the donor emission spectrum and the acceptor absorption spectrum. FRET is distance-dependent and the donor and acceptor must be in close proximity (typically 1–10 nm). Each fluorophore has its own dipole moment due to non-uniform distributions of positive and negative charges on the atoms. The donor and acceptor transition dipole orientations must be approximately parallel for efficient energy transfer via dipole–dipole interactions. Consequently, the FRET efficiency (E) is expressed as a function of the donor–acceptor distance R, with an inverse 6th power law:

\[
E = \frac{1}{1 + (R/R_0)^6},
\]

where \(R_0\) is the Förster distance of this pair of donor and acceptor, the distance at which the energy transfer efficiency is 50%. This value is determined according to the optical characteristics and
spatial orientations of the two fluorophores. For good donor–acceptor pairs, it is typically 3–7 nm.

![Graph showing FRET efficiency vs. distance](image)

**Fig. 2.2.** The FRET efficiency, $E$, depending on the distance, $R$. The value of $R_0$ is 5 nm. $E$ changes drastically between 2–8 nm.

Fig. 2.2 is a graph based on the above equation with $R_0$ of 5 nm. A notable characteristic is that $E$ changes drastically from approximately 0.05 to 1.0 between 2 to 8 nm. For this reason, FRET has been widely used as a “spectroscopic ruler” with the resolution below a nanometer\(^2\). The size of a biomolecule is typically in the range of several tens of nanometers to one thus, structural folding and interaction dynamics can be measured through FRET methods.
FRET E value can be calculated experimentally from the fluorescence intensities of the two fluorophores:

$$E = I_\Lambda / (\gamma I_D + I_\Lambda),$$

where $I_D$ is the fluorescence from the donor, $I_\Lambda$ from the acceptor. $\gamma$ is the detection correction factor. FRET can be measured at ensemble level but single–molecule measurements provide much more information about molecular structural dynamics, statistics, and kinetics.

To study the dynamics of biomolecules using FRET, the donor and acceptor should be properly labeled. In the case of DNA or RNA, an amine–modified thymine is usually used to react with an NHS (N–hydroxysuccinimidy)–ester form of a fluorophore. In the case of proteins, the reaction between a thiol group of cysteine and a maleimide form of a fluorophore is most popular\(^3\). Popular FRET pairs include cyanines, ATTO Dyes, and Alexa Fluor dyes.
2.2 Total Internal Reflection Fluorescence Microscopy (TIRFM)

2.2.1 Total internal reflection and evanescent wave

Total internal reflection fluorescence microscopy (TIRFM) is a type of fluorescence based analysis method which utilizes a near-field wave, evanescent wave, to illuminate very small objects such as biological cells or single protein and DNA molecules near the interface between two media. The idea of using total internal reflection to illuminate objects contacting the surface of glass was first proposed by E.J. Ambrose in 1956\(^1\). This idea was adapted to the fluorescence microscopy by Daniel Axelrod in the early 1980s as TIRFM\(^5\).

Total internal reflection happens when a propagating wave strikes a medium boundary at the incident angle larger than the critical angle. It occurs only when the refractive index of the first medium is higher than that of the second medium located on the other side of the boundary. At these conditions, the light is totally reflected and cannot pass through the second medium (Fig. 2.3).

According to Snell’s law, the refractive indices and the angles of incidence and refraction have the following relationship:

\[
\frac{n_1 \sin \theta_1}{n_2} = \sin \theta_2
\]
The critical angle is equal to the incident angle when the refracted angle becomes $90^\circ$ and thus $\sin \theta_2 = 1$. Then the critical angle is

$$\theta_c = \theta_1 = \arcsin(n_2/n_1).$$

For example, if visible light passes from quartz ($n_1 \approx 1.55$) into water ($n_2 \approx 1.33$), the critical angle is: $\theta_c = \arcsin(1.33/1.55) \approx 59.1^\circ$.

**Fig. 2.3.** Illustration of total internal reflection and the evanescent wave. Black arrows indicate the light ray. $n_1$ is the refractive index for the first medium (quartz) and $n_2$ for the second medium (water) ($n_1 > n_2$). Total internal reflection occurs when the incident angle is higher than the critical angle. The intensity of an evanescent wave decays exponentially as a function of the distance from the boundary.

When total internal reflection occurs, evanescent electromagnetic waves are generated at the boundary between two media (Fig. 2.3). In quantum mechanics, this phenomenon is
explained by the Schrödinger equation representing that the electric and magnetic fields cannot be discontinuous at a boundary. The evanescent wave is found in the near-field region within one third of a wavelength of the incident light. It propagates parallel to the surface with an intensity $I$ that decays exponentially as a function of the perpendicular distance $z$ from the interface:

$$I = I_0 \exp\left(-\frac{z}{d}\right)$$

The characteristic penetration depth (or exponential decay depth) $d$ is expressed as:

$$d = \frac{\lambda}{4\pi n_2} \left(\frac{\sin^2 \theta_1}{\sin^2 \theta_c} - 1\right)^{-1/2},$$

where $\lambda$ is the wavelength of incident light in vacuum. The wave penetrates into the second medium only to a depth of on the order of $\lambda$ or smaller except for $\theta_1$ close to $\theta_c$. The decay depth $d$ decreases as increasing $\theta_1$.

As mentioned above, the immanent nature of the evanescent wave enables selective excitation of fluorescently labeled single molecule in an aqueous solution near the glass interface. It excludes unwanted excitation of the molecules which are not surface-immobilized, resulting in a high signal-to-noise ratio well-suited for single-molecule observations. Combined with proper optical configurations and a CCD camera, simultaneous real-time observation of hundreds of single-molecules is possible. FRET is
typically used to observe the structural changes of the molecule with
the time resolution no less than 10 msec.

2.2.2 Prism-type TIRFM setup

TIRFM can be set up in two ways, either using an oil-immersion
(objective-type) or a water-immersion (prism-type) objective lens.
We used a home-made prism-type TIRF microscope setup for
single-molecule studies in this thesis. Prism-type TIRFM has been
a major imaging tool for smFRET since it was first introduced. The
setup in detail is discussed below (Fig. 2.4).

Biomolecules which are to be observed should be labeled
properly and immobilized on the surface of a quartz slide (PQ-2040-
03, Prism Research Glass) (see the next section for surface
preparation). For FRET measurements, the wavelength of the
excitation laser should be selected to excite the donor of a FRET pair.
We used a 532-nm diode-pumped solid-state laser (Samba™
0532-04-01-0100-500, Cobolt) to excite Cy3 molecules. A Pellin
Broca Prism (PLBC-5.0-79.5-SS, CVI Laser Optics) was used to
adjust the shallow angle with respect to the refractive boundary (≤
30°) more easily which is needed for TIR. The space between the
prism and quartz slide was filled with immersion oil to exclude air.
The evanescent wave is generated at the interface between the
quartz slide and sample solution to excite immobilized molecules. Background signal is much reduced in this way. The fluorescence from Cy5 (acceptor) and Cy3 (donor) was collected by a water-immersion objective lens (x 60, NA = 1.2, Olympus), separated from the excitation light using a long-pass filter (HQ545LP, Chroma), spatially filtered using a mechanical slit, split by a dichroic mirror (645DCXR, Chroma), and focused by lenses into the EMCCD (DU-897E-CS0-#BV, Andor). The two separated beams are focused onto the different area of the EMCCD, respectively.

The data acquisition software was downloaded from the group website of Taekjip Ha (http://bio.physics.illinois.edu/). Coordinates were corrected to distinguish the position of an individual FRET pair using IDL software. Then we can measure the FRET E value of a single FRET pair in real-time.
Fig. 2.4. The schematic illustration of a prism–type TIRFM. TIR occurs at the interface between a quartz slide and water. Immobilized biomolecules are excited by the evanescent wave. The fluorescence from the donor and acceptor is separated by a 645-nm dichroic mirror. The two separated beams are focused onto the different region of the EMCCD. After the correction of coordinates, we can calculate the FRET E value of the single FRET pair in real-time.
2.2.3 Quartz surface preparation for sample immobilization

Surface immobilization is important since molecules must be localized in space over a period of time for imaging. We used polyethylene glycol (PEG)–coating method for sample immobilization.

Firstly, clean quartz (or glass) slides must be prepared. The hydroxyl group of quartz was reacted with amino–silane (N-[3-(trimethoxysilyl)propyl]ethylenediamine) to expose the amine group on the surface. Then the slides were incubated with mPEG–SVA (MW 5000, Laysan Bio) in 100 mM sodium bicarbonate for 3 hr. The NHS–ester group connected to PEG reacts with the amine–modified surface. The PEG solution contained 98 % of mPEG–SVA and 2 % of biotin–PEG–SVA, both purchased from Laysan Bio. This sparsely populated biotin (vitamin B7) is the key for low concentration sample immobilization. Biotin can interact with NeutrAvidin protein. NeutrAvidin is a kind of avidin protein that contains four binding sites for biotin with a high degree of affinity and specificity \((K_d = 10^{-15} \text{ M})\). Using biotinylated samples, samples can be attached to the surface by making biotin–NeutrAvidin–biotin complex for more than an hour.
2.3 10–23 Deoxyribozyme

Deoxyribozymes are DNA molecules that act like enzymes. They are also called as DNA enzymes or DNAzymes. Unlike ribozymes, which were first discovered in nature\(^8,9\), the first catalytic DNA sequence was obtained artificially by \textit{in vitro} selection\(^10\) and no natural deoxyribozyme has been found yet. Various types of deoxyribozymes that exhibit enzymatic activities such as RNA cleavage\(^10–12\), RNA ligation\(^13\), and a growing range of other chemical reactions have been identified using \textit{in vitro} selection techniques\(^14\). Due to their target specificity, stability, and modest cost, deoxyribozymes have been widely used \textit{in vitro} for applications such as biosensors\(^15\) and nanodevices\(^16\), and also \textit{in vivo} for therapeutic applications\(^17\).

10–23 Deoxyribozyme is an RNA–cleaving DNA enzyme first developed in 1997\(^11\). The name “10–23” refers to the 23\(^{\text{rd}}\) clone obtained after the 10\(^{\text{th}}\) round of \textit{in vitro} selective amplification. 10–23 Enzyme catalyzes divalent metal ion–induced cleavage of RNA by transesterification at phosphorus located between an unpaired purine and paired pyrimidine residue. It is a single–stranded DNA with its catalytic core composed of 15 deoxynucleotides which is flanked by two substrate–recognition domains on either side (Fig. 2.5). The substrate–recognition domains recognize an RNA substrate by binding via Watson–Crick base pairing and the sequence
can be changed in a complementary manner without loss of catalytic activity. The sequence change in catalytic domain may lead to dramatic loss of catalytic activity except for some sites typically thymidine at position $^{8,11,18-20}$. Although at first it was developed to be optimized to Mg$^{2+}$ ion, it also shows a modest level of activity in the presence of other divalent metal cations such as Mn$^{2+}$ (EPPS), Pb$^{2+}$, Ca$^{2+}$ and Cd$^{2+}$ (Tris) at physiological conditions$^{18}$. Modifications for more efficient 10–23 deoxyribozyme analogues have also been carried out$^{21,22}$.

**Fig. 2.5.** One example of the nucleotide composition of 10–23 deoxyribozyme (red: catalytic domain, black: substrate–recognition domain) with a bound complementary substrate. R is a purine residue (A or G) and Y is a pyrimidine residue (C or U). The hierarchy of RY reactivity is known to follow the scheme GU $\geq$ AU $>$ GC $>$ AC$^{21}$. 

![Diagram of deoxyribozyme structure]
Since 10–23 deoxyribozyme is easily synthesized, chemically stable, generalizable to any target sequence, and highly efficient in cleaving the target DNA, it can be a substitute for antisense oligodeoxynucleotides and ribozymes. Many investigators have been utilized the 10–23 enzyme both \textit{in vitro} and \textit{in vivo}\textsuperscript{14,16,17,23}, but there have been still unclear characteristics in the structure and chemical mechanism. Studies in single-molecule level provided some structural and mechanism information that was hidden in ensemble level measurements\textsuperscript{20,24}.
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3. The Effects of the Temperature, pH, and Unpaired RNA Residue on the Kinetics of the Four Reaction Sub-steps of 10-23 Deoxyribozyme

3.1 Introduction

10-23 Deoxyribozyme is generally known to pass through a series of reaction sub-steps. These include enzyme-substrate binding, substrate cleavage, and dissociation of the products. One can easily consider the feasibility of this reaction scheme, and many ensemble studies use steady-state approximation and the Michaelis-Menten equation to calculate the substrate cleavage rate and catalytic efficiency. However, in the ensemble measurement, assumptions such as the enzyme-substrate binding event occurring as soon as the enzyme and substrate solutions are mixed and that the dissociation of the products are extremely rapid compared to the cleavage reaction are necessary.

The TIRF-FRET method has enabled us to observe the structural changes of 10-23 deoxyribozyme in real time with a time resolution of ~100 msec, indicating that we can dissect each reaction sub-step and analyze the kinetics without the assumptions that are
necessary when conducting an ensemble kinetic analysis (Fig. 3.1). Moreover, the binding and product dissociation reaction can be divided into two steps. The half-binding of the substrate to one free substrate-recognition domain of the 10–23 enzyme is followed by a full-substrate binding reaction which is much faster than the first one. Product dissociation also occurs in a sequential manner; the dissociation of one cleaved substrate followed by that of another one.¹

This chapter deals with a kinetic analysis of each reaction sub-step of 10–23 deoxyribozyme. Given that the full-binding reaction was much faster than the half-binding reaction under the conditions studied, this type of reaction was infrequently recognizable in our time resolution. Hence, we neglected the two binding steps and considered this as a single reaction. Therefore, the four steps of binding, cleavage, the first dissociation, and the second dissociation, were analyzed. We varied three conditions: the temperature, the pH and the type of substrate RNA residue. A temperature-dependence investigation (temperature range: 26–34 °C) provides information about the transition state of each reaction from kinetic parameters of the enthalpy, entropy, and free energy of activation. In addition, a study of the degree of pH dependence (pH range: 7.33–7.81) confirmed that the rate-limiting step for the cleavage reaction is the deprotonation of a single proton and that only the cleavage step is influenced by the pH. Five types of substrate RNA residues (GU, AU, CU, UU, and −U) were also studied to determine the effect of the unpaired residue during every reaction.
step. These kinetic analyses helped us to gain a better understanding of the detailed reactions occurring in relation to the enzymatic mechanisms of 10–23 deoxyribozyme.

**Fig. 3.1.** Schematic illustration of single-molecule detection of 10–23 deoxyribozyme using TIRFM. The freely diffusing substrate meets the immobilized 10–23 enzyme and bind. After cleavage, each of the products dissociate sequentially and randomly. Structural changes of 10–23 deoxyribozyme can be detected by changes of FRET E values.
3.2 Experimental

3.2.1 Sample preparation: oligonucleotides

The sequence for 10–23 deoxyribozyme is the following: 5’ – /5Cy3/TAA CCT CTT CAG GCT AGC TAC AAC GAA TAG TGG ATA/iCy5/TAA AAA AAA AAA AAA AAA AAA A–3’ . Cy3 (donor) is attached to the d(deoxy)T base at 5’ –end and Cy5 (acceptor) to the internal dT base. The poly–A(18) chain at the 3’ –end is pre–annealed with the complementary sequence which is biotin–modified for attachment to the quartz surface: 5’ –TTT TTT TTT TTT TTT TTT/3Bio/–3’ . Substrate sequence for temperature and pH dependence studies is the following: 5’ –TAT CCA CTA TrGrU GAA GAG GTT–3’ . Only the cleavage site, rGrU, is RNA bases and others are DNA bases. RNAs are more difficult to handle (and quite expensive) and we assumed that although DNA–RNA heteroduplexes are more stable than DNA–DNA duplexes², the cleavage activity would not significantly be affected by the type of duplex of the substrate recognition arm. For substrate RNA residue dependence test we used four more sequences: 5’ –TAT CCA CTA TrArU GAA GAG GTT–3’, 5’ –TAT CCA CTA TrCrU GAA GAG GTT–3’, 5’ –TAT CCA CTA TrUrU GAA GAG GTT–3’, and 5’ –TAT CCA CTA TrU GAA GAG GTT–3’ . The last one has no
unpaired purine residue (Fig. 3.2). All customized nucleic acids were ordered from Integrated DNA Technologies (Coralville, IA).

Annealing was carried out in 25 mM Tris–HCl pH 8.0 (AM9856, Ambion), 250 mM NaCl after heating for 2 min at 94 °C and cooling to room temperature overnight. Quartz modification is described in Chapter 2.2.3.

![Diagram of 10-23 Deoxyribozyme](image)

**Fig. 3.2.** Oligonucleotides used in this study. Only the cleavage site of substrate is composed of RNA. Poly thymine and poly adenine chains are pre–annealed. Biotin is attached for sample immobilization.
3.2.2 Imaging buffer

Triplet state quencher and oxygen scavenger system

Overcoming the limitation of the short imaging time because of photophysical bleaching of fluorophores is important in single-molecule experiments. Bleaching of the fluorophore is normally considered to take place from the excited singlet, or from the triplet state of the dye since excited molecules are more prone to have chemical reactions with radical oxygen species. Blinking, a phenomenon that appears as if the fluorophore is transiently switched off, also hinders the sensitivity and resolution of fluorescence measurements. Blinking is observed when the fluorophore randomly enters transient, nonfluorescent states such as radical ion states. To reduce the photobleaching effect, oxygen scavenger systems are employed to create anaerobic conditions. For antiblinking and also antibleaching, triplet state quenchers such as Trolox are used to rapidly deplete the relatively long-lived triplet state.

We used Trolox (Sigma–Aldrich) for quenching triplet states. Approximately 2.4 mM of Trolox solution was prepared which was titrated to pH 7.0 with 10 M NaOH solution. The final concentration of NaOH was 2 mM. Before the addition of NaOH solution, Trolox does not dissolve well thus precipitates in distilled water. After the addition of NaOH and thorough vortexing, Trolox dissolves well. This Trolox solution was filled in a transparent Falcon tube and nutated
for 12 hr under a fluorescent lamp to generate proper amount of Trolox-quinone. Then the solution was filtered with a syringe filter of pore size less than 0.2 um (Anotop, Whatman). The final solution becomes slightly yellow in color.

For oxygen scavenger system, we used pyranose oxidase from *Coriolus* sp. (Sigma–Aldrich) in combination with catalase from bovine liver (Sigma–Aldrich) (POC) since unlike glucose oxidase with catalase (GOC) system, POC system does not generate acidic compounds thus does not alter the pH of the buffer for at least 1 hr. We underwent several trials to find the optimal concentration of POC. The ultimate condition of POC system for imaging was 3 unit/mL (0.3 mg/mL) pyranose oxidase, 0.4 μg/μL catalase, and 0.8 % w/v D-glucose.

**Adjusting pH of imaging buffer at reaction temperature**

We expected that the rate of 10–23 deoxyribozyme would be sensitive to slight variations in pH. Due to the sensitive nature of this work, determining and fixing pH of imaging buffer as precise as possible was necessary. Known by experience, pH of Tris buffer changes according to temperature. Thus, we mixed Ambion® 1M Tris–HCl pH 8 and 1 M Tris–HCl pH 7 (AM9851) in a proper ratio into the imaging buffer to adjust the desired pH at reaction temperature. We made imaging buffer to contain 82 % v/v saturated and filtered Trolox solution (approximately 2 mM Trolox), 50 mM
Tris–HCl, 100 mM MgCl₂ (Fluka), 50 mM NaCl, 0.1 mg/mL bovine serum albumin (BSA; New England Biolabs), 1 mM DL–dithiothreitol (DTT; Sigma–Aldrich), POC system, and 200 nM substrate. The volume ratio of Ambion® Tris–HCl pH 8 and Tris–HCl pH 7 for conditions studied are listed in Table 3.1.

<table>
<thead>
<tr>
<th>Volume ratio of Ambion® Tris</th>
<th>pH (± 0.02) of imaging buffer at each temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8</td>
<td>pH 7</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>8.5</td>
<td>1.5</td>
</tr>
<tr>
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<td>2.75</td>
</tr>
<tr>
<td>6.75</td>
<td>3.25</td>
</tr>
<tr>
<td>5.5</td>
<td>4.5</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4.5</td>
<td>5.5</td>
</tr>
<tr>
<td>3.25</td>
<td>6.75</td>
</tr>
</tbody>
</table>

Table 3.1. The pH of imaging buffer. Total concentration of Tris–HCl in imaging buffer was 50 mM. Conditions for pH dependence study are marked with yellow, temperature dependence study with pink, and RNA residue dependence study with gray.

We also tested for 10 mM Tris–HCl but the buffering capacity was lower. The pH of imaging buffer was measured using Orion 720Aplus pH meter and Orion ROSS Ultra 8103BN pH electrode (Thermo Scientific). The total volume of imaging buffer was 200 μL or 400 μL (both gave the same results). Temperature of the imaging buffer was maintained using a digital dry bath and a thermometer while measuring the pH. The pH was recorded every 10 min for 1 hr.
and averaged over time, and this was conducted at least twice. Considering the experimental and mechanical errors of measurements, the possible error range of pH is plus or minus 0.02. For the temperature dependence study, the averaged pH is denoted as 7.52.

### 3.2.3 Data acquisition and analysis

TIRFM setup, described in Chapter 2.2.2, should be aligned good and ready. Prior to the TIRF imaging, a sample chamber with immobilized 10–23 deoxyribozyme in imaging buffer should be prepared. The procedure is as follows.

1) Drill six small holes in each of a quartz slide (75 mm x 25 mm x 1 mm, Prism Research Glass) that is going to be PEGylated (two holes per sample chamber). The size of a hole must fit in with the size of the pipette tip used. PEGylate perforated quartz slides and glass coverslips as in Chapter 2.2.3.

2) By affixing a glass coverslip to a quartz slide with double-sided tape, partition the area into three sections, each for one sample chamber. Seal the gaps in the edges with epoxy. In this way one quartz slide can be used three times for imaging.

3) Inject 35 μL of 0.1 mg/mL NeutrAvidin (Sigma–Aldrich) in T50 (50 mM NaCl, 10 mM Tris–HCl pH 8.0) buffer into an
empty chamber and incubate for 10 min. Wash out the excess NeutrAvidin with T50 buffer thoroughly.

4) Inject 180 µL of 25~6.25 pM 10–23 deoxyribozyme in T50 buffer and incubate for 10 min. Wash with T50 buffer thoroughly.

5) Make 200 µL (or 400 µL) of imaging buffer and inject 180 µL of it (twice for 400 µL). Incubate the quartz slide for 10 min at the heating plate of the temperature controller (CU201, Live Cell Instrument) installed in TIRF microscope. Now it is ready to be illuminated.

All reactions were recorded with 100 msec time resolution and each record was obtained for more than 500 seconds. FRET efficiency E was calculated from $E = I_A / (\gamma I_D + I_A)$ after the removal of crosstalk and background signal. We assumed 10~5 % of leakage from donor to acceptor. The gamma factor, $\gamma$, was adjusted for every single dye pair.

The value of each FRET state is determined by the position of the center of the peak in Gaussian function. Dwell times of each FRET state were collected using MATLAB. Each sub-reaction, binding, cleavage, first dissociation and second dissociation, is an elementary reaction within the time scale of our measurement, which is a single step reaction with one transition state. The binding reaction is considered as a pseudo-first-order reaction since the concentration of the substrate remains almost constant (Otherwise,
it is approximately second-order\textsuperscript{d}). Other three reactions are unimolecular reactions which obeys first-order kinetics. The occurrence of reaction dwell times was expressed in a histogram and fitted with single-exponential curve using Microsoft Origin to get the lifetimes and kinetic rates (Fig. 3.3). The number of bins and width of the histogram was selected for the best fitted one based on the range of common theories\textsuperscript{7}.

![Graph showing dwell time histogram and fitted curve.](image)

**Fig. 3.3.** An example of the dwell time histogram (pH 7.33, 30 °C). 1455 traces were analyzed and the normalized occurrence (divided by maximum value) depending on the dwell time for the reaction fitted well to a single exponential decay function. The lifetime of the binding reaction for this condition is 31.7 sec (rate = 0.0315 sec\textsuperscript{-1}).
3.3 Results and Discussion

3.3.1 Reaction sub-steps: binding, cleavage, dissociation1, and dissociation2

In this study, we analyzed the kinetics of four reaction sub-steps: binding, cleavage, dissociation1, and dissociation2 (Fig. 3.4). Since the persistence length of ssDNA is less than 10 nm, the 37-mer single-stranded DNA enzyme sample is flexible enough to make randomly coiled structure with the aid of Mg$^{2+}$ and Na$^+$ which reduce electrostatic repulsion between the negatively charged phosphate groups of the DNA backbone ($E, E=0.82$). When a substrate binds the enzyme the two arms form double-strand, which is more rigid, thus straightens the structure ($ES, E=0.35$). We do not know the exact structure of the catalytic core, however, it is somehow interacting with the magnesium ion. With the Mg$^{2+}$ cofactor, the substrate is cleaved which results in an upstream fragment terminating in a 2',3' -cyclic phosphate and a downstream fragment terminating in a 5' -hydroxyl ($EP_1P_2, E=0.30$). The lower FRET value indicates that the distance between the two dyes become even more distant. This can explain the preference for cleavage over ligation. The 2',3' -cyclic phosphate is enthalpically unfavorable relative to the 3',5' -phosphodiester. Thus, there should be a larger entropic gain that compensates for the enthalpy loss, such as
a loss of structural integrity\textsuperscript{12}. It appears that after cleavage, the reactive termini of the two products are not sufficiently in close proximity to facilitate rapid ligation. After that, spontaneous dissociation of one of the products occurs (\(EP, E=0.49\)) and subsequently, the remaining product dissociates to recover the original structure of 10–23 deoxyribozyme (\(E, E=0.82\)).

![Diagram of reaction sub-steps](image)

**Fig. 3.4.** Illustration of 10–23 deoxyribozyme reaction sub-steps (\(E\) for enzyme, \(S\) for substrate, and \(P\) for product). A red arrow at \(ES\) state is the cleavage site.

The smFRET trajectory clearly shows the structural changes due to the enzymatic reaction (Fig. 3.5). The dwell time for \(E, ES, EP_1P_2,\) and \(EP\) corresponds to the dwell time for the binding, cleavage, dissociation\textsuperscript{1}, and dissociation\textsuperscript{2} reaction, respectively. Each reaction has different characteristics depending on the different molecular environment such as temperature, pH, and the type of residue on the cleavage site.
We treated the binding step as a single step because the binding of the second arm is too fast to be fully distinguished in our experimental condition. Strictly speaking, $EP_1$ and $EP_2$ show slightly different FRET E values since the two arms have different sequence\(^1\). In the kinetic analysis of dissociation1 and dissociation2, we did not considered which one of the product dissociated first. The dwell time of dissociation1 and dissociation2 well fitted to the single exponential function. We analyzed only the forward reactions because the reverse reactions rarely occurred in our experimental condition. 10–23 deoxyribozyme is selected in vitro based on its ability to cleave, thus detached from the column. Therefore, it has a high preference for cleavage over ligation. Re-binding of the dissociated products would be probabilistically unfavorable due to the low concentration of the products.
**Fig. 3.5.** Representative time trajectory of Cy3 and Cy5 fluorescence and the corresponding FRET E values (26 °C, pH 7.52). Randomly coiled free enzyme shows high FRET (E, E=0.82). Substrate binding causes the two dyes to get far away from each other (ES, E=0.35) and substrate cleavage makes them more distant (EP₁P₂, E=0.30). After the first dissociation of one of the products, the freed arm randomly coils again (EP, E=0.49) and subsequently, full dissociation happens to recover the free enzyme state, E. The dwell time for the binding, cleavage, dissociation1, and dissociation2 reaction corresponds to the dwell time for E, ES, EP₁P₂, and EP state, respectively.
3.3.2 The transition states for binding, cleavage, and sequential dissociation reactions

Transition state theory and Eyring–Polanyi equation

Transition state theory (TST) explains the reaction rates of elementary chemical reactions by assuming a chemical equilibrium between reactants and activated transition state complexes. TST relates the reaction rate to temperature and it is derived from statistical thermodynamics in the kinetic theory of gases. If the rate constant of a reaction is experimentally determined, the enthalpy of activation ($\Delta H^\ddagger$), entropy of activation ($\Delta S^\ddagger$), and Gibbs free energy of activation ($\Delta G^\ddagger$) can be calculated by TST, giving insight into the chemical mechanism.

Assuming the transmission coefficient $\kappa$ as unity (which means the transition state does not go back to the reactant but always proceeds to the product), the TST equation can be written as:

$$k = \left(\frac{k_B T}{h}\right) \exp\left(-\frac{\Delta G^\ddagger}{RT}\right) = \frac{1}{\tau}$$

where:

- $k = \text{reaction rate constant (or rate)}$
- $k_B = \text{Boltzmann constant}$
- $T = \text{absolute temperature}$
\[ h = \text{Planck's constant} \]

\[ R = \text{gas constant} \]

\[ \tau = \text{reaction lifetime.} \]

In the case of single-molecule measurement of an elementary reaction, the reciprocal of the reaction lifetime equals the reaction rate constant (or rate). From the definition of Gibbs free energy

\[ \Delta G^\ddagger = \Delta H^\ddagger - T \Delta S^\ddagger. \]

Thus, the TST equation can be rewritten as:

\[ k = \frac{1}{\tau} = \left( \frac{k_B T}{h} \right) \exp \left( \frac{\Delta S^\ddagger}{R} \right) \exp \left( -\frac{\Delta H^\ddagger}{RT} \right) \]

Taking natural logarithms of both sides, we can obtain Eyring–Polanyi equation:

\[ \ln \left( \frac{k}{T} \right) = \ln \left( \frac{1}{\tau} \right) = \left( \frac{1}{R} \right) \ln \left( \frac{\Delta H^\ddagger}{T} \right) + \ln \left( \frac{k_B}{h} \right) + \frac{\Delta S^\ddagger}{R} \]

If a chemical reaction is performed at different temperatures and the reaction rate is determined, by plotting \( \ln(k/T) \) versus \( 1/T \), the enthalpy of activation can be derived from the slope and the entropy of activation can be derived from the y-intercept.

The transition state corresponds to the state of highest enthalpy. The enthalpy of activation is approximately equal to the activation energy used in the Arrhenius equation. The conversion of one into the other depends on the molecularity, \( \Delta H^\ddagger = \Delta E^\ddagger - (\Delta n)RT \), where \( \Delta E^\ddagger \) is the activation energy and \( \Delta n \) is the number difference.
between transition state complexes and reactants (for reaction $A + B \rightleftharpoons (AB)^{\dagger}$ the value of $\Delta n$ is $-1$)$^{13}$. At room temperature, RT is approximately 0.6 kcal/mol. The entropy of activation provides clues about the molecularity or orderliness of the transition state. If the reaction is associative and the complex is more ordered at the transition state, $\Delta S^{\dagger}$ will be a negative quantity. If the reaction is dissociative, $\Delta S^{\dagger}$ will be positive. The magnitude of $\Delta G^{\dagger}$ is, in effect, the “energy barrier” to the reaction, and by reducing the $\Delta G^{\dagger}$ value, the chemical reaction occurs at higher rate$^{14}$. The contribution of the enthalpy and entropy of activation to $\Delta G^{\dagger}$ will differ according to the chemical reactions.

Interpretation of kinetic parameters of 10–23 deoxyribozyme

Each reaction rate of binding, cleavage, dissociation1 and dissociation2 in the enzymatic reaction pathway of 10–23 deoxyribozyme shows a temperature dependency in the range of 26–34 °C. All reactions become faster as temperature increases (Table 3.2). The data were fitted to the Eyring–Polanyi equation (Fig. 3.6) and the enthalpy, entropy, and free energy of activation were obtained (Table 3.3).
Table 3.2. Lifetimes of the four sub-reactions of 10–23 deoxyribozyme depending on temperature. The cleavage reaction is the slowest reaction in the overall enzymatic process, which means it is the rate-determining step at the condition studied here.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Binding</th>
<th>Cleavage</th>
<th>Dissociation1</th>
<th>Dissociation2</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>33.4</td>
<td>45.3</td>
<td>22.2</td>
<td>20.5</td>
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<td>28</td>
<td>24.7</td>
<td>34.7</td>
<td>20.2</td>
<td>15.6</td>
</tr>
<tr>
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<td>34.5</td>
<td>13.4</td>
<td>12.4</td>
</tr>
<tr>
<td>32</td>
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<td>26.7</td>
<td>9.4</td>
<td>9.1</td>
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<tr>
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<td>14.1</td>
<td>23.7</td>
<td>5.0</td>
<td>6.3</td>
</tr>
</tbody>
</table>

**Fig. 3.6.** The Eyring–Polanyi plot for each sub-reaction of 10–23 deoxyribozyme. The data are linearly fitted in the range of 26–34 °C (pH 7.52). All reactions are accelerated as the temperature increases. The slope provides $\Delta H^+$, and the y-intercept provides $\Delta S^+$ (Error bars are fitting errors for lifetime).
<table>
<thead>
<tr>
<th></th>
<th>$\Delta H^\dagger$ (kcal/mol)</th>
<th>$\Delta S^\dagger$ (J/(mol · K))</th>
<th>$\Delta G^\dagger$ at 26 °C (kcal/mol)</th>
<th>$\Delta G^\dagger$ at 34 °C (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding</td>
<td>17.5</td>
<td>−29.3</td>
<td>19.6</td>
<td>19.7</td>
</tr>
<tr>
<td>Cleavage</td>
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<td>−82.0</td>
<td>19.8</td>
<td>19.9</td>
</tr>
<tr>
<td>Dissociation1</td>
<td>35.2</td>
<td>219.1</td>
<td>19.5</td>
<td>19.1</td>
</tr>
<tr>
<td>Dissociation2</td>
<td>25.9</td>
<td>92.2</td>
<td>19.3</td>
<td>19.1</td>
</tr>
</tbody>
</table>

Table 3.3. Thermodynamic activation parameters for the four reaction steps of 10–23 deoxyribozyme.

The formation of the enzyme–substrate complex for 10–23 deoxyribozyme is a hybridization reaction of nucleic acids. Hybridization is generally temperature-sensitive. The rate of hybridization goes to zero at the melting temperature, $T_m$. As the temperature decreases below $T_m$, the rate first increases, then reaches a flat maximum, and finally decreases, showing a bell-shaped profile\(^{15}\). In a molecular point of view, each of the bases of the two oligonucleotide strands will stochastically collide and interact by the time-scale of femto seconds to establish the sequence-specific, non-covalent bonds which results in a single duplex. Hybridization of randomly coiled oligonucleotides is known to form a transient intermediate with a few base pairs called nucleus. This nucleation step is the rate-limiting step of hybridization and the rest of the base pairings and helix formation happen quickly\(^6\).

From our results, the sign of the entropy of activation is minus ($-29.3 \text{ J/(mol} \cdot \text{K})$), which means the activation for binding is an associative mechanism. Because of negative $\Delta S^\dagger$, $\Delta G^\dagger$ at 34 °C
slightly increased compared to that at 26 °C. This will result in the bell-shaped rate constant profile as the temperature increases further. The binding step of 10–23 deoxyribozyme proceeds in a two-step manner. If one of the binding arms manages to form a nucleus, full binding of that arm and the next arm occurs rapidly. \( \Delta H^\ddagger \) is obtained by 17.5 (kcal/mol), which means the activation energy of the binding reaction is a positive value. This agrees well with the previous observations at temperatures well below \( T_m^{16,17} \) (\( T_m \) for the substrate studied is \( \sim 50 \) °C). We concluded that the binding step is diffusion-controlled nucleation reaction and hybridization of a few bases is enough to cross the barrier.

The next event in the catalytic process of 10–23 deoxyribozyme is the conversion of the enzyme–substrate complex into an activated complex that can decay into \( EP_1P_2 \) complex, which is the most important step. We measured the kinetic constant of the individual cleavage reaction without typical assumptions (such as binding comes to equilibrium as soon as adding substrate and dissociation is very fast that can be ignored) and ensemble averaging. The cleavage reaction is the rate-limiting reaction in the overall enzymatic reaction at the temperatures studied here (Table 3.2). \( \Delta S^\ddagger \) showed negative value (\( -82 \) J/(mol·K)) suggesting that the transition state complex is associative. The transition complex should be forming an effective structure to cleave the substrate. It is also interacting with magnesium ion(s). Thus, the enzyme–substrate complex is considered to have a higher bond order in the transition
state although cleavage of the phosphodiester bond is a dissociative reaction. We suggest that the coordination number of phosphorus is promoted from four to five by the formation of the transition state\textsuperscript{18} as described in Fig. 3.9. $\Delta H^\ddagger$ for the cleavage reaction of 10–23 (13.9 kcal/mol) is quite smaller than the apparent $\Delta H^\ddagger$ for reactions of other RNA cleaving enzymes such as 8–17 deoxyribozyme (27–28.7 kcal/mol)\textsuperscript{19} and the hammerhead ribozyme (30.3 kcal/mol)\textsuperscript{20}. This might be due to the limitation of ensemble measurements or indicating a different mechanism. At any rate, it is clear that the structure of the catalytic core plays a key role in catalysis of RNA cleavage.

The last part of the enzymatic process is the dissociation of the product(s). In the case of 10–23 deoxyribozyme, there are two products with different sequences. In general, to measure the rate of catalytic activity in ensemble experiments, the rate of product release should be much faster than the catalytic rate thus the design of the sample is limited. In our single-molecule measurements, cleavage rate and dissociation rate can be dissected hence sample design for fast dissociation is unnecessary. Simultaneous dissociations of the two products are probabilistically uncommon. One of the two products dissociates first at random (dissociation1), and the consequent structural change affects the next dissociation reaction (dissociation2). Dissociations show high positive values of $\Delta S^\ddagger$ (219.1 and 92.2 J/(mol·K)) which means a high amount of disorder takes place at the transition states compared to the reactant
states, especially for dissociation. As the temperature increases, the free energy barrier of dissociations will decrease, making the rate faster. At 34 °C, it takes only a few seconds to dissociate. The value of the dissociation rates could be underestimated because of the 0.1 sec of temporal resolution. According to the nearest-neighbor model, $\Delta H^\dagger$ for dissociations (35.2 and 25.9 kcal/mol) are close to the enthalpy change of the breaking of hydrogen bonding and hydrophobic stacking attractions of three to four base pairs. We suggest that flapping of 3~4 base pairs is enough for the full dissociation of the single product of 10 bases.

### 3.3.2 The effects of the pH and unpaired RNA residue on reaction rates

Dependence of sub–reaction rates on the pH

The pH dependence of sub–reaction rates in the presence of Mg$^{2+}$ was studied at 30 °C by varying the pH from 7.33 to 7.81 (Fig. 3.7). Only the cleavage reaction shows a distinct change in the reaction lifetime: the lifetime rapidly decreases from 48.1 to 20.8 sec as the pH increases. The log of cleavage rate increased linearly with a slope of 0.76 as the pH increased. The pH dependence of 10–23 deoxyribozyme had been studied in ensemble by Santoro and Joyce under somewhat different conditions in the pH range 6.1–9.7, and an analogous linear pH–dependence of the rate of substrate cleavage
had been observed in the pH range 6.5–8.5\textsuperscript{12}. However, the data had been ensemble averaged thus it actually shows a pH dependency on the turnover rate: the log-linear relationship only had held when the cleavage step is the rate-determining step. In our results, cleavage is slowest at pH 7.33 and 7.51 but as the pH increases, the binding step becomes the slowest step. We can directly measure the pH dependence on the cleavage rate regardless of the switch of the rate-determining step. The near unity slope of the log-linear plot of the cleavage rate on pH suggests that single deprotonation within the ES complex is part of the rate-limiting step of substrate cleavage. Most likely, the 2’-hydroxyl of the guanosine residue at the cleavage site must be deprotonated in order to perform an efficient nucleophilic attack on the adjacent phosphorus (Fig. 3.9).

Binding and dissociation rates show no clear dependency on pH within the studied region. It is general that the hybridization of nucleic acids is independent on pH at neutral region (pH 6–8)\textsuperscript{6}. This is thought to be due to the pK\textsubscript{a} values of the nucleotides (3.88 for N1 site of adenosine, 4.54 for N3 site of cytidine, 10.00 for N1 site of guanosine, and 10.47 for N3 of thymidine\textsuperscript{22}). In both extremes of low pH and high pH, the nucleotide bases are either all protonated or deprotonated thus affecting the stability of the duplex. There have been many thermodynamic studies on the stability of nucleic acid duplexes and triplexes depending on pH in ensemble level but the rate of hybridization and especially dissociation are still poorly understood, owing to the difficulty of directly observing the
hybridization and dissociation reactions. Our results show the hybridization and dissociation rates directly; however, the pH range is too narrow to clarify the effect of pH.

**Fig. 3.7.** The pH dependence on the lifetime of each sub-step. A reciprocal of the lifetime is the rate. Error bars are S.E.M.
Effects of an unpaired RNA residue in the substrate on the reaction rates

As mentioned previously, the reactivity of 10–23 deoxyribozyme depends on the type of residue at the cleavage site. We prepared five types of the substrates with the same two arms and different RNA residues at the cleavage site (GU, AU, CU, UU, and U without an unpaired residue) to confirm how the single unpaired RNA residue of the substrate affects the reaction rates of the four individual enzyme reaction sub-steps. Since the unpaired residue does not directly participate in duplex formation and dissociation, we expected that only the cleavage rate would be significantly changed. The result for 5’–GU–3’ and 5’–AU–3’ is showed on Fig. 3.8. As we expected, only the cleavage step is affected by the type of unpaired RNA residue. The cleavage rate for the G residue is ~5 times faster than that for the A residue and the turnover rate is ~2 times faster. Other residues, CU, UU, and −U, show very slow turnover rates that are usually not detectable within the detection time limited to ~10–15 min due to the photobleaching of dyes.
Fig. 3.8. Effects of an unpaired RNA residue on the average reaction time of the sub-reactions of 10–23 deoxyribozyme. A reciprocal of the lifetime is the rate. The cleavage rate of 5’–GU–3’ is approximately 5.4-fold faster than that of 5’–AU–3’.
Fig. 3.9. Hypothetical mechanism of the RNA cleavage reaction in 10–23 deoxyribozyme. U is uridine and B is the unpaired base residue. The conjectured transition state is marked with the double dagger symbol. Magnesium ion(s) and the catalytic core structure would play a critical role in the catalysis of cleavage which is not clearly revealed at this writing.
3.4 Conclusion

We examined the effects of temperature, pH, and substrate RNA residue on the rates of the four distinct reaction sub-steps of 10–23 deoxyribozyme by single-molecule TIRF-FRET method. Within our experimental conditions, the enzyme-substrate binding reaction is a diffusion-controlled nucleation reaction limited by the hybridization of a few bases. The substrate cleavage reaction possesses an associative mechanism at the transition state due to the higher bond order formed at the phosphate in the backbone. The reaction shows a clear log-linear increase with increasing pH and a slope close to 1, implying single deprotonation of the 2′-hydroxyl is part of the rate-limiting step and the unpaired residue of the substrate affects the activity of the cleavage step significantly. The hypothetical cleavage mechanism is described in Fig 3.9. The dissociation of 3~4 base pairs seems to be sufficient to dissociate the remaining 6~7 base pairs in the product-enzyme duplex. We hope that our work will be helpful to understand the mechanism and kinetics of 10–23 deoxyribozyme and other DNA enzymes, ribozymes, and even protein enzymes.
3.5 References


4.1 Introduction

In 1913, Leonor Michaelis and Maud Leonora Menten proposed a mathematical enzyme reaction model, which is now referred to as the classic Michaelis–Menten (MM) kinetics\(^1\). The MM mechanism takes a quite simple form, but it is often highly applicable to ensemble-averaged enzyme kinetics. It involves an enzyme \(E\) binding reversibly to a substrate \(S\) to form an enzyme–substrate complex \(ES\), which in turn undergoes unimolecular decomposition to form a product \(P\) while regenerating the original enzyme \(E\).

\[
E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow{k_2} E + P
\]

Under quasi-steady-state approximation, one can easily show the turnover rate (i.e., the rate of product formation), as follows:

\[
V = \frac{k_2[E]_T[S]}{K_M + [S]}, \quad (K_M = \frac{k_{-1} + k_2}{k_1})
\]

This shows hyperbolic dependence on the substrate concentration \([S]\) (where \([E]_T\) is the total enzyme concentration).
Often, there are several enzymatic reactions in biochemistry that do not follow the form of the MM equation. These include multi-substrate reactions (e.g., ping-pong mechanisms) and reactions that require cooperative binding of the substrate. Their enzymatic reaction schemes are far more complicated than the MM scheme.

In the ensemble-averaged kinetics, the turnover rates for ribozymes and deoxyribozymes are known to follow the MM equation under conditions in which \([S] \gg [E]\)\(^5-^9\). For substrate cleavage enzymes, the minimal kinetic scheme for catalysis is as follows:

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} EP_1P_2 \xrightarrow{k_3} E + P_1 + P_2
\]

Generally, the rate of product release is considered to be much faster than the rate of cleavage; hence \(k_3 \gg k_2\). The enzymes usually have a strong preference for cleavage over ligation. Therefore, \(k_2 \gg k_3\). Consequently, it is reduced to an MM scheme.

According to our study, 10–23 deoxyribozyme, a RNA-cleaving DNA enzyme, exhibits a quite different enzymatic reaction mechanism, including two characteristic sub-steps known as shortcut binding and strand displacement. Shortcut binding, as we refer to it, is a binding reaction of a new substrate before the last product dissociates from the enzyme. Strand displacement of the remaining product by the half-bound substrate occurs after shortcut binding, which is much faster than the spontaneous dissociation of nucleic acids\(^10\). We experimentally distinguished and identified the two sub-steps using the TIRF-FRET method and analyzed the
kinetics as we varied the substrate concentration. We calculated the rate equation and compared the turnover rate of the realistic shortcut–binding–included mechanism with that of the hypothetical mechanism with shortcut binding excluded for 10–23 deoxyribozyme to determine how the former case differs from the MM mechanism and looked to ways to design more systematic enzymes to improve the turnover rate.
4.2 Experimental

Preparation of oligonucleotides

HPLC purified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). For the experiments of verifying shortcut binding and strand displacement, 10−23 deoxyribozyme is labeled with Cy3 and the substrate with Cy5 as described in Fig. 4.1. The poly−A(18) chain and the lateral one binding arm of 10−23 deoxyribozyme are pre−annealed with a biotinylated complementary sequences which has a rG−overhang at the 3’−end. Annealing process are in Chapter 3.2.1. The complete sequences are as follows.

10−23: 5’−TAA CCT CTT CAG GCT AGC TAC AAC GAA TAG TGG ATA /iCy3/TAA AAA AAA AAA AAA AAA A−3’.

Biotinylated chain: 5’−TTT TTT TTT TTT TT/iBiodT/ ATA TCC ACT ATrG−3’.

Substrate: 5’−/5Cy5/TAT CCA CTA TrGrU GAA GAG GTT−3’.

For the kinetic analysis of each reaction sub−steps, we used the same sequence of 10−23 deoxyribozyme, the poly−T(18) chain, and 5’−GU−3’ substrate as the previous study (see Chapter 3.2.1).
Fig. 4.1. Sequences and structures of the substrate and 10-23 deoxyribozyme for the experiments of verifying shortcut binding and strand displacement. Residues in blue are RNAs and others are DNAs. Sequences in the dotted box are pre-annealed. The sequence in red are the catalytic core domain of 10-23 deoxyribozyme.

**Ensemble experiments**

Measurements were performed on a steady-state spectrofluorometer (QM-4/2005SE, PTI) at room temperature. Sample solutions were excited by 532 nm of light. Two different procedures were conducted (50 mM Tris–HCl pH 8.0, 100 mM MgCl₂, 150 mM NaCl). (1) The fluorescence of 100.5 nM Cy3-labeled 10-23 enzyme was measured. Subsequently, without changing the cuvette, Cy5-labeled substrate was added to the solution to give final concentrations of 10-23 enzyme of 100 nM and substrate of 500 nM.
(Fig. 4.6 left). (2) The direct excitation of 505 nM Cy5–labeled substrate was measured and subsequently Cy3–labeled enzyme was added to the cuvette to give the same final concentrations as (1) (Fig. 4.6 right).

**Single-molecule experiments**

The single-molecule fluorescence was measured in an imaging buffer which contains 100 mM MgCl₂, 50 mM NaCl, 50 mM Tris–HCl, saturated and filtered Trolox, 0.1 mg/mL BSA, 1 mM DTT, and an oxygen scavenger system. The pH of the imaging buffer was adjusted by mixing 1 M Tris–HCl, pH 7.0 and 1 M Tris–HCl, pH 8.0 in a proper ratio at the reaction temperature. The oxygen scavenger system contains 3 U/mL of pyranose oxidase from Coriolus sp., 0.4 mg/mL of catalase from bovine liver, and 0.8 % of D-glucose. See Chapter 3.2.2 and 3.2.3 for more detailed procedures and data analysis methods. The verification of shortcut binding and strand displacement was carried out at 34 °C, pH 7.52 with 50 nM Cy5–labeled substrate. The kinetic analysis of the sub-steps of 10–23 deoxyribozyme was carried out at 26 °C, pH 7.52 with 50 nM, 200 nM, and 800 nM substrate.
4.3 Results and Discussion

4.3.1 TIRF measurements of reaction pathways including shortcut binding and strand displacement

In the single-molecule experiments, we have previously investigated many turnovers going along the normal pathway of 10–23 deoxyribozyme (see Chapter 3.3.1). On the other hand, it is probable that a different path for 10–23 deoxyribozyme to meet the substrate and release its product exists. One possible enzymatic pathway is described in Fig 4.2 which is designated as “shortcut binding (SB)” . It starts a new turnover of the enzyme by binding of a new substrate to the one free arm prior to the dissociation of the remaining bound product. The dissociation of the remaining product and simultaneous full-binding of the half-bound substrate, designated as “dissociation’ ”, could occur thereafter to recover the enzyme–substrate complex, ES, if it is faster than the dissociation of the half-bound substrate from the enzyme. Dissociation’ would occur mainly by the strand-displacement mechanism (Fig. 4.3). The strand-displacement reaction replaces one oligonucleotide bound to a complementary strand by another oligonucleotide with the same sequence which forms a branched nucleation complex with the initial duplex followed by progressive migration of the branch point and eventual replacement. Since it requires only the partial melting of the
initial duplex to start the reaction, it is typically much faster than the spontaneous dissociation under physiological conditions\textsuperscript{10}. In this way dissociation2 for pre-turnover and binding for post-turnover overlap.

\textbf{Fig. 4.2.} Scheme of reaction steps for 10–23 deoxyribozyme. The red arrow indicates the cleavage site. After dissociation1 occurs, a new substrate can approach the enzyme and bind to its free arm before dissociation2 occurs, which we named a “shortcut binding (SB)” process. The same would occur for either arm.
**Fig. 4.3.** Scheme of the strand-displacement mechanism. The branch point moves randomly until the complex restores the original $E_P S$ duplex (the displacement failed) or creates the displaced $E_S$ duplex.

In the smFRET time trajectory, we found fairly enough reaction pathways which appear to include one or more shortcut binding reaction (Fig. 4.4). A free DNA enzyme, in the presence of Mg$^{2+}$, is randomly coiled thus the distance between Cy3 and Cy5 is very close ($E$ state, $E\sim 0.84$). When a substrate binds, two arms become double-strands and the two dyes get far away from each other showing low FRET ($E_S$ state, $E\sim 0.29$). After substrate cleavage, the structural change makes the two dyes to get more far away from each other ($E_P P_S$ state, $E\sim 0.23$). When one product from either arm dissociates (dissociation1) the single-stranded free arm randomly coils again ($E_P$ state, $E\sim 0.44$). Unless shortcut binding occurs, the dissociation2 reaction will continue to recover the initial $E$ state (in Fig. 4.4, it is designated as normal pathway and also see Fig. 3.5.). Several traces showed a lower FRET state ($E\sim 0.21$) after the $E_P$ state followed by subsequent FRET states of $E\sim 0.29$, 0.23,
0.43, and 0.85, the same FRET values as the $ES$, $EP_1P_2$, $EP$, and $E$ states respectively. Consequently, it is reasonable to regard the $E \sim 0.21$ state as the $EPS$ state. The structural difference between the $EP_1P_2$ and $EPS$ state might result in the slightly different dye-to-dye distances nevertheless, the FRET difference is in the error range. Since the strand-displacement reaction is probabilistically more favorable than the spontaneous dissociation reactions, we assume that fast strand displacement takes place (dissociation') to accomplish the $ES$ state after shortcut binding. After that, reactions follow the same order as the pre-turnover pathway (cleavage and dissociation1) and finally after dissociation2, the enzyme regains its original structure. In some reaction pathways, the FRET drop after the $EP$ state appeared more than once before the recovery of the free enzyme (Fig. 4.5).
Fig. 4.4. Representative smFRET time trajectory showing an one-shortcut-binding-included reaction pathway and a normal reaction pathway (26 °C, pH7.52, 200nM of substrate). The $EPS$ state has almost same FRET E value as the $EP_1P_2$ state. From the time “Waiting for binding” until the time “Dissociation2”, two sets of products were dissociated thus two turnovers occurred.
Fig. 4.5. Representative time trajectory showing a two-shortcut-binding-included reaction pathway (26 °C, pH7.52, 200nM of substrate). Right after the 1st and 2nd shortcut bindings, dissociation’ terminates the 1st and 2nd turnovers (two sets of products are released). Dissocation2 terminates the 3rd turnover and makes the enzyme to recover the original structure in this pathway (overall, three sets of products are released).

4.3.2 Verifying shortcut binding and strand displacement

Ensemble measurements

To verify that shortcut binding and sequential strand displacement could take place after dissociation1, we designed a different set of DNA sample as in Fig. 4.1. The 3’—side of the binding arm in 10–23 deoxyribozyme was pre–annealed with a half substrate as the $EP_2$ complex. Prior to the single–molecule TIRF measurement, we checked that this DNA enzyme and substrate can be hybridized with
each other using steady-state fluorometer (Fig. 4.6). If they associate, FRET should be measured.

![Graph showing fluorescence spectra]

**Fig. 4.6.** Ensemble experiments using fluorometer. Sample solutions were excited by 532 nm of light. The “10–23 + substrate” solutions have final concentrations of 500 nM substrate and 100 nM 10–23 which show FRET (dotted orange lines). Substrate only solution shows direct excitation of Cy5 (red line).

The green line shows the fluorescence spectrum from Cy3 labeled on the 10–23 enzyme (~100 nM) which has a maximum intensity peak at ~564 nm and a minor hill around 600 nm. After mixed with 5-fold more Cy5-labeled substrate, the fluorescence intensity at 564 nm decreased by half and a new peak arose at ~664 nm (Fig. 4.6 left). This new peak is from Cy5 and the fluorescence owing to FRET and the direct excitation by 532 nm of light should be
merged. The intensity drop at 564 nm may be due to FRET and also photobleaching of Cy3.

In order to examine the effect of direct excitation, the fluorescence spectrum from Cy5 excited by 532 nm of light which was labeled on substrate (~500 nM) was obtained (Fig. 4.6 right). The fluorescence from direct excitation at 664 nm was measured to some extent. In order to examine the effect of photobleaching of Cy3, the solution containing Cy3-labeled 10–23 enzyme was added to the cuvette to give the same final concentrations as the former measurement. The peak intensity at 664 nm increased and a new peak which is from Cy3 arose at 564 nm as in the former case (dotted orange line). From this ensemble result, we concluded that the photobleaching of dyes was not significant in this experiment and apart from the direct excitation of Cy5 the mixture containing 10–23 enzyme and substrate showed FRET which means the substrate can bind the $EP_2$ complex.

**Single-molecule experiments**

As we observed in the ensemble experiments, the direct excitation of Cy5 by 532 nm of light exists to a considerable extent. In order to check the impact of direct excitation in our TIRF setup, we first did not immobilized the Cy3-labeled 10–23 enzyme, which is pre-annealed with the biotinylated chain, and added the Cy5-labeled substrate in the imaging buffer. The green channel showed only the
debris molecules. The red channel showed overall higher background signal than that of the green channel which is due to the direct excitation of freely diffusing Cy5. Secondly, we immobilized the Cy3-labeled 10–23 enzyme and added no substrate in the imaging buffer. The green channel distinctly showed single Cy3 molecules. The red channel was quite dark compared to that of the former case. A smFRET time trajectory of the immobilized 10–23 enzyme is shown in Fig. 4.7. Only the fluorescence from Cy3 is exhibited.

![Graph showing fluorescence over time](image)

**Fig. 4.7.** Representative time trajectory of the immobilized Cy3-labeled 10–23 enzyme. The leakage from donor to acceptor was 10 %.
Finally, when we added the Cy5–labeled substrate to the enzyme–immobilized chamber, direct excitation was shown on the red channel and we could obtain smFRET time trajectories with fluctuating fluorescence intensities of Cy3 and Cy5 (Fig. 4.8). Because of the high background signal, we reduced the concentration of substrate from 200 nM to 50 nM.

The substrate unbound state shows no FRET since only Cy3 was localized. The low FRET state (E~0.4) occurs after substrate half–binding which indicates shortcut binding. The fast replacement of the pre–annealed arm (gray strand in Fig. 4.8) through branch migration leads to the substrate full–bound state which shows higher FRET (E~0.8) and the substrate can be displaced vice versa (half–dissociation). The full–dissociation reaction after half–binding was also observed. The trace proceeding from the high FRET state to no FRET state is due to the fast half– and full–dissociation of the substrate when the two steps are not distinguishable in our time resolution or the dissociation of Cy5–labeled product after cleavage. We consider the latter case is more likely but we cannot conclude with our sample. The result of this experiment suggests that shortcut binding and subsequent strand displacement can occur in the enzymatic reaction of 10–23 deoxyribozyme.
Fig. 4.8. Reaction scheme for the sample and a representative time trajectory (34 °C, pH 7.52, 50 nM substrate). Half binding can be followed by full binding which replaces the annealed arm through branch migration. The background fluorescence of acceptor is due to direct excitation of Cy5 diffusing freely in solution.

We did not tested the case of $EP_1$ however, we suppose the $EP_1$ state has sufficient potential for shortcut binding occurrence since the two arms have same length and the GC content does not vary that much. We concluded that the lower FRET states after the $EP$ state as in Fig. 4.4 and 4.5 are due to the shortcut-binding reaction.
4.3.3 Dependence of rates for each reaction sub-step on the substrate concentration

Dependence of shortcut-binding-occurrence on the substrate concentration

Since shortcut binding is an association reaction of complementary oligonucleotides, it is highly likely that the ratio of shortcut-binding-occurrence and the rate of shortcut binding are dependent on the concentration of DNA strands. We analyzed the ratio of shortcut-binding-occurrence at three substrate concentrations: 50 nM, 200 nM, and 800 nM (Fig. 4.0). More than 600 pathways were collected at each case and they were largely classified into two categories: the normal reaction path which includes no shortcut binding and the shortcut-binding (SB)-included reaction path which passes through more than one shortcut binding between the times the enzyme starts to wait for substrate binding and the enzyme gets free again. SB-included pathways were further categorized according to the number of shortcut binding per reaction pathway. At 50 nM substrate, more than 90% of enzymatic reactions were conducted through the normal pathway and among the few SB-included pathways, shortcut binding conducted just once per path largely dominated. At 200 nM substrate, the percentage of the SB-included path increased approximately 4-fold than at 50 nM substrate and shortcut binding occurring more than twice before the enzyme gets free also increased. At 800 nM
substrate, the percentage of the SB–included path almost doubled than that of the normal path and most of SB–included pathways contained the shortcut–binding reaction twice. Overall, shortcut binding occurred more frequently at higher substrate concentration. We suggest that as the substrate concentration gets higher, the probability of a new substrate forming a nucleation complex with the $EP$ state before dissociation2 increases resulting the higher ratio of shortcut–binding–occurrence.

**Fig. 4.9.** The percentage of the normal pathway, which does not go through the shortcut–binding reaction, decreased while the percentage of the shortcut–binding (SB)–included trace increased at higher substrate concentration. At higher substrate concentration, shortcut binding occurred more frequently before the enzyme gets free.
The effects of the substrate concentration on the rate of each reaction sub-step

Dwell times of each forward reaction illustrated in Fig. 4.10 were collected as we varied the substrate concentration. We neglected reverse reactions which are probabilistically unfavorable thus rarely seen in our experimental system. We did not differentiated between the two substrate-recognition arms. The distribution of dwell times of six individual reaction sub-steps, binding, cleavage, dissociation1, shortcut binding, dissociation’, and dissociation2, fitted well with single-exponential decay function. The lifetimes of each reaction steps are represented as a function of substrate concentration (Fig. 4.11). The reciprocal of lifetime is the rate which means when lifetime decreases, the rate becomes faster, and vice versa.

![Reaction pathway of 10-23 deoxyribozyme. Analyzed forward rate constants are represented in black.](image-url)

*Fig. 4.10. Reaction pathway of 10-23 deoxyribozyme. Analyzed forward rate constants are represented in black.*
Fig. 4.11. Lifetimes of each sub-step in the enzymatic process of 10–23 deoxyribozyme by varying substrate concentrations. Lifetimes of the sub-steps showing no clear dependence on substrate concentration are represented in dashed lines (cleavage, dissociation1 and dissociation’1) and those showing dependence represented in solid lines (binding, shortcut binding and dissociation2). Error bars are S.E.M.

Since the rate-limiting step of enzyme-substrate hybridization is a diffusion-controlled nucleation reaction\textsuperscript{11}, the binding reaction is dependent on the substrate concentration. Under the conditions studied, the reaction obeys pseudo-first order kinetics. Cleavage and dissociation1 show no clear dependence on the substrate concentration. It is reasonable because they are unimolecular reactions of the ES and EP\textsubscript{1}EP\textsubscript{2} complex, respectively.
Another unimolecular reaction, the strand-displacement reaction, also seems to be independent on the substrate concentration. Strand displacement is quite fast (lifetime ~ 4.8 sec) hence it is also possible that we missed the dependency under the limited temporal resolution of 0.1 sec, however, we suppose this is not the case. Strand displacement might be much faster than we calculated because we neglected many possible shortcut-binding-pathways if the dwell time of the \( EPS \) state was too short to be distinguished from other states.

The notable points are the rate of shortcut binding and dissociation2, both of which are dependent on the substrate concentration according to our data. Shortcut binding is an association reaction between the two oligonucleotides hence we expected the rate is dependent on the substrate concentration. At 50 nM substrate, the rate of shortcut binding is 1.9-fold faster than the rate of binding and similar at other conditions. However, as we mentioned in Chapter 3.3.1, collected dwell-times of the binding reaction is actually more close to that of the substrate half-binding reaction since the full-binding to the next free arm of the enzyme is too fast to be recognizable. Thus, the 1.9-fold difference seems unusual. Dissociation2 is a unimolecular reaction of the \( EP \) complex but the substrate molecule is affecting the rate of it. We suggest that the two reactions, shortcut binding and dissociation2, compete with each other.
The $EP$ complex has two possible destinations. One is to proceed to dissociation2 and return to the free enzyme and another is to meet the new substrate (shortcut binding) and release the remaining product by strand displacement. Because of these crossroads, we cannot measure the intrinsic rate constants for both reactions and the observed reaction times, which are averaged, would be the same statistically at the system we studied. This can be easily shown by calculating the probability density functions of each species. For convenience, the product released later will be designated as $P_1$ from now on. If we only take the two reactions into account, the reaction mechanism can be written as below.

$$EP_1 \xrightarrow{k_{d2}} E' + P_1$$

$$EP_1 + S' \xrightarrow{k_{sb}^0} EP_1 S'$$

$k_{d2}$ and $k_{sb}$ are the rate constants of dissociation2 and shortcut binding, respectively. $k_{sb}^0 = k_{sb}[S]$ and $S'$ is the new substrate. $E'$ is designated to distinguish from $E$. If shortcut binding occurs at time $t$, it means dissociation2 did not occur until the time $t$. The normalized probability density function for the observed shortcut binding reaction, $f(t; k_{sb,obs})$, can be written as follows.

$$f(t; k_{sb,obs}) = \frac{dP_{EP_1S'}(t)}{dt} = k_{sb,obs} \exp(-k_{sb,obs}t)$$

$$= Nk_{sb}^0 \exp(-k_{sb}^0t) \left\{ 1 - \int_0^t k_{d2} \exp(-k_{d2}x) \, dx \right\}$$

$$\Rightarrow k_{sb,obs} \exp(-k_{sb,obs}t) = Nk_{sb}^0 \exp(-(k_{sb}^0 + k_{d2})t)$$
\[ N = \frac{k_{sb,obs}}{k_{sb}^0} \]

And vice versa, the normalized probability density function for the observed dissociation2 reaction, \( f(t; k_{d2,obs}) \), is deduced similarly therefore, we obtain \( k_{sb,obs} = k_{d2,obs} = k_{sb}^0 + k_{d2} \). The observed reaction times for both shortcut binding and dissociation2 converge to the same value \( 1/([S]k_{sb} + k_{d2}) \). The data set for shortcut binding at 50 nM substrate was too small (57 reactions) hence it might be statistically inaccurate. If we make an assumption that

\[ k_{sb}^0 \approx k_b^0 \]

then, we can approximately calculate the intrinsic rate constant of dissociation2

\[ k_{d2,obs} = k_{sb}^0 + k_{d2} \approx k_b^0 + k_{d2} \]

\[ \therefore k_{d2} \approx k_{d2,obs} - k_b^0 \]

According to our data, the intrinsic rate of dissociation2 must be much slower (~0.012 sec\(^{-1}\)) than the skewed rate of dissociation2 (0.0276 to 0.0791 sec\(^{-1}\)).
4.3.4 The rate equations: implications on the catalytic turnover of 10–23 deoxyribozyme

Derivation of single–molecule rate equations

To fully understand the kinetics of 10–23 deoxyribozyme, we derived the rate equations of single–molecule versions that describe each of the enzyme sub–reactions based on the probabilities, P(t), of finding the single enzyme molecule in each state\textsuperscript{12,13}. We attempted to compare the single turnover rate of three kinetic models: (1) the conventional normal pathway, (2) the hypothetical pathway that does not have dissociation\textsuperscript{2} but always proceed to shortcut binding, and (3) the realistic shortcut–binding–included pathway. For all three models, we neglected the reverse reaction of binding which is not necessary for comparing the three models.

According to (1) the conventional model of 10–23 deoxyribozyme, there exist only binding, cleavage, dissociation\textsuperscript{1} and dissociation\textsuperscript{2}:

$$
E + S \xrightleftharpoons{[S]k_b} ES \xrightarrow{k_c} EP_1 P_2 \xrightarrow{k_{d_1}} EP_1 + P_2 \xrightarrow{k_{d_2}} E' + P_1 + P_2 \quad (E' \xrightarrow{\delta} E)
$$

where $k_{d_0} = [S]k_b$ and $E'$ converts back to $E$ instantaneously. The turnover rate of this model gives a similar result as MM equation which shows hyperbolic dependence on the substrate concentration.

(2) The hypothetical mechanism that $EP_i$ proceed only to the shortcut–binding reaction can be written as follows.
\[
E + S \xrightarrow{[S]k_b} ES \xrightarrow{k_c} EP_1 P_2 \xrightarrow{k_{d_1}} EP_1 + P_2 + S' \xrightarrow{k_{d_2}} EP_1 S' + P_2 \xrightarrow{k_{sd}} ES' + P_1 + P_2
\]

\[(ES' \xrightarrow{\delta} ES)\]

We included the reverse reaction of shortcut binding. In this case, the reverse reaction of shortcut binding competes with the strand-displacement reaction. \(ES'\) also converts back to \(ES\) instantaneously.

Finally, (3) the realistic model which includes both dissociation2 and shortcut binding is written below.

\[
E + S \xrightarrow{[S]k_b} ES \xrightarrow{k_c} EP_1 P_2 \xrightarrow{k_{d_1}} EP_1 + P_2 \xrightarrow{k_{d_2}} E' + P_1 + P_2 \quad (E' \xrightarrow{\delta} E)
\]

\[
EP_1 + P_2 + S' \xrightarrow{k_{d_2}} EP_1 S' + P_2 \xrightarrow{k_{sd}} ES' + P_1 + P_2
\]

\[(ES' \xrightarrow{\delta} ES)\]

In this model, dissociation2 and shortcut binding compete with each other and also, the reverse reaction of shortcut binding and strand displacement compete with each other.

All three mechanisms share the same front part written in gray letters. From the start point of the enzymatic reaction to the generation of the \(EP_1\) complex, the averaged rate is same for mechanism (1), (2), and (3). Thus, we calculated the mean dwell-time \(<t>\) (the reciprocal of \(<t>\) is an average rate) of initial part (the gray part) \(<t>_{ini}\) and the characteristic latter parts \(<t>_{(1),lat}, <t>_{(2),lab}\) and \(<t>_{(3),lat}\) separately. Then, the turnover rate of each mechanism can be obtained by the reciprocal of \(<t>_{ini} + <t>_{lat}\). The
concentration of substrate \([S]\) in single-molecule experiments is
considered nearly constant. \(<t>_{ini}\) can be deduced easily as:

\[
<t>_{ini} = \frac{k_ck_d1 + k_b(k_c + k_d1)[S]}{[S]k_bk_ck_d1}
\]

For the latter part of mechanism (1),

\[
EP_1 \xrightarrow{k_d2} E' + P_1 \quad (E' \xrightarrow{\delta} E)
\]

\[
\frac{dP_{EP_1}(t)}{dt} = -k_d2P_{EP_1}(t)
\]

\[
\frac{dP_{P_1}(t)}{dt} = \frac{dP_{E'}(t)}{dt} = k_d2P_{EP_1}(t)
\]

\[
P_{EP_1}(0) = 1, P_{P_1}(0) = P_{E'}(0) = 0
\]

where \(t\) is the elapsed time from the beginning which starts from \(EP_1\).

It is easily shown that

\[
P_{EP_1}(t) = \exp(-k_d2t)
\]

\[
\frac{dP_{P_1}(t)}{dt} = k_d2P_{EP_1}(t) = k_d2\exp(-k_d2t)
\]

\[
<t>_{(1),lat} = \int_0^\infty t \frac{dP_{P_1}(t)}{dt} dt = k_d2
\]

Since it is an elementary reaction, the result is obvious. Now we can
calculate a single turnover rate of normal reaction pathway (1)

\[
V_{(1)} = \frac{1}{<t>_{ini} + <t>_{(1),lat}}
\]

\[
= \frac{[S]k_bk_d1k_c}{k_d2k_d1k_c + [S](k_bk_d2k_c + k_bk_d2k_d1 + k_bk_d1k_c)}
\]

which resembles the MM equation.
For the latter part of mechanism (2),

\[ EP_1 + S' \xrightarrow{k_{sb}} \underset{k_{-sb}}{EP_1 S'} \xrightarrow{k_{sd}} ES' + P_1 \quad (ES' \xrightarrow{\delta} ES) \]

\[ \frac{dP_{EP_1}(t)}{dt} = -[S]k_{sb}P_{EP_1}(t) + k_{-sb}P_{EP_1 S'}(t) \]

\[ \frac{dP_{EP_1 S'}(t)}{dt} = [S]k_{sb}P_{EP_1}(t) - (k_{sd} + k_{-sb})P_{EP_1 S'}(t) \]

\[ \frac{dP_{P_1}(t)}{dt} = \frac{dP_{ES'}(t)}{dt} = k_{sd}P_{EP_1 S'}(t) \]

The result is

\[ \frac{dP_1(t)}{dt} = \frac{k_{sd}(A + [S]k_{sb})(B + [S]k_{sb})}{k_{-sb}(B - A)} \left\{ \exp(At) - \exp(At) \right\} \]

\[ < t >_{(2),lat} = \frac{k_{sd}(A + [S]k_{sb})(B + [S]k_{sb})}{k_{-sb}(B - A)} \left( \frac{1}{A^2} - \frac{1}{B^2} \right) \]

where \( A \) and \( B \) are given by

\[ A = \frac{-(k_{sd} + k_{-sb} + [S]k_{sb}) + \sqrt{(k_{sd} + k_{-sb} + [S]k_{sb})^2 - 4[S]k_{sb}k_{sd}}}{2} \]

\[ B = \frac{-(k_{sd} + k_{-sb} + [S]k_{sb}) - \sqrt{(k_{sd} + k_{-sb} + [S]k_{sb})^2 - 4[S]k_{sb}k_{sd}}}{2} \]

However, the resulting \( V_{(2)} = 1/(<t>_{in} + <t>_{(2),lat}) \) also shows hyperbolic dependence on the substrate concentration as the MM equation with different maximum velocity (it will be discussed in more detail in the next section).

For the latter part of mechanism (3),

\[ EP_1 \xrightarrow{k_{d2}} E' + P_1 \quad (E' \xrightarrow{\delta} E) \]
\[
EP_1 + S' \xrightarrow{[S]k_{sb}} EP_1S' \xrightarrow{k_{sd}} ES' + P_1 \quad (ES' \rightarrow ES)
\]

\[
\frac{dP_{EP_1}(t)}{dt} = -([S]k_{sb} + k_{sd})P_{EP_1}(t) + k_{sb}P_{EP_1S'}(t)
\]

\[
\frac{dP_{EP_1S'}(t)}{dt} = [S]k_{sb}P_{EP_1}(t) - (k_{sd} + k_{sb})P_{EP_1S'}(t)
\]

\[
\frac{dP_{P_1}(t)}{dt} = \frac{dP_E(t)}{dt} + \frac{dP_{ES'}(t)}{dt} = k_{d2}P_{EP_1}(t) + k_{sd}P_{EP_1S'}(t)
\]

The result is

\[
\frac{dP_{P_1}(t)}{dt} = k_{d2}\{C\exp(\lambda_1 t) + (1 - C)\exp(\lambda_2 t)\} + k_{sd}D\{\exp(\lambda_1 t) - \exp(\lambda_2 t)\}
\]

\[
\langle t \rangle_{(3),lat} = k_{d2}\left(\frac{C}{\lambda_1^2} + \frac{1 - C}{\lambda_2^2}\right) + k_{sd}D\left(\frac{1}{\lambda_1^2} - \frac{1}{\lambda_2^2}\right)
\]

where \(C\), \(D\), \(\lambda_1\), and \(\lambda_2\) are given by

\[
C = \frac{a + \sqrt{a^2 - 4b}}{2} - 2(k_{d2} + [S]k_{sb}), \quad D = \frac{[S]k_{sb}}{\sqrt{a^2 - 4b}}
\]

\[
\lambda_1 = \frac{-a + \sqrt{a^2 - 4b}}{2}, \quad \lambda_2 = \frac{-a - \sqrt{a^2 - 4b}}{2}
\]

\[
a = k_{d2} + [S]k_{sb} + k_{sd} + k_{sb}, \quad b = k_{d2}k_{sb} + k_{sd}k_{d2} + [S]k_{sb}k_{sd}
\]

The arranged \(V_{(3)} = 1/(\langle t \rangle_{ini} + \langle t \rangle_{(3),lat})\) takes the form of

\[
V_{(3)} = \frac{C_1[S]^2 + C_2[S]}{C_3[S]^2 + C_4[S] + C_5}
\]

where \(C_1\), \(C_2\), \(C_3\), \(C_4\), and \(C_5\) are composed of the rate constants. \(V_{(3)}\) does not take the form of the MM equation. How the values of the rate constants affect the turnover rate of each mechanism will be discussed in the next section with simulated graphs by MATLAB.
Simulated results: implications on turnover rate

We made two assumptions $k_b \approx k_{sb}$ and $k_{dl} \approx k_{-sb}$ when simulating the turnover rate. The reverse reaction of shortcut binding is a half-dissociation reaction in the presence of the remaining bound product. $k_{sb}$ and $k_{-sb}$ can be experimentally determined by our samples which were used to verify the presence of shortcut binding and strand displacement. The experiment will be progressed later on.

First, we compared the derived rate equations with our experimental data (Fig. 4.12). The rate constants for plotting the turnover rates of three mechanisms were given as $k_b = k_{sb} = 1.56 \times 10^5$ M$^{-1}$s$^{-1}$, $k_c = 1/44.1$ s$^{-1}$, $k_{dl} = k_{-sb} = 1/29.4$ s$^{-1}$, $k_{d2} = 1/74$ s$^{-1}$, and $k_{sd} = 1/4.8$ s$^{-1}$ based on our data obtained at 26 °C, pH 7.52, with 100 mM of Mg$^{2+}$ and 50 mM of Na$^+$. Note that the intrinsic rate of dissociation$2$ is slower than the rate of cleavage.

The turnover rate of the normal pathway which does not have the shortcut binding process, $V_{(1)}$, shows a hyperbolic dependence on the substrate concentration with the lowest maximum velocity $\sim 0.0068$ s$^{-1}$. This maximum velocity is exactly the same as that of the MM equation.

The turnover rate of the pathway proceeding only to shortcut binding, not to dissociation$2$, $V_{(2)}$, is slower than the normal mechanism till the substrate concentration reaches $\sim 107.5$ nM. This is because, under lower substrate concentration, the rate of dissociation$2$ is faster than the rate of product formation by shortcut
binding process. In addition, since the rate of shortcut binding is dependent on the substrate concentration, \( V_{(2)} \) can accelerate even further. As the substrate concentration increases, \( V_{(2)} \) accelerates more than \( V_{(1)} \) reaching the maximum velocity of \( \sim 0.0128 \) s\(^{-1}\), almost doubled compared to the maximum velocity of \( V_{(1)} \).

The turnover rate of the pathway of the dissociation2 and shortcut binding process coexist, \( V_{(3)} \), acts differently relative to the MM equation. \( V_{(3)} \) and \( V_{(1)} \) is almost the same when the substrate concentration is lower. However, \( V_{(2)} \) gradually increases more than \( V_{(1)} \) and comes close to \( V_{(2)} \). This can be explained in the following manner. Under lower substrate concentration, shortcut binding is sufficiently slower than dissociation2, thus, the occurrence of shortcut binding is low (Fig. 4.9). Therefore the mechanism is reduced to the normal pathway, having the almost same value of the turnover rate. As the substrate concentration increases, shortcut binding occurs more frequently and rapidly. When the substrate concentration becomes high enough, most of the \( EP_1 \) complex proceed to shortcut binding. Hence the mechanism is reduced to the mechanism (2), showing the identical maximum velocity \( \sim 0.0128 \) s\(^{-1}\). Our experimental data of the turnover rate of 10–23 deoxyribozyme is more close to \( V_{(3)} \) rather than \( V_{(1)} \) indicating that shortcut binding and strand displacement must be considered in the enzymatic mechanism of 10–23 deoxyribozyme.
Fig. 4.12. (Top) The turnover rate of normal pathway \( V_1 \), shortcut-binding-only pathway \( V_2 \), realistic pathway including shortcut binding \( V_3 \) which had been derived beforehand were plotted using MATLAB. The rate constants were given as \( k_{b}=k_{sb}=1.56 \times 10^5 \text{ M}^{-1}\text{s}^{-1}, k_{c}=1/44.1, k_{d}=k_{sb}=1/29.4, k_{d2}=1/74, k_{sd}=1/4.8 \text{ s}^{-1} \), based on our data. (Bottom) Comparison of the data from our single-molecule experiment with the simulated \( V_3 \). Error bars are S.E.M.
What if dissociation2 is even slower? The situation can be made if one of the substrate-recognition domain of the DNA enzyme is even longer than 10 bases, e.g. 15 bases. It is known that even if the duplex of nucleic acids becomes more than 15 base pairs, it hardly dissociates ($k < 10^{-4}$ s$^{-1}$)$^{14,15}$. The hybridization rates of 25-base sequences and 20-base sequences would not be significantly different$^{16}$. The rate of strand displacement would become approximately 4-fold slower$^{10}$. When we substitute $k_{d2}=1/10000$ s$^{-1}$, and $k_{s2}=1/19.2$ s$^{-1}$ normal turnover hardly occurs (Fig. 4.13). The turnover only occurs through the shortcut binding process, thus, $V_{(3)}$ becomes identical to $V_{(2)}$ regardless of the substrate concentration. If we considered the mechanism of 10–23 deoxyribozyme as the MM mechanism, designing the 25mer substrate would be hard to think of. However, if we consider the shortcut-binding and strand-displacement process, asymmetric design of the binding arms could be possible. Thus, we suggest strand displacement enables the turnover of deoxyribozymes that have longer substrate sequences.
Fig. 4.13. The plot of the turnover rates when $k_{d2}=1/10000$ s$^{-1}$ and $k_{sf}=1/19.2$ s$^{-1}$ was substituted.
4.4 Conclusion

We observed and verified the existence of the two additionally important reaction process, shortcut binding and strand displacement, in the enzymatic mechanism of 10–23 deoxyribozyme in real time, using the TIRF–FRET method. Shortcut binding pathway occurs more frequently as the substrate concentration increases. Owing to the competition between shortcut binding and dissociation, the observed rates of shortcut binding and dissociation is skewed thus the observed rate of dissociation also depends on the substrate concentration. The mechanism and turnover rate equation of 10–23 deoxyribozyme differ from the classic MM equation because of the shortcut–binding and strand–displacement process. The result of simulation gives us a hint for the validity of the DNA enzyme with longer substrate–binding arm. DNA enzymes with asymmetric arm substrate–binding domain might improve the enzymatic turnover activity. Other ribozymes and deoxyribozymes may have similar mechanism as 10–23 deoxyribozyme and more systematic design of these enzymes could be possible.
4.5 References


5. Appendix: The Effects of Glycerol on the Kinetics of the Four Reaction Sub–Steps of 10–23 Deoxyribozyme

5.1 Introduction

Glycerol is a viscous, non–toxic liquid which is composed of three hydroxyl groups responsible for its solubility in water. Glycerol and its aqueous solutions have been commonly used to mimic the viscous cellular environments\(^1\)–\(^6\). Many cellular processes, such as the diffusion of biomacromolecules, the binding and interaction between different biomolecules, the conformational dynamics of proteins, and reaction rates are influenced by the viscosity of solutions. Apart from the effect of the viscosity, the physico–chemical effect of solutes added to the solution cannot be neglected. The stability of macromolecules such as DNA duplex and proteins are influenced by nonspecific interactions with molecules and ions in the surrounding medium\(^7\)–\(^8\).

Herein, we studied the effect of glycerol on the reaction rates of four individual enzymatic steps, binding, cleavage, dissociation1, and dissociation2, for 10–23 deoxyribozyme by single molecule total internal reflection fluorescence measurement. We conducted the
single-molecule experiments at glycerol concentration of 0 % and 25 %. The viscosity of the 25 % glycerol solution is almost double compared to the 0 % glycerol solution. The reaction rates of each step showed significant differences at different conditions. The results can be explained by the effects of both viscosity and interactions between glycerol and DNA. On the other hand, polyacrylamide gel electrophoresis showed little glycerol dependence for the enzymatic turnover rate at glycerol concentration of 0 %, 12 %, 20 %, and 25 %, which shows that single-molecule measurement can reveal kinetic factors that are averaged and often hidden in an ensemble measurement.
5.2 Experimental

Single-molecule measurement

We used the same TIRF setup as previous studies. The designs of oligonucleotides were same as those used in Chapter 3.2.1 and 5’-GU-3’ substrate was used. The imaging buffer commonly contained 114 μL of saturated and filtered Trolox solution, 8 μL of 1 M Tris–HCl (pH 8; pH 7=6:4), 10 μL of 2 M MgCl₂, 2 μL of 10 mg/mL BSA, 8 μL of pre-imaging buffer (PIM; pH 8; pH 7=6:4), 4 μL of 10 μM substrate diluted with 10 mM Tris–HCl (pH 8), and 4 μL of 40 % w/v D–glucose. The pH value in brackets indicates the pH of Ambion® main stock at room temperature. PIM contains 10 μg/μL catalase, 75 unit/mL pyranose oxidase, 25 mM DTT, 1.25 M NaCl, and 250 mM Tris–HCl. For 0 % glycerol solution, 50 μL of distilled water was added and for 25 % v/v glycerol solution, 50 μL of 99.5 % glycerol (Sigma–Aldrich, 49767) was added to the imaging buffer. The final volume of imaging buffer was 200 μL. The experiments were conducted at 30 ℃ and the pH value of the imaging buffer was 7.53 (±0.02) on average.

The viscosity of the solution at 30 ℃ is taken from the tabulated viscosity of water/glycerol mixture⁹. According to the table, viscosities of 0, 12, 20, and 25 % v/v glycerol solutions are 0.800, 1.082, 1.360, and 1.590 cP, respectively. Buffer and salt
concentrations are considered to have only a minor effect on the viscosity.

**Ensemble measurement**

We used denaturing polyacrylamide gels. The protocol to make 5 mL x 4 gels is as follows. We made sufficient amount of gel mixture (40 mL).

(1) Prepare the clean glass plates, spacers, and combs (CAVOY; 10 x 8 cm gel size, 0.75 mm thickness, 10-well). Rinse the plates with distilled water and ethanol and dry completely. Assemble the glass plates with spacers in gel caster.

(2) Put 19.2 g of urea (Ambion®; RNase free) into a 50 mL falcon tube. The final concentration of urea is 8 M.

(3) Add 4 mL of 10x TBE (Ambion®).

(4) Add 16 mL of 40 % acrylamide solution (BIO-RAD; acrylamide:bisacrylamide=29:1). The final concentration of acrylamide is 16 %. Acrylamide is a neurotoxin thus handle with care.

(5) Add distilled water to make 40 mL.

(6) Heat the solution in the microwave until it gets warm and the solutes completely dissolve.

(7) Put the falcon tube into the ice box and wait for 15 min to cool down to room temperature.
(8) Add 280 μL of 10 % w/v ammonium persulfate (APS). The final concentration of APS is 0.07 % w/v.

(9) Add 28 μL of TEMED. Immediately, pour the gel mixture slowly into the prepared gel form and insert the comb into the gel carefully. Let the acrylamide to polymerize for 1 hr at room temperature.

(10) Make 1x TBE (900 mL of distilled water + 100 mL of 10x TBE) for gel running buffer. Wash the wells thoroughly by pipetting the buffer up and down using a syringe. Pour all of the remaining buffer into the gel cabinet.

(11) Pre-electrophorese gels for more than 1 hr at a voltage of 100 V. This is to warm up the gels. Now it is ready for polyacrylamide gel electrophoresis (PAGE). Before sample loading, turn off the high voltage and wash the wells one more time.

We compared the turnover rate of 10–23 deoxyribozyme at glycerol concentration of 0 %, 12 %, 20 %, and 25 %. The substrate was labeled with Alexa Flour 488 for imaging using a gel imager. The sequence is the following: 5’-/5Alex488N/TAT CCA CTA TrGrUGAA GAG GTT-3’. The 10–23 enzyme sample was the same one used for TIRF study which were pre-annealed with the biotinylated poly-T(18).

The total volume of the reaction solution was 200 μL for each reaction. All reactions were carried out at 30 °C, pH 7.53. The reaction solutions commonly contained 114 μL of Trolox solution, 8 μL of 1 M Tris–HCl (pH 8:pH 7=6:4), 10 μL of 2 M MgCl₂, 2 μL
of 10 mg/mL BSA, 8 μL of PIM (pH 8; pH 7 = 6:4), 2 μL of 100 μM substrate diluted with 10 mM Tris–HCl (pH 8), 1.5 μL of 10 mM Tris–HCl (pH 8), and 4 μL of 40% w/v D-glucose. For 0%, 12%, 20%, and 25% v/v glycerol conditions, 50 μL of distilled water, 26 μL of distilled water + 24 μL of glycerol, 10 μL of distilled water + 40 μL of glycerol, and 50 μL of glycerol were added to the reaction solution, respectively. The solutions were incubated in a dry bath for 10 min at 30 °C before the addition of the enzyme. To initiate the enzyme reaction, 0.5 μL of 20 μM 10–23 was added to the solutions, incubated for 15 min, then magnesium ions were quenched with EDTA to terminate the reaction. The loading solution was composed of 16 μL of 0.5 M EDTA (pH 8), 9.6 μL of 6x orange DNA loading dye (Thermo Scientific), and 32 μL of the reaction solution. 6x orange DNA loading dye includes 10 mM Tris–HCl (pH 7.6), 0.15% orange G, 0.03% xylene cyanol FF, 60% glycerol, and 60 mM EDTA. The loading solutions were put into the ice box immediately after mixing.

The loading solutions were loaded to the wells with a volume of 10 μL, and the electrophoresis started to run at a voltage of 100 V for 3 hr. At the end of each well, residual loading solutions were loaded. The gels were illuminated with UV light, photographed, and analyzed using a gel imager (Syngene, G:BOX).
5.3 Results and Discussion

Single-molecule TIRF measurements

![Graph showing average reaction times for different steps at 0% and 25% glycerol](image)

Fig. 5.1. Average reaction times for sub-steps, binding, cleavage, dissociation1, and dissociation2, and for the turnover of the 10–23 enzyme at different conditions. The reciprocal of the average reaction time becomes the average rate. The error bars are S.E.M.

The result of the TIRF measurement is described in Fig. 5.1. The rate of enzyme–substrate binding, which is a hybridization reaction between two nucleic acids, shows no critical difference depending on
the glycerol concentration. The rate of cleavage gets slower (≈0.7-fold) when glycerol is added. The rate of sequential dissociation both become approximately four-fold faster when 25 % v/v glycerol is added. The turnover rates for both conditions are almost the same. These results can be explained by both the viscosity effect and the physico-chemical effect of glycerol.

Since the rate of a reaction in solution is affected by the solvent cage effect\textsuperscript{10}, we consider the mechanism for enzyme-substrate binding composed of the following steps:

\[ E + S \xrightleftharpoons[k_{\text{esc}}]{k_{\text{enc}}} ES_{\text{cage}} \xrightarrow[k_r]{k_r} ES, \]

where \( E \) is the immobilized 10–23 enzyme, \( S \) is the substrate in solution, \( ES_{\text{cage}} \) represents the solvent cage, \( ES \) is the product of the duplex formation, \( k_{\text{enc}} \) is the rate constant at which \( E \) and \( S \) encounter, \( k_{\text{esc}} \) is the rate constant at which the reactants escape the solvent cage, and \( k_r \) is the rate at which they form a duplex when within the cage. The \( ES_{\text{cage}} \) state cannot be distinguished by our experimental setup. When \( k_r << k_{\text{esc}} \), the first reaction will be at equilibrium, so the rate constant of the overall reaction will be given by \( (k_{\text{enc}}/k_{\text{esc}})k_r \). On the other hand, when \( k_r >> k_{\text{esc}} \), the rate of the overall reaction is controlled by the encounter rate constant, \( k_{\text{enc}} \). Such reaction is called a diffusion-controlled reaction and a simple model for neutral particles gives the overall rate constant as \( k_{\text{enc}} = 4\pi R(D_E + D_S) \). \( D \) is the translational diffusion coefficient and \( R \) is the radius of the cage.
If the enzyme–substrate binding reaction were a diffusion-controlled reaction, the observed rate constant calculated from our experimental data would be of the same order of magnitude as the encounter rate constant $k_{\text{enc.}}$. The experimental values are measured as $k_{b,0\%} = 3.03 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $k_{b,25\%} = 2.80 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, depending on the glycerol concentration. In solution the diffusion coefficient can be approximated by the Stokes–Einstein relation, $D = k_B T/6\pi \eta r$, where $\eta$ is the viscosity coefficient of the solvent and $r$ is the radius of the diffusing spherical particle. In our experiments the enzyme $E$ is immobilized, thus $D_E$ is neglected. To calculate $D_s$, $r$ is assumed as 1 nm, the distance of a phosphorus atom from the DNA fiber axis. Substituting $T = 303 \text{ K}$, $\eta = 0.8 \text{ cP}$, and $r = 1 \text{ nm}$ gives $D_{s,0\%} = 2.77 \times 10^{-10} \text{ m}^2\text{s}^{-1}$. At 25% glycerol condition, the viscosity is almost doubled, thus $D_{s,25\%} = 1.39 \times 10^{-10} \text{ m}^2\text{s}^{-1}$. The radius of the cage $R$, the contact distance of the two reactants, is assumed as 2 nm. The calculated encounter rate constants are $k_{\text{enc.},0\%} = 4.19 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{enc.},25\%} = 2.10 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, which are almost 10$^4$-fold higher than the experimental values of the binding rate constants. Hence, we concluded that the rate of the binding reaction in the system studied is not a diffusion-controlled reaction, and therefore there is little viscosity dependence. Wetmur & Davidson (1968) have also explained that the nucleation rate constant for DNA renaturation is not controlled by the translational diffusion of the molecules as a whole. As mentioned in Chapter 3.3.2, the nucleation reaction when within the solvent cage is considered to be the rate-limiting reaction.
The stability of duplex polynucleotides is mainly controlled by base-stacking interaction which involves hydrophobic components\textsuperscript{13}. The hydrophobic interaction is caused by an incompatibility of the OH groups of the water molecules with the nonpolar parts\textsuperscript{14}. Both water and glycerol have high concentrations of OH groups per unit volume, however, water has greater propensity to form hydrogen bonds with itself than glycerol does\textsuperscript{15}. Therefore, when 25 \% v/v glycerol is added, the number of water molecules associated within the duplex chain decreases, and thus could make the hydrophobic interactions weaker. This would destabilize the helical structure. There have been many studies regarding the effects of various cosolvents, including glycerol, on the melting of duplex DNA. As the concentration of glycerol increases, the melting temperature of the duplex decreases, which means glycerol destabilizes the structure of duplex DNA\textsuperscript{15-17}. This could explain the acceleration of dissociation rates in our results.

The cleavage reaction is the key chemical reaction of the catalytic system studied. The mechanism has not been fully understood, but as mentioned in Chapter 3.3.2, a specific structure of the catalytic core part of 10–23 deoxyribozyme may play an important role in cleaving the phosphate bond. Because of glycerol, the stability or conformation of the secondary structure of the core part may be affected. The viscosity effect on vibrational frequency of the bonds may also cause the deceleration of the cleavage rate.
Ensemble measurements

According to the single-molecule result we obtained, the turnover rates of 10–23 deoxyribozyme at 0 % and 25 % v/v glycerol conditions are of comparable values despite the difference in the cleavage and dissociation rates. We conducted polyacrylamide gel electrophoresis to compare only the turnover rates at different glycerol concentrations. As the glycerol concentration increases from 0 % to 25 %, the solution becomes more viscous (Fig. 5.2 Top). Fig. 5.2. Bottom shows the ratio of cleaved substrate depending on the glycerol concentration. The turnover rates show no clear dependency on the glycerol concentration. Using TIRFM, kinetic factors of each reaction sub-step can be revealed but using ensemble measurements, the characteristics of each sub-step depending on the glycerol concentration are hard to be distinguished.
Fig. 5.2. Polyacrylamide gel electrophoresis data of 10–23 deoxyribozyme cleaving the substrate at different v/v glycerol concentration. (Top) A typical gel image. The upper line is the substrate part and the lower line is the 5’-labeled product part. (Bottom) An averaged result of four independent experiments. Error bars are S.D.
5.4 Conclusion

Using single-molecule TIRF microscope, we could determine each enzymatic reaction step of 10–23 deoxyribozyme by tracking conformational changes in real-time. Single-molecule kinetic analysis revealed that the cleavage and dissociation steps are significantly affected by glycerol when it is increased to 25 % v/v but the binding step has no clear difference. It is because there are many factors that contribute and often compete with each other to give a final rate of a reaction. Since 10–23 deoxyribozyme and its substrate are composed of polynucleotides, the enzyme-substrate binding and product release steps correspond to the hybridization and denaturation of nucleic acids, respectively. The rate of the binding step seems to have little dependence on the translational diffusion factor. Glycerol seems to affect the stability and conformation of DNA duplex structure. On the other hand, ensemble measurements using PAGE did not showed any significant difference in enzymatic turnover rate depending on glycerol. Accordingly, the single-molecule TIRF measurement can reveal hidden kinetic factors that are indistinguishable in ensemble measurements.
5.5 References


국문초록

형광 공명 에너지 전달 (FRET) 현상을 이용한 전반사 형광 (TIRF) 현미경법은 형광분자가 표지된 단일 생물분자의 구조적인 변화를 나노미터 수준으로 실시간으로 관측하는 것을 가능하게 해준다. 이러한 관측 결과로부터 양상별 실험이에서는 알 수 없었던 특징들을 알 수 있다. 이 논문은 전반사 형광 현미경법을 이용하여 10-23 디옥시라이보자임의 효소 반응 메커니즘을 각 단계별로 연구한 내용을 담고 있다. 10-23 디옥시라이보자임은 RNA 를 자르는 역할을 하는 DNA 로 이루어진 효소이며 현재 다양한 응용 과학 분야에서 널리 연구되고 있다. 이 논문에서 다룬 연구 내용은 크게 두 부분으로 나뉜다.

첫 번째 연구는 전반사 형광 현미경법을 이용하여 10-23 디옥시라이보자임의 내 가지 일반적인 반응 세부 단계의 효소와 기질의 결합, 기질 자르기, 생성물 해리 첫 번째 과정, 그리고 생성물 해리 두 번째 과정에 대한 동역학적 속도를 구분해서 분석한 내용을 담고 있다. 반응의 속도에 따라 영향을 줄 수 있는 온도, 용액의 pH, 그리고 칼리는 부위의 염기 종류와 같은 분자적인 환경을 달리해서 분석해본 결과, 분자의 환경이 달라짐에 따라 효소 반응의 속도는 각 단계별로 다른 양상을 보이고, 동역학적인 매개변수도 각각 다르게 나타났다. 이러한 분석을 통하여 각각의 반응 세부 단계들의 메커니즘과 그 특징들이 더 풍부한 해석을 해볼 수 있었다.

두 번째 연구는 일반적으로 추측되고 있던 10-23 디옥시라이보자임의 효소 반응 세부 단계와는 조금 다른 양상을 보이는 메커니즘을 발견한 것으로 대한 내용을 담고 있다. 10-23
디옥시라이보자임의 생성물 해리 두 번째 과정이 일어나기 전에 새로운 기질이 효소의 비어있는 한 쪽 결합 부위에 붙어서 그 다음 전환 과정으로 넘어갈 수가 있는 것을 전반사 형광 현미경법으로 관측하였다. 마지막 생성물이 떨어지기 전에 새로운 기질이 붙어버리는 과정은 본 연구진은 “지름길 결합”이라고 명명하였다. 지름길 결합 과정 이후에는 새로 붙은 기질이 효소와의 상호작용의 염기 서열을 이용하여 아직 떨어지지 않고 남아있는 생성물을 밀어내어 붙여버리는 가닥 대체 반응이 일어날 수 있다. 본 연구에서는 지름길 결합과 가닥 대체가 일어날 수 있다는 것을 전반사 형광 현미경법과 양상불 실험으로 입증하고, 이 두 세부 단계를 포함하여 동역학적인 분석을 하였다. 기질의 농도가 각 세부 단계의 동역학적인 속도에 어떠한 영향을 미치는지 분석해본 결과, 기질의 농도가 증가할수록 지름길 결합은 더 자주 발생하였고, 효소와 기질의 결합 과정과 함께 생성물 해리 두 번째 과정 또한 기질 농도의 영향을 받는다는 결과가 나왔다. 지름길 결합과 생성물 해리 두 번째 과정이 서로 경쟁적인 관계에 있기 때문에 헤보기 속도가 기질의 농도가 증가함에 따라 둘 다 빨라지게 되는 것이다. 마지막으로, 지름길 결합이 있는 메커니즘이 없는 메커니즘에 대한 속도 수식을 둘어서 지름길 결합과 가닥 대체 과정이 효소의 전환 속도를 빠르게 한다는 것을 설명하고 있다.

종합하여 보자면, 본 연구에서는 전반사 형광 현미경법을 사용하여 10-23 디옥시라이보자임의 메커니즘적인 특징들을 밝혔고, 이러한 연구가 10-23 디옥시라이보자임의 응용뿐만 아니라 다른 효소 작용을 하는 핵산들의 화학적인 메커니즘과 응용을 연구하는데 유용할 수 있기를 기대한다.
주요어: 10-23 디옥시라이보자입, 동역학, 효소적 메커니즘, 단분자
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