**In vitro** Determination of Rapamycin-triggered FKBP-FRB Interactions Using a Molecular Tension Probe

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As protein–protein interactions (PPI) have been mostly investigated in cellulo or in vivo, it is unclear whether the PPI-based imaging schemes are practically valid in a bioanalytical means in vitro. The present study exemplifies the PPI in vitro inside a unique single-chain probe, named TP2.4, which carries a full-length artificial luciferase (ALuc) sandwiched in between two model proteins of interest, e.g., FKBP and FRB, expressed in *E. coli*, and purified. We found that the TP2.4 efficiently recognizes its ligand in vitro and varies its molecular kinetics: i.e., rapamycin boosts the enzymatic affinities \(K_m\) of TP2.4 to its substrates, but does not or only weakly influences the turnover rates \(K_{cat}\) and the maximal velocity \(V_{max}\). The corresponding circular dichroism (CD) study shows that rapamycin weakly contributes to the enhancement of the \(\alpha\)-helical contents in TP2.4. Kinetic constants according to the substrates revealed that a coelenterazine derivative, 6-N\(_3\)-CTZ, exerted the best catalytic efficiency and the greatest variance in the total photon counts. The present study is the first in vitro example that demonstrates how intramolecular PPI works in a purified single-chain bioluminescent probe and what factors practically influence the biochemistry.

**Keywords** Molecular tension probe, protein–protein interactions, artificial luciferase, bioluminescence, *in vitro* assay, imaging

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**Introduction**

Protein–protein interactions (PPIs) are the most fundamental and principal occurrence among intracellular molecular events in cells. PPI initiates most of the biological processes including post translational protein modifications (phosphorylation, methylation, acetylation, etc.), proteolysis, conformational changes in proteins, and trafficking of proteins filling intracellular signaling cascades.\(^1\)\(^\text{—}\)\(^2\) Currently, PPIs have been studied using many optical probes, including fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), and protein-fragment complementation assay (PCA).\(^3\)\(^\text{—}\)\(^4\) We previously introduced a novel single-chain bioluminescent probe of a concept derived from PCA for studying hormone-induced conformation changes in steroid receptors, phosphorylation in signaling proteins, PPIs, and probes measuring cytotoxicity in cells.\(^6\)\(^\text{—}\)\(^8\) Although the single-chain bioluminescent probes are recently emerging as a novel technology, it is still unclear whether the probes practically work even in a cell-free environment, and if works, what kind of molecular dynamics acts behind their functions. As the molecular dynamics in a cell-free condition is completely different from those in intact cells, and in vivo living animals, the probes may face unexpected issues upon their applications to *in vitro* assays.

The present study exemplifies a model PPI system *in vitro* using a molecular tension probe, named TP2.4, in which a full length artificial luciferase 23 (ALuc23) is sandwiched between the FK506-binding protein (FKBP) and the FKBP-rapamycin-binding domain of mTOR (FRB) as a model PPI pair. We chose this rapamycin-mediated FKBP–FRB interaction system, because the mammalian Target Of Rapamycin (mTOR) signaling pathway is known to be deeply related with a number of human diseases such as cancer, diabetes, obesity, and genetic disorders.\(^1\)\(^\text{—}\)\(^3\) The authors previously proved that the basic concept on “strain probes” is applicable to other luciferases and PPI models.\(^4\)\(^\text{—}\)\(^5\) The rapamycin mediated

**Abbreviations**

PPI: Protein-protein interaction. ALuc: Artificial luciferase. TP2.4: Molecular tension probe version 2.4. FRB: FKBP-rapamycin-binding domain of mTOR. FKBP: FK506-binding protein.
intramolecular FRB-FKBP interaction (conformational change) appends molecular tension to the sandwiched ALuc23, which in turn enhances the ALuc23 bioluminescence intensity more greatly than from its relaxed form. The molecular chemistry behind the PPI in purified TP2.4 is investigated, which includes the sensorial response to the ligand, circular dichroism (CD) spectra, and ligand-driven properties of the kinetic constants like the turnover rates and the Michaelis–Menten constant. This study provides a new insight on how the PPI works in in vitro assay systems and what factors practically work behind its biochemistry.

Materials and Methods

Construction of a plasmid vector encoding TP2.4 fusion protein, expression, and the purification

The molecular tension probe, TP2.4, is a single-chain probe, which was made by a tandem linkage of FRB (11 kD, PDB access number: 1AUE_A), artificial luciferase 23 (ALuc23; Genbank access number: MF817968),12 and FKBP (12 kD, Genbank access number: AAP36774.1), the basal design of which was described in our previous study.15 The genetic engineering and purification procedures of TP2.4 are summarized in Suppl. Experimental Procedure 1 (Supporting Information). The workflow is briefly illustrated in Fig. 1(A). The stock concentration of TP2.4 for the other studies hereafter was set to be 22.5 μM. The purity and molecular weight of the obtained TP2.4 was determined by resolving in acrylamide gel electrophoresis and staining with Coomassie Brilliant Blue (CBB).

Determination of rapamycin-driven optical intensities of TP2.4 in varying pH levels in vitro

Rapamycin-driven optical intensities of the purified TP2.4 were determined in a universal buffer (0.2 M boric acid, 0.05 M citric acid, 0.1 M trisodium orthophosphate) with varying pH levels (from pH 5 – 12 (Fig. 1(B)). The pH was adjusted with a NaOH solution. An aliquot (10 μL) of the stock TP2.4 solution (9.7 μM) was initially diluted with 33 μL of a universal buffer with different pHs. Twenty microliters of the mixture were further diluted in 10-fold with the same universal buffer with or without rapamycin (final concentration: 10–6 M). Twenty microliters of the adjusted solution were transferred into each well of a fresh 96-well optical bottom plate (Thermo Scientific) and simultaneously mixed with 50 μL of the same buffer (Promega) containing native coelenterazine (nCTZ) using a multichannel pipette (Gilson). The plate was immediately placed into the chamber of an image analyzer (LAS-4000, Fujifilm) equipped with a cooled charge-coupled device (CCD) camera system (–25°C). The optical intensities were determined with the image acquisition software (Image Reader v2.0) and analyzed with the specific image analysis software (Multi Gauge v3.1). The software provides relative luminescence intensities (RLU) per time (s) and area (mm²). The error bars hereafter indicate the standard deviation (SD) of each mean in two sigma.

Multivalent cation-driven optical properties of TP2.4 in vitro

As we already introduced the multivalent cation-driven optical intensities of ALuc16 in a previous study,16 here, we focused on the contribution of multivalent cations on the signal-to-background intensity ratios (S/B ratios) of TP2.3. An aliquot of the stock TP2.4 solution was first incubated with a PBS solution carrying 10–6 M rapamycin (final concentration) or the vehicle for 10 min. Separately, each multivalent cation was diluted with a cation-free Tris–HCl buffer, where the absence of cation was previously proved by the authors using an inductively coupled plasma mass spectrometry (ICP-MS).16 The rapamycin-incubated TP2.4...
solution was further diluted 100-fold with the Tris-HCl buffer carrying different kinds of multivalent cations. The counter anions were chloride. The final concentrations of TP2.4 and multivalent cations were 10 μg/mL and 10^{-6} M, respectively.

The concentration of multivalent cations, 10 μg/mL, was chosen from the knowledge of the effective concentration (EC) to ALucs in the authors’ previous study. An aliquot (20 μL) of the solutions was transferred into each well of a fresh 96-well optical bottom plate (Thermo Scientific) (0.2 μg per well) and simultaneously mixed with 50 μL of the Tris-HCl buffer dissolving nCTZ with a multichannel pipette (Gilson). The corresponding optical intensities were determined with the same method as that of Fig. 1(B).

The luminescence intensities are expressed as a fold intensity of relative luminescence units (RLU), i.e., RLU (+/-) ratios, where RLU (+) and RLU (-) represent the luminescence intensities with the column-purified TP2.4 after it is incubated with and without rapamycin, respectively; the RLU is an amplified value of photon counts generated from the image analyzer (arbitrary unit) (Fig. 2(A)).

The time courses of TP2.4 according to divalent cations were investigated with a luminometer after auto-injection of the substrate, native CTZ, in a 96-well plate reader (SH-9000, Corona). The optical intensities were recoded every 0.1 s during the first 60 s (Fig. 2(B)).

Circular dichroism (CD) spectra for reasoning the rapamycin-driven optical intensities of TP2.4 in vitro

We further investigated the circular dichroism (CD) spectra for reasoning the multivalent cation-driven feature of S/N ratios of TP2.4 in vitro (Fig. 2(C)).

The measurement solutions were prepared by mixing aliquots of Co(II) (0.17 mM) or Ca(II) (0.25 mM) in pure water with the stock TP2.4, and the CD diagrams were immediately carried out with a CD spectrometer (Jasco J-815, Japan). The spectropolarimeter used a 0.1-cm path length quartz cuvette. The stock solution of TP2.4 (9.7 μM) was dialyzed into a PBS buffer and diluted to a concentration of 2.5 μM. Spectra were acquired from 260 – 200 nm using the following set of instrument parameters: continuous scan mode; 50 nm/min scan speed; 0.5 nm data pitch; 2 nm bandwidth; 2 s integration time; 5 accumulations.

Separately, we investigated the CD spectra of TP2.4 for explaining the rapamycin-driven elevation of the optical intensities (Fig. 2(D)).

The experiment solution was made by mixing an aliquot of
Vehicle or two different concentrations of rapamycin, i.e., $10^{-6}$ and $10^{-5}$ M with the stock TP2.4, where the final concentration of TP2.4 was adjusted to be $45.0 \mu$M. The corresponding CD spectra were determined with the same method as that of Fig. 2(C).

**Kinetic constants of purified TP2.4 with and without rapamycin**

The kinetic constants of purified TP2.4 with selected substrates were determined in the presence or absence of rapamycin (Fig. 3(B) inset a, Table 1). The investigated kinetic constants of purified TP2.4 include maximal reaction rate ($V_{max}$), total photon counts, Michaelis–Menten equation ($K_m$), turnover rates ($K_{cat}$), and the relative total photon counts.

$K_m$, $V_{max}$, and $K_{cat}$ were calculated according to the following protocol: the substrates, nCTZ, 6-N3-CTZ, eCTZ, and vCTZ were first dissolved with methanol and further diluted with HBSS buffer (Gibco) to 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 250, and 500 $\mu$M, respectively. Separately, the TP2.4 stock solution was diluted with the HBSS buffer carrying rapamycin or vehicle to be $10^{-7}$ M (final concentration of TP2.4) and $10^{-7}$ M (final concentration of rapamycin). The TP2.4 solutions with or without rapamycin were primed in the automatic injector of a microplate reader (SH-9000 lab, Corona), whereas 50 $\mu$L of the substrate solutions were set on a black-frame, 96-well optical bottom microplate (Nunc). The bioluminescence intensities over time were immediately recorded for 30 s after injection of 50 $\mu$L of the TP2.4 solution into the prepared microplate.

The kinetic constants, $V_{max}$, $K_m$, $K_{cat}$, were calculated on the basis of the kinetic data using a specific software, Prism 7.04 (GrapPad). The graph showing the fitting is specified in Fig. 3(C).

**Fig. 3 Initial kinetics of TP2.4 according to the substrates in the presence of rapamycin.** (A) The chemical structures of the selected substrates, nCTZ, 6-N3-CTZ, eCTZ, and vCTZ. (B) The initial optical intensities of TP2.4 treated with rapamycin or the vehicle ($n = 4$). The intensities were integrated for 0.5 s after the addition of the specific substrates, nCTZ, 6-N3-CTZ, or eCTZ. The unit “RLU” is an arbitrary unit and means Relative Luminescence Unit. The values were independently measured in quadruplicates from a single experiment ($n = 4$). The error bars are expressed in two sigma values. The $p$-values (student’s T-test) are *** = < 0.001 and ** = < 0.01. Inset “a” shows the variance of the S/B ratios (rapamycin (+)/rapamycin (–)) according to the substrate concentrations. The fold intensity values were obtained by dividing the intensity values of rapamycin (+) condition with rapamycin (–) condition. (C) The graphic information of the kinetic fitting. The kinetic constants, $V_{max}$, $K_m$, $K_{cat}$, were determined on the basis of the kinetic fitting using specific software, Prism 7.04 (GrapPad). The rapa (+) and rapa (–) mean the experimental conditions with and without rapamycin, respectively. The closed and open circles represent the conditions with and without rapamycin, respectively.

Exact determination of the quantum yields (QYs) of the substrates requires knowledge of the exact purities of the substrates and a light-integrating sphere equipped with a photon-counting device equally sensitive to all the wavelengths.17 Alternatively, we determined the relative total photon counts generated by the reaction between TP2.4 and the selected substrates using a microplate reader (SH-9000 lab, Corona) (Table 1).

The relative total photon counts of TP2.4 according to the substrates were determined based on the suggested method for determining QYs.18 In summary, if a luciferase level is in excess compared to luciferins (E >> S), QYs are defined by $P_{tot}/S_{tot}$, where $P_{tot}$ and $S_{tot}$ indicate the total amount of emitted photons and consumed substrates, respectively. As all of the luciferins are consumed in this system, the initial level of luciferin is equivalent to $S_{tot}$.

For the measurement, purified TP2.4 (0.1 $\mu$M in HBSS buffer) was stimulated with vehicle or rapamycin (final concentration: 0.1 $\mu$M). The selected substrates, nCTZ, 6-N3-CTZ, eCTZ, vCTZ were initially diluted with HBSS buffer to 1 pmol per 50 $\mu$L (final concentration). Fifty microliters of the substrate solutions were set in a black-frame, 96-well optical bottom microplate (Nunc), whereas the TP2.4 solution was primed in the automatic injector of the microplate reader. Immediately after automatic injection of 50 $\mu$L of TP2.4 into each well, the total photon emission was integrated for 10 min at room temperature.
Results

Bacterially-expressed TP2.4 was successfully purified with functional maintenance of its binding affinity to rapamycin.

The initial issue regarding the purification of TP2.4 expressed in E. coli is whether the probe is properly expressed in fully folded form in bacterial cells and maintains its enzymatic activities in vitro. This concern originated from the fact that copepod luciferases like Gaussia luciferase (GLuc) and ALuc23 used in TP2.4 single chain probe are cysteine-rich proteins with multiple disulfide bonds, and thus have been considered not suitable for expressing in bacterial systems. To address this issue, TP2.4 was over-expressed in the bacterial strain SHuffle T7 Express lyS with IPTG induction (Fig. 1(A)). The cells were sonicated, and the lysate was centrifuged at 18000 rpm for 40 min. TP2.4 in the subsequent supernatant was consecutively purified using a HisTrap HP column (GE Healthcare) and an ÄKTA Purifier system (GE Healthcare). After purification using two consecutive affinity columns, the chromatogram showed two solid peaks. The TP2.4 samples were obtained from the second peak and subjected to acrylamide gel electrophoresis followed by Coomassie Brilliant Blue (CBB) staining. The staining revealed the presence of expected molecular weight (~45kDa) of TP2.4. The presence of a single band indicates that TP2.4 is successfully expressed and was efficiently purified from bacterial cells without any impurity.

The purified TP2.4 shows enhanced bioluminescence upon exposure to rapamycin in vitro.

The relative optical intensities of purified TP2.4 were determined in a universal buffer (pH range = 5 – 12) in the presence or absence of rapamycin (final concentration: 10^{-6} M, Fig. 1(B)). The optical intensities were greatly influenced by pH. In the presence of rapamycin, TP2.4 emitted ~2- to 4-fold stronger bioluminescence than the vehicle at any pH level tested for the study. The signal-to-background (S/B) ratios, according to the concentrations of the metal cations, Co(II) and Ca(II) (Fig. 2(B)). The addition of the metal cations generally negatively influences the absolute optical intensities. Co(II) and Ca(II) at 10 μg/mL (i.e., 0.17 and 0.25 mM) concentration interestingly induced increased activity with sustained reduction in background signals, resulting in increasing S/B ratios by time. Considering the molar concentrations of TP2.4 and Ca(II), an excess amount of Ca(II) was applied to TP2.4 (0.2 μM) in the experiment. In contrast Co(II) at 1.70 mM (i.e., 100 μM/mL) concentration completely suppressed the optical intensities of TP2.4, whereas the same concentration of Ca(II) partially retained them. The time course analyses show that TP2.4 stimulated with metal cations and rapamycin commonly increase the enzymatic activities of ALucs (Fig. 2, Fig. S2 (Supporting Information)). In contrast with the pH that varies only the enzymatic activities of ALucs (Fig. 2, Fig. S2 (Supporting Information)).

The purified TP2.4 showed differential response in the presence of various ionic strength as measured by optical intensities and S/B ratios.

The molecular dynamics of purified TP2.4 was investigated in the presence or absence of varying multivalent metal cations and ionic strength, because the authors previously found that an excess amount of multivalent cations can modulate the enzymatic activities of ALucs (Fig. 2, Fig. S2 (Supporting Information)). In contrast with the pH that varies only the absolute intensities in an equal proportional magnitude (Fig. 1(B)), divalent metal cations and ionic strength interestingly modulate not only the absolute intensities, but also the enzymatic kinetics, and S/B ratios. The multivalent cations showed much dominant S/B ratios. The best S/B ratios were observed with Co(II), which showed ca. 6-fold higher S/B ratio by virtue of reduced backgrounds (Fig. 2A, Fig. S2). In contrast, Al(III) exerted the poorest S/B ratio, which was caused by the elevated background signals.

We further investigated the kinetics of optical intensities according to the concentrations of the metal cations, Co(II) and Ca(II) (Fig. 2(B)). The addition of the metal cations generally negatively influences the absolute optical intensities. Co(II) and Ca(II) at 10 μg/mL (i.e., 0.17 and 0.25 mM) concentration interestingly induced increased activity with sustained reduction in background signals, resulting in increasing S/B ratios by time. Considering the molar concentrations of TP2.4 and Ca(II), an excess amount of Ca(II) was applied to TP2.4 (0.2 μM) in the experiment. In contrast Co(II) at 1.70 mM (i.e., 100 μM/mL) concentration completely suppressed the optical intensities of TP2.4, whereas the same concentration of Ca(II) partially retained them. The time course analyses show that TP2.4 stimulated with metal cations and rapamycin commonly increase the optical intensities, but interestingly this feature is not observed without metal cations and rapamycin. The comparison is interpreted such that metal cations or rapamycin alone does not necessarily boost the optical intensities by time in vitro, but only when they are combined together.
Furthermore, it should be noted that the S/B ratios are influenced by the buffer additives (Fig. 2(A) vs. Fig. S3 (Supporting Information)) and the species of multivalent cations (Fig. S2). We found that PBS buffers have a character to elevate the background BL intensities with TP2.4 (Fig. 2(A), Fig. S2), whereas the universal buffer keeps the backgrounds low (Fig. S3). The universal buffer consists of three different buffer species and should have higher ionic strength than the PBS buffer. These species may have an effect to stabilize and suppress the background intensities of the purified TP2.4 in the experiment.

In the experimental setup, it is unlikely that the proton level (pH) or other cations are the dominating contributor to the bioluminescence elevation. It is because the proton and other cation levels were fixed with buffers throughout the experiments.

The ionic effects by the counter anions and monovalent cations were not examined in this study, because the counter anions are commonly chloride and the authors already reported that they are less influential to the enzymatic activities of TP2.4.

The effects of rapamycin and metal cations on the structure of TP2.4 were further investigated with circular dichroism (CD) (Fig. 2(C)). The molar ellipticity in the CD spectra at 222 nm reveals that the α-helical portion of TP2.4 is slightly decreased, but did not show significant variation by an addition of 0.25 mM Ca(II) (Fig. 2(B)). This result corresponds with the slightly suppressive optical intensities of TP2.4 by the addition of Ca(II) (Fig. 2(B)).

Does the PPI in TP2.4 influence the α-helical contents?

It has been said that fragmented luciferases are folded in the way of protein-fragment complementation assays (PCA), not beforehand. On the other hand, there is a notion that luciferases are enzymatically active immediately after expression, unlike fluoroscent proteins where, the fluorophores require maturation time. These two ideas conflict and it is still unclear whether the folding occurs beforehand or after PPI. If folding afterward may be a unique phenomenon only of fragmented luciferases.

To address these questions, we further conducted a circular dichroism (CD) study with the purified TP2.4 (Fig. 2(D)). The results show that the molar ellipticity at 222 nm is gradually increased with the addition of rapamycin: i.e., the α-helix ratios of TP2.4 with vehicle, 10⁻⁶ M rapamycin, and 10⁻⁵ M rapamycin in TP2.4 were 39.1, 39.6, and 43.1%, respectively. The α-helical contents vary ca. 4% between vehicle- and rapamycin-stimulated TP2.4. TP2.4 consists of ALuc23, FRB and FKBP. Among the components, it is considered that ALuc23 is the main contributor of the variance in the α-helical ratios: i.e., it is because (i) rapamycin-triggered FRB-FKBP binding does not change the secondary structures, no matter whether the binding has metal ions or not in the system; and (ii) we previously proved that ALuc has a character to change it’s α-helical content by the addition of multivalent cations.

The results show that a small portion of the ALuc23 in TP2.4 remains unfolded after expression and gets folded after addition of rapamycin. However, it remains unclear whether the greatly enhanced bioluminescence of TP2.4 by PPI happens by virtue of further folding or the relieving of steric hindrance by the linkage of FRB and FKBP that hampers the access of the substrates.

Kinetics of purified TP2.4 in the presence and absence of rapamycin

The kinetic constants (Kₐ, Vₘₕₖ, Kᵦ) of TP2.4 were further investigated in the presence and absence of rapamycin (Fig. 3(B) inset a, Table 1, Fig. 3(C)). The substrates, such as nCTZ, 6-N₃-CTZ, eCTZ, and vCTZ, were selected for the measurement of kinetic constants; 6-N₃-CTZ was chosen because this substrate is precisely known to be highly specific to ALuc23. eCTZ and vCTZ were selected because they have a unique chemical structure linking at the C-5 and C-6 positions of the substrate backbone and are known to emit red-shifted bioluminescence with marine luciferases.

The optical intensities of TP2.4 were determined between 0 to 0.5 s (T₀) after addition of nCTZ, 6-N₃-CTZ, or eCTZ (Fig. 3(B)). The optical intensities at T₀ were greatly variant according to whether TP2.4 is treated beforehand with rapamycin or not. The largest S/N ratio between TP2.4 treated with rapamycin or the vehicle was found with nCTZ; i.e., 33.6-fold for 0.1 μM nCTZ, 22.8-fold for 0.25 μM CTZ, and 11.0-fold for 0.5 μM CTZ. The S/N ratios were also significantly influenced by the concentrations of the substrates applied (Fig. 3(B), inset a). In contrast, the optical intensities at T₀ between rapamycin-treated and mock-stimulated TP2.4 were almost invariant in the presence of 6-N₃-CTZ. In case of eCTZ, the optical intensity gaps at T₀ were in the middle of those of nCTZ and 6-N₃-CTZ; i.e., 2.8-fold for 0.1 μM nCTZ, 4.6-fold for 0.25 μM CTZ, and 5.9-fold for 0.5 μM CTZ.

The optical intensities of TP2.4 increased dose-dependently to substrate nCTZ and almost saturated at 50 μM (Fig. S4, Supporting Information). TP2.4 reached the maximal optical intensities 0.5 s after the addition of nCTZ. The S/N ratios of rapamycin-treated and mock-activated TP2.4 were gradually decreased by increasing the concentrations of nCTZ. The inset “α” of Fig. S4 highlights the nCTZ concentration-driven kinetics of TP2.4.

Among the kinetic constants, the Kₐ values were the most dramatically changed parameter for both with and without rapamycin treatment conditions (Table 1). The decreasing Kₐ values of nCTZ and 6-N₃-CTZ indicate the increasing affinity between TP2.4 and the substrates, which were ca. 1.7-fold in the presence of rapamycin. Interestingly, the Kₐ value of eCTZ was dramatically decreased from 101.1 to 12.9 μM. This means that the affinity of TP2.4 with eCTZ is significantly increased by the addition of rapamycin, up to ca. 9-folds. In contrast, the Kₐ of vCTZ was almost invariant by the addition of rapamycin. Taken together, the TP2.4 affinity of the substrates in the presence of rapamycin was as follows: 6-N₃-CTZ > nCTZ > vCTZ > eCTZ.

The Vₘₕₖ values of all the substrates showed no considerable variances between rapamycin- and mock-treated TP2.4. The absolute values of Vₘₕₖ were decreased in the order of nCTZ > 6-N₃-CTZ > eCTZ > vCTZ.

It is well known that the Kₐ values frequently are recited as the turnover rates, which were decreased in the order of nCTZ > 6-N₃-CTZ >> eCTZ > vCTZ. The Kₐ/Kₐ values were further calculated for evaluating the overall catalytic efficiency (Table 1). The Kₐ/Kₐ values decreased in the order of 6-N₃-CTZ > nCTZ >> eCTZ > vCTZ with rapamycin-treated TP2.4. The highest Kₐ/Kₐ values were found with the TP2.4-6-N₃-CTZ pair, which was ca. 12.34 in the presence of rapamycin.

We further determined the total photon counts of TP2.4 in the condition of complete consumption of the substrates by applying a low concentration of the substrates to a large amount of TP2.4 (Table 1). The total photon counts of the rapamycin-activated and mock-stimulated TP2.4 are summarized in the relative values. The total photon counts of rapamycin-activated TP2.4 were generally higher than a mock-treated protein for any substrates. The ratios decreased with substrates in the order of 6-N₃-CTZ > nCTZ = eCTZ. The highest contrast in the presence or absence of rapamycin was found with 6-N₃-CTZ, which
raised the total photon counts by 1.8-fold. 

Taken together, the overall kinetics studies mentioned above can be summarized as follows: (i) rapamycin generally boosts the optical intensities by increasing the $K_\text{m}$ values in the TP2.4-substrate reactions in vitro. The optical enhancement is mainly contributed by the smaller $K_\text{m}$ values and the larger total photon fluxes in the presence of rapamycin and (ii) in contrast, rapamycin has less influence on the $K_\text{cat}$ and $V_{\text{max}}$ values.

**Discussion**

Protein-protein interactions (PPI) are the most popular and diverse molecular events that regulate signaling networks in animal cells. In this study, we investigated sensorial and kinetic properties of the TP2.4 in vitro at various conditions.

We found that TP2.4 basically maintains the rapamycin-binding and PPI properties in a variety of reaction conditions in vitro, which include diverse buffer conditions, pHs, cation circumstances, and the substrate properties and concentrations.

It is interesting to note that the S/B ratios of TP2.4 are predominantly controlled by cation contents (Fig. 2). The cation-driven optical intensities may be explained as follows: the authors previously demonstrated that ALuc protein carries an EF-hand-like structure,16 which basically comprise a Ca(II)-binding motif, hence the results in Fig. 2 can be interpreted as the multivalent cations having the potential to bind or perturb the EF-hand-like structure of ALuc23 inside TP2.4. As the result, it may modulate the optical intensities as discussed in the authors’ previous study with the CD spectra of ALuc with Al(III) and Pb(II).16 The completely suppressed luciferase activities in the highest ionic strength can be interpreted as a result of the ionic strength degrading TP2.4.

It has not been made clear why the optical intensity of TP2.4 is greatly enhanced by intramolecular PPI. As the optical intensities basically adhere to the enzymatic activities of ALuc23 that is present inside TP2.4, we investigated the apparent kinetic properties ($K_\text{m}$, $V_{\text{max}}$, $K_\text{cat}$, $K_\text{cat}/K_\text{m}$ and the total photon counts) of TP2.4 in response to the treatment of rapamycin or vehicle using native CTZ or its selected analogues as substrates.

Table 1 addresses TP2.4 activities in the presence of rapamycin, and at the mock-stimulated condition. The apparent $K_\text{cat}$ values (the turnover rates) had no significant variation before and after rapamycin treatments, whereas the apparent $K_\text{m}$ values showed the tendency of becoming lower with rapamycin treatment. We found that the optical enhancement of rapamycin-treated TP2.4 is basically substrate-dependent and dominated by the $K_\text{m}$ values rather than the turnover rates ($K_\text{cat}$). The overall range of $K_\text{cat}$ values is reasonable when compared with those in earlier literature: e.g., the $K_\text{cat}$ values of Renilla luciferase (RLuc) are 3.9 - 4.9 s⁻¹.26

The kinetic constants ($K_\text{m}$, $V_{\text{max}}$, $K_\text{cat}$) of rapamycin-treated and mock-stimulated TP2.4 are dominated by the applied substrates. nCTZ is the smallest in size and the only natural substrate among the selected substrates. nCTZ exerted the highest maximum velocity ($V_{\text{max}}$) and turnover rates ($K_\text{cat}$). The apparent $K_\text{m}$ values of TP2.4 with nCTZ are 31.9 μM in the absence of the molecular tension by vehicle stimulation, and 7.2 μM in the presence of the molecular tension induced by rapamycin stimulation. The values are in the reasonable range, considering the previous study, where the $K_\text{m}$ values of NanoLuc and RLuc with nCTZ were ca. 10 and 15 μM, respectively.27 The overall results can be interpreted such that ALuc23 present inside TP2.4 sensitively alters its enzymatic performance in response to rapamycin, although the N- and C-terminals are fused with FRB and FKBP, respectively.

It is interesting that the $K_\text{m}$ values of the 6-N-CTZ/TP2.4 pair were 5- to 19-fold smaller than those of the nCTZ/TP2.4 pair; i.e., 1.7 and 31.9, respectively. This result indicates that ALuc23 in TP2.4 has much higher affinity to 6-N-CTZ than to nCTZ. This high affinity corresponds with the results of our previous study where 6-N-CTZ showed strong specificity to the ALuc family with high bioluminescence intensities.24

The most dramatic dynamics of $K_\text{m}$ values was found with eCTZ. The $K_\text{m}$ values of TP2.4 are 8-fold improved after rapamycin treatment in the presence of eCTZ; i.e., from 101.1 (without tension) to 12.9 μM (with tension). In contrast, the $V_{\text{max}}$ and $K_\text{cat}$ are merely decreased: i.e., only by ca. 4 - 6% of the original values after appending strain with rapamycin. This significant variation may be interpreted as the bridged side chain at the C-6 position of eCTZ that basically works negatively on the enzymatic performance, but it may also positively contribute to the enzymatic affinity with tensed ALuc23 located inside TP2.4.

Table 1 also indicates that the overall enzymatic performance ($K_\text{cat}/K_\text{m}$) is significantly influenced by the variance of $K_\text{m}$ values, rather than $K_\text{cat}$ values. The results theoretically explain why the 6-N-CTZ/TP2.4 pair emits stronger bioluminescence than the nCTZ/TP2.4 and eCTZ/TP2.4 pairs.

Comparison of the total photon counts (TPC) reveals that only the 6-N-CTZ/TP2.4 pair significantly enhances its photon counts in the presence of rapamycin. It should be noted that the experiment was conducted with a small amount of the substrates, and thus, all the substrates might be consumed within the long measurement time (10 min). Therefore, the elevated TPC of the 6-N-CTZ/TP2.4 pair is interpreted as the improvement of the quantum yield (QY) in the presence of rapamycin.

The S/B ratios are a typical parameter for gauging sensorial efficiency of probes. The overall results indicate that the S/B ratios are greatly variable in vitro according to (i) the buffer ingredients like divalent cations and detergents (Figs. 2 and 3), (ii) the chemical structures and the amounts of substrates applied (Fig. 2 and Table 1), and (iii) even the signal-collection timings (Fig. 3(B) and Fig. S4). These results look natural considering that the bioluminescence of TP2.4 is generated by an enzymatic reaction and thus potentially influenced by any known factors influencing other enzymatic reactions. It means that researchers can freely control and modify the enzymatic reactions in vitro, different from imaging in live cell-based assays.

Taken together, the present study is the first example where PPIs are investigated in vitro with a single-chain probe. Conventionally, it was unclear how molecular tension probes enhance the optical intensities via PPI and what mechanism works behind it. The present study also answers the question on the molecular dynamics: i.e., rapamycin enhances the catalytic efficiency of TP2.4 via boosting the substrate affinity ($K_\text{m}$) and QY of TP2.4, but unlikely via elevation of the turnover rates ($K_\text{cat}$). The CD data supports the idea that the molecular tension probe may be incompletely folded in a portion and matured afterwards via PPI.

We previously assumed that the great optical variations observed before and after PPI might be partly attributable to the relaxation of steric hindrance, which is created by the fusion of FRB and FKBP to the N- and C-terminals of the ALuc backbone.13 However, this assumption is unlikely because the turnover rates ($K_\text{cat}$) of TP2.4 are almost invariant before and after rapamycin treatment.

The present study provides new insight on how the PPI practically works in in vitro assay systems and what mechanisms exist behind it.
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Author Contributions

S. B. K., R. N., and K. S. conceived and designed the experiments; S. B. K., R. F. and R. N. performed the experiments; S. B. K. and N. R. analyzed the data; S. B. K., N. R., and R. P. wrote the paper. All authors proofread the manuscript.

Conflict of Interest

The authors declare no conflict of interest. The founding agency had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Supporting Information

Supporting information (SI) describes the BL variance according to pH, multivalent cations, detergents, and substrate concentrations. It also includes the experimental procedure for constructing the gene encoding TP2.4 and its expression. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References

1. J. Westermarck, J. Ivaska, and G. L. Corthals, Mol. Cell. Proteomics, 2013, 12, 1752.
2. A. L. Barabasi, N. Gulbahce, and J. Loscalzo, Nat. Rev. Genet., 2011, 12, 56.
3. D. W. Piston and G. J. Kremers, Trends Biochem. Sci., 2007, 32, 407.
4. Z. Xia and J. Rao, Curr. Opin. Biotechnol., 2009, 20, 37.
5. A. Bolbat and C. Schultz, Biol. Cell, 2017, 109, 1.
6. T. Xu, D. Close, W. Handagama, E. Marr, G. Sayler, and S. Ripp, Front Oncol., 2016, 6, 150.
7. S. S. Pillavi, H. Yukawa, D. Onoshima, V. Biju, and Y. Baba, Anal. Sci., 2017, 33, 137.
8. C. Hu, X. J. Kong, R. Q. Yu, T. T. Chen, and X. Chu, Anal. Sci., 2017, 33, 783.
9. S. B. Kim, M. Awais, M. Sato, Y. Umezawa, and H. Tao, Anal. Chem., 2007, 79, 1874.
10. S. B. Kim, T. Ozawa, H. Tao and Y. Umezawa, Anal. Biochem., 2007, 362, 148.
11. S. B. Kim, Y. Takenaka, and M. Torimura, Bioconjugate Chem., 2011, 22, 1835.
12. S. B. Kim, M. Torimura, and H. Tao, Bioconjugate Chem., 2013, 24, 2067.
13. S. B. Kim, Y. Ito, and M. Torimura, Bioconjugate Chem., 2012, 23, 2221.
14. J. Li, S. G. Kim, and J. Blenis, Cell Metab., 2014, 19, 373.
15. S. B. Kim, R. Nishihara, D. Citterio, and K. Suzuki, Bioconjugate Chem., 2016, 27, 354.
16. S. B. Kim, S. Miller, N. Suzuki, T. Senda, R. Nishihara, and K. Suzuki, Anal. Chem., 2015, 31, 955.
17. Y. Ando, K. Niwa, N. Yamada, T. Enomot, T. Irie, H. Kubota, Y. Ohmiya, and H. Akiyama, Nat. Photonics, 2008, 2, 44.
18. H. H. Seliger and W. D. Mcelroy, Arch. Biochem. Biophys., 1960, 88, 136.
19. G. A. Stepanyuk, H. Xu, C. K. Wu, S. V. Markova, J. Lee, E. S. Vysotski, and B. C. Wang, Protein Expr. Purif., 2008, 61, 142.
20. I. Remy and S. W. Michnick, Nat. Methods, 2006, 3, 977.
21. S. W. Michnick, Curr. Opin. Struct. Biol., 2001, 11, 472.
22. A. Mazo-Vargas, H. Park, M. Aydin, and N. E. Buchler, Mol. Biol. Cell, 2014, 25, 3699.
23. S. Y. Lee, H. Lee, H. K. Lee, S. W. Lee, S. C. Ha, T. Kwon, J. K. Seo, C. Lee, and H. W. Rhee, ACS Central Sci., 2016, 2, 506.
24. R. Nishihara, E. Hoshino, Y. Kakudate, S. Kishigami, N. Iwasawa, S. Sasaki, T. Nakajima, M. Sato, S. Nishiyama, D. Citterio, K. Suzuki, and S. B. Kim, Bioconjugate Chem., 2018, 29, 1922.
25. A. M. Loening, A. Dragolescu-Andrasi, and S. S. Gambhir, Nat. Methods, 2010, 7, 5.
26. A. M. Loening, T. D. Fenn, A. M. Wu, and S. S. Gambhir, Protein Eng. Des. Sel., 2006, 19, 391.
27. M. P. Hall, J. Unch, B. F. Binkowski, M. P. Valley, B. L. Butler, M. G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T. Machleidt, M. B. Robers, H. A. Benink, C. T. Eggers, M. R. Slater, P. L. Meisenheimer, D. H. Klaubert, F. Fan, L. P. Encell, and K. V. Wood, ACS Chem. Biol., 2012, 7, 1848.