Dynamical regulations of protein-ligand bindings at single molecular level

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Abbreviations

Ab: Antibody

Ag: Antigen

DXT: Diffracted X-ray tracking

Fab: Fragment antigen binding

mAb: Monoclonal Ab

NP: (4-hydroxy-3-nitrophenyl)acetyl

NP-Cap: NP- ϵ -amino caproicacid

Abstract

We present new quantitative regulations of the binding-affinity using dynamical single-molecule detection system with X-rays. In the study of antigen-antibody interactions, we found that structural fluctuations of single-molecules were negatively regulated by antigen-binding. Although strategies to produce ligand-induced stability have been well studied from the macro aspect both theoretically and experimentally, our dynamical single-molecular experimental results are first observations with angstrom accuracy in the real-time and space. It is considered that those negative regulations of protein structural fluctuations with binding event are related to biological functions. In addition, we clarified that ratio between antigen-binding condition and no-binding one in observed structural fluctuations are extremely relative to the binding-affinity. These results indicate that the phenomena of protein-ligand interactions considered as stable states can be defined as results of dynamical processes at the single-molecule level. Such new quantifications from angstrom-level structural fluctuations can be applied to various biological science and biotechnologies.

Introduction

Protein-protein and protein-ligand interactions are essential for most cellular processes, including signal transduction, gene regulation, and enzyme reaction [1, 2]. Understanding the mechanisms of biomolecular interactions would significantly improve our understanding of biological systems. Such mechanisms have been researched intensively using X-ray crystallography, nuclear magnetic resonance, surface plasmon resonance biosensors, and isothermal titration calorimetry etc [3-9]. Although those methods provide us with attractive information such as averaged molecular structures, kinetics and thermodynamics parameters, and binding affinity, many molecules (at least 10^{13} molecules) are needed for those methods to be useful due to the limitation of the detection sensitivity.

Here we show the quantitative determination of the binding affinity at the single-molecule level using dynamical single-molecule detection system with X-rays. We called Diffracted X-ray Tracking (DXT), which monitors X-ray diffraction spots from a gold nanocrystal, labeling an individual single molecule in biological systems. Time-resolved DXT can analyze behaviors of individual biomolecules in real time and space.

Recently, there are many single molecular techniques and imaging system using visible light. Especially, the methodology that utilizes a fluorescent probe is general techniques. In these probe methods, there are many discussions about both effects of mass and size of these

probes. For example, Yasuda et al. investigated the load dependence of the F_1 -ATPase rotational rate on the labeled actin filament [10], and observed the viscous friction on the labeled colloidal gold (40 nm) or polystyrene beads (108, 196, or 291 nm) [11]. And they confirmed that 40-nm colloidal gold rotated at the full speed (saturating speed) of the F_1 motor [11]. This result indicated that the 40-nm colloidal gold was not an impeding load for F_1 motor.

In the DXT measurement, labeled gold nanocrystals are 20~30 nm. Thus the influence of the mass of the gold nanocrystal in DXT measurement is estimated to be very small factor. In previous studies, we have observed the structural fluctuations of several biomolecules such as DNA, myosin, actin filament, bacteriorhodopsin (BR), and β 2-microglobulin by DXT [12-17]. In the study of BR, we observed the momentary jump of 0.73 ± 0.48 Å due to the expression of BRs function [17]. The value of this jump agreed with estimated moments from known X-ray crystallography data of BR. It is indicated that DXT has been proven to be able to observe internal single molecular motions to an angstrom accuracy.

In general, radiation damage in all X-ray observations is the biggest obstacle. Experiments on biological or inorganic crystals show significant damage at around 10^7 Gy [18], we are called Henderson limit. Our single molecular observations are observed in this dose regime. However, the following points show that X-ray destruction phenomena to the nanocrystals or the observed single biomolecules have hardly happened during X-ray exposure

time. There are no changes in the intensities of observed X-ray diffraction spots from the labeled nanocrystal while measuring it (from 1s to 3s). Additionally, there is little time dependence in the width of the average movements in the observed diffraction spots.

X-ray damage process has been investigated by using many large biomolecular crystals [19]. Our experiments are single molecular observations, and the size of the sample is ultimately smaller than that of the normal crystals. Therefore, the concept of Henderson limit has the possibility not to be able to adjust as an analysis of single molecular observations [20]. Recently, when the size of the crystal is reduced, it was reported that the effect of X-ray radiation damages is decreased [21]. DXT method has a possibility of quantitatively appreciable of the X-ray destruction characteristic at single molecule level. We think evaluation of X-ray radiation damages as important application field of DXT observations.

In this study, we investigated the effects of ligand binding to the fluctuation of a single protein molecule. Although effects of ligand binding to the fluctuation of a single protein molecule have been measured using enzyme-substrate systems with atomic force microscope [22], these systems are not necessarily suitable for analyzing the interactions due to the inherent instability of enzyme-substrate complexes. Therefore, in this study, we analyzed an antigen (Ag)-antibody (Ab) interaction from the perspective of the stability of Ag-Ab complexes, using anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) monoclonal Abs (mAbs) such as N1G9, C6, 9T13,

and E11.

Materials and Methods

Preparation of mAbs and Fab fragments

Four anti-NP mAbs (N1G9, C6, 9T13, and E11) were used in the present study. Fab fragments of these mAbs were prepared by digestion with papain (Worthington Biochemical Co.) with an enzyme/substrate ratio of 1/100 (w/w) in 0.1 M phosphate buffer (pH 7.0) containing 10 mM cysteine and 2 mM EDTA for 4 h at 37 °C and purified by NP-BSA column and gel filtration on a Superdex 200 column. The preparation of mAbs and Fabs were more than 95% pure, as judged by SDS-PAGE. The protein concentrations of mAbs and Fab fragments were determined at an OD of 280 nm and were calculated using molar absorption coefficients of $2.1 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ for mAbs and $8.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for Fab fragments, respectively.

Preparation of Ags

NP- ϵ -amino caproicacid (NP-Cap) was prepared as described previously [23]. The concentration of NP was determined at an OD of 430 nm and calculated using a molar absorption coefficient of $4.23 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at pH 8.4 [24].

Diffraction X-ray tracking (DXT)

We used gold nanocrystals fabricated by vacuum evaporation on a NaCl (100) surface.

The gold nanocrystals can be separated from the NaCl substrate by 50 mM MOPS 50 mM CHAPS (pH 7.0). In order to immobilize Fab fragments on the substrate, Cr (40 Å) and Au (100 Å) were deposited on the quartz substrate (10 × 10 × 0.05 mm, ATOCK. Inc.). And the Succinimidyl 6-[3'-(2-pyridyldithio)-propionamido]hexanoate (PIERCE Biotechnology) was coupled to the surface of the quartz substrate. The thickness of the solution (50mM HEPES pH 7.0) on the substrate (~ 7 μm) was controlled with the thickness of the spacer between the substrate and the polyimide film. We used the white X-ray mode (Laue mode) of beam line BL44B2 (RIKEN Structural Biology II, Spring-8, Japan) to record Laue diffraction spots from gold nanocrystals. Photon flux at the sample position is estimated to be about 10¹⁵ photon/s/mm² in the energy range from 7 kV to 30 kV. The X-ray focal beam size is 0.2 mm (horizontal) × 0.2 mm (vertical). The diffraction spots were monitored with an X-ray image intensifier (Hamamatsu Photonics, V5445P) and a CCD camera (Hamamatsu Photonics, C4880-82) with 656 × 494 pixels.

Isothermal titration calorimetry (ITC)

ITC experiments were carried out on a Microcal MCS calorimeter. All samples in 50mM HEPES (pH 7.0) were carefully degassed before measurement. The NP-Cap solution (200 μM) was titrated into the Fab solution (6 μM) using a 100 μl syringe. Each titration

consisted of a preliminary 2 μ l injection followed by 19 subsequent 5 μ l additions at 20 °C. The heat for each injection was subtracted from the heat of dilution of the injectant, which was measured by injecting the NP-Cap solution into 50 mM HEPES (pH 7.0). Each corrected heat value was divided by the moles of NP-Cap injected and data were analyzed with Microcal Origin software supplied by the manufacturer.

Results and discussions

In order to avoid the complicatedness resulted from involving two Fab arms of an Ab molecule, we measured the structural fluctuations of Fab fragments, using the DXT system. Figure 1A shows schematic illustration of the detection system for an individual single Fab molecule with X-rays. First, the Fab fragments were immobilized on the quartz substrates using amine-coupling method [7]. Since there are ~ 20 reactable Lys residues on the surface, the molecular directions of Fab fragments conjugated on the quartz substrates are random. Next, a gold nanocrystal was labeled with either Cys128 (of a heavy-chain constant domain) or Cys214 (of a light-chain constant domain). In addition to these, an Fab fragment possesses the Cys residues (22, 96, 147, and 202 heavy-chain residues and 22, 90, 137, and 196 light-chain residues), however, these residues are buried in the interior by forming intrachain disulfide bonds and not available for labeling of nanocrystal.

We used the white X-ray mode (Laue mode) of beam line BL44B2 (RIKEN Structural Biology II, SPring-8, Japan) to record Laue diffraction spots from labeled gold nanocrystals. Since the irradiation of protein with X-rays induces the destruction of protein structure, pulse X-rays were used to decrease the real expansion time, in this study the measuring time was less than 2 sec. In this time range, we have never detected any attenuation or expansion of observed movements from diffracted spots.

Figure 1B shows representative images of Laue spots moving in position from frame to frame at 20 °C. It was observed that Laue diffraction spots from the gold nanocrystal on each Fab fragment vibrated randomly. These are the first single-molecular observations of Ab structural fluctuations in the real-time and space. The movements of observed spot were mainly in the 2θ direction (Fig. 1) not χ direction [15]. The diffraction angles of those spots were 50~90 mrad/sec in the absence of Ag, NP-Cap. If Fab fragments were rigid, those changes in diffraction angle can be converted to 6~11 Å/sec of translational movement (maximum value). However, since protein molecules are considered to be flexible, we expected that a lower value of translational movement (~6 Å/sec) was detected by DXT.

Next, we compared Laue diffraction spots of free Abs to their complexes with NP-Cap. The results revealed that the vibration movements in the presence of NP-Cap were apparently lower than that of free Abs. Figure 2 shows 45 motions ($\Delta 2\theta$) of representative gold nanocrystals on Fab fragments along the 2θ direction of the diffraction angle in the presence or absence of NP-Cap at 20°C. It was clearly shown that the gold nanocrystal motions of free Fab fragments were about four times larger than those of the complexes with NP-Cap. Such decreasing in the motions of gold nanocrystals was observed in all Fab fragments (N1G9, C6, 9T13, and E11) so far studied.

Ligand-induced stability in proteins has been investigated from the macroscopic

viewpoint both theoretically [25-27] and experimentally [28, 29]. Miller and Dill investigated the ligand-protein interaction using the HP lattice model [25]. They calculated the thermal motions in the model as the Boltzman-average of the fluctuations, which has a resemblance to a *B* factor in X-ray crystallography. The *B* factors are often taken as a measure of the protein's flexibility. Furthermore, they argued that ligand binding could restrict the thermal motions. Our results agree with Miller and Dill's theory at the single-molecule level.

Figure 3A shows the mean square displacements (MSD) of the gold nanocrystals on Fab fragments as a function of the time interval for analysis of Brownian motions. In simple Brownian diffusion, the $(\Delta\theta^2)L^2-\Delta t$ plots are linear with a slope of $4D$, where D and L are the two-dimensional diffusion coefficient and the length of the Fab fragment, respectively. Thus, the simple diffusion mode can be expressed as

$$(\Delta\theta^2)L^2 = 4D(\Delta t) \quad (\text{Eq. 1}).$$

However, the $(\Delta\theta^2)L^2-\Delta t$ plots are parabolic in these results. This relationship is known as a directed diffusion mode [30]:

$$(\Delta\theta^2)L^2 = 4D(\Delta t) + V^2(\Delta t)^2 \quad (\text{Eq. 2})$$

Eq. 2 indicates that particles move in a certain direction at a constant drift velocity V with a diffusion coefficient of D . We demonstrated that these directed diffusion modes are due to the X-ray radiation pressure forces [16]. Thus the value of V indicates a molecular softness.

Table 1 shows the values of D and V that were determined from the MSD curves (Fig. 3A). It was found that each Ab has a variable D value, in particular N1G9, C6, and 9T13 are of the same IgG subclass (IgG1) and have identical constant domain (C_H1 and C_λ) sequences but different variable domain (V_H and V_L) sequences; the number of mutation of N1G9, C6, 9T13, and E11 are 0, 12, 18, and 28 respectively [31, 32]. The results indicate that the variable region (as shown Fig. 1A) affects the structural fluctuations of Fab fragments.

Incidentally 9T13 has the smallest D and V values. In our previous study, we demonstrated that the binding affinity of 9T13 is matured by reducing the negative entropy change [32], and considered that the Ag recognition mechanism of 9T13 is shifted from the induced-fit mechanism to the lock-and-key mechanism. In the lock-and-key mechanism, the binding site is complementary to the Ag, so the flexibility of the Ag binding site is lower than that in the induced-fit mechanism. The results for 9T13 in the present study are comparable with those of the Ag-recognition mechanism of 9T13.

Next we investigated the relationship between Ab binding affinity (K_a) and the decreasing rate of D values. Figure 3B depicts the relationship between $\ln(D_b/D_f)$ and the Gibbs free energy change (ΔG). ΔG was calculated using the equation

$$\Delta G = -RT \ln K_a \quad (\text{Eq. 3}),$$

Where R is the gas constant and T is the absolute temperature. D_b and D_f are the values of D in

the Ag bound state and free state respectively. The plots in Fig. 3B confirm the extremely linear relationship between $\ln(D_b/D_f)$ and ΔG . Thus the equation is shown as follow,

$$\Delta G = -RT \ln K_a = \alpha \ln(D_b/D_f) + \beta \quad (\text{Eq. 4})$$

where α depends on Ag concentration and β depends on experimental condition such as temperature and Fab immobilization. In this study, α and β are 3.5 kcal/mol and -7.2 kcal/mol respectively. And Eq. 4 is converted as follow,

$$\ln K_a = -(\alpha/RT) \ln(D_b/D_f) - (\beta/RT) \quad (\text{Eq. 5})$$

These results show that the binding affinity can be determined from the changes in the structural fluctuations of Fab at the single-molecule level. The D values decreased linearly with increasing Ag concentration, indicating that the values of D_b were determined in equilibrium (Fig. 3B inset). The very important thing is that the value of $\ln(D_b/D_f)$ correlated with K_a in spite of the difference of the variable domain sequence described above. These negative regulations of Fab structural fluctuation with Ag binding are independent from the amino acid sequence of the variable region. Surprisingly, those regulations are probably observed not only in the local regions of Fab but also across the entire Fab area because the directions of immobilized Fab fragments are not controlled in our experiments. If it is possible to control strictly our experimental conditions of Fab immobilization, we can directly discuss the relationship around D_f , D_b , and K_a .

Although we observed Fab structural fluctuations in this study, it is considered that those structural regulations due to Ag-binding can be observed in the intact Ab. In the previous studies, we have shown that Ag-binding to the binding site of Ab changes the Ab structure at not only variable regions (binding sites) but also at constant region. It was suggested that those results are related to the mechanisms of B cell receptor (BCR; membrane-form Ab) signal transduction [7, 8]. It would be of very interest to investigate whether the structural regulation due to Ag-binding is related to the BCR signal transduction mechanism. If it is supposed that this dynamical structural regulation can be observed in other biomolecular interaction systems, we can discuss several topics at the single-molecule level; for example, relationships between biomolecular interaction and molecular function or the ultimate single-pair analysis.

To obtain statistically valid data in a measuring time of 2 sec, in this study we analyzed diffracted spots from 45 molecules under each condition described above. However we would be able to analyze the biomolecular interaction from only single molecules using fast X-ray detectors or by long time-range observations using fast pulse X-rays. We are now investigating in detail those super-fast ($\sim \mu\text{s}$) single-molecule experimental systems.

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Figure legends

Figure 1, (A) Schematic drawing of the detection system for single Fab molecule with X-rays.

The Ag binding region (variable region) and constant region represent red and blue respectively. The size of gold nanocrystal is approximately 20-30 nm. Structure data were taken from PDB (accession numbers are 1NGP and 1IGT). (B) The representative images of Laue spots moving in position frame to frame at 20 °C. The Laue spots from individual gold nanocrystals appeared as brightly shining dots (white or yellow).

Figure 2, Motions ($\Delta 2\theta$) of representative nanocrystals along the direction of the diffraction angle at each Fab (C6: A and C; 9T13: B and D) in the presence (0.18 mM) (C and D) or absence (A and B) of Ag.

Figure 3, (A) Mean square displacements of the gold nanocrystals as a function of time interval in each Fab. Ag free and bound (0.18 mM) states are indicated by open and closed square respectively. (B) The relationship between $\ln(D_b/D_f)$ and Gibbs free energy change and Ag concentration dependency of the C6 D values (inset). D_b : the D values of Ag binding state, D_f : the D values of Ag free state.

A

Fig. 1A

B

Observed Laue pattern

Fig. 1B

Fig. 2

A **B**

Fig. 3

Table 1 The values of D and V were determined from the plots (Fig. 3A) of $(\Delta\theta^2)L^2$ vs the interval times by χ^2 minimization fit for Eq. 2. The measured spots are 45 trajectories.

$(\Delta\theta^2)L^2 = 4Dt + (Vt)^2$ (Eq. 2) ($L = 70\text{\AA}$)

The values of ΔG and K_a were taken from ITC measurements.

Name	$D \times 10^{-1} (\text{\AA}^2/\text{s})$	$V (\text{\AA}/\text{s})$	$\Delta G (\text{kcal/mol})$	$K_a (\text{M}^{-1})$
N1G9 free	2.65 ± 0.03	1.67 ± 0.01		
N1G9 + Ag	2.11 ± 0.03	1.18 ± 0.01	-8.0	$8.5 \pm 1.3 \times 10^5$
C6 free	3.97 ± 0.03	1.61 ± 0.01		
C6 + Ag	1.54 ± 0.03	1.01 ± 0.01	-10.6	$8.2 \pm 3.8 \times 10^7$
9T13 free	2.35 ± 0.03	1.26 ± 0.01		
9T13 + Ag	0.65 ± 0.03	1.09 ± 0.01	-11.7	$5.1 \pm 6.9 \times 10^8$
E11 free	9.49 ± 0.07	1.94 ± 0.01		
E11 + Ag	1.93 ± 0.03	1.13 ± 0.01	-12.7	$3.0 \pm 7.2 \times 10^9$

The S.D. were based on fitting errors.