BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

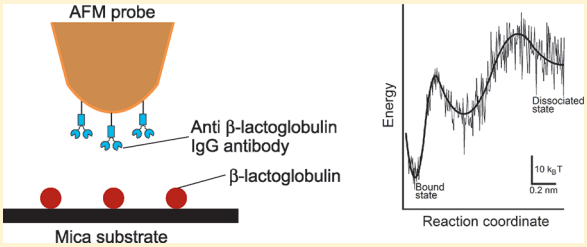
Evaluation of Temperature Effect on the Interaction between β-Lactoglobulin and Anti-β-lactoglobulin Antibody by Atomic Force Microscopy

Jun'ichi Wakayama and Shigeru Sugiyama*

Nano-Biotechnology Laboratory, Food Engineering Division, National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

Supporting Information

ABSTRACT: Molecular recognition such as antigen−antibody interaction is characterized by the parameters of kinetics and the energy landscape. Examinations of molecules involved in the interaction at different temperatures using atomic force microscopy (AFM) can provide information on not only the effects of temperature on the unbinding force between a molecule of interest and a complementary molecule but also the parameters of kinetics and the energy landscape for dissociation of the molecular complex. We investigated the effect of temperature on the dissociation process of the complex of β-lactoglobulin and anti-bovine β-lactoglobulin IgG polyclonal antibody using AFM. Measurements of the unbinding forces between β-lactoglobulin and the antibody were performed at 25, 35, and 45 °C. The following results were obtained in our present study: (i) The unbinding forces decreased as temperature increased, suggesting that the binding force between β-lactoglobulin and the antibody includes the force originating from temperature-dependent interactions (e.g., hydrogen bonding). (ii) At each temperature, the unbinding force exhibited two linear regimes in the force spectra, indicating that the dissociation process of the β-lactoglobulin−antibody complex passes at least two energy barriers from the bound state to the dissociated state. (iii) The dissociation rates at zero force and the position of energy barriers increased as temperature increased. (iv) The heights of the two energy barriers in the reaction coordinates were 49.7 k_BT and 14.5 k_BT. (v) The values of roughness of the barriers were ca. 6.1 k_BT and 3.2 k_BT. Overall, the present study using AFM revealed more information about the β-lactoglobulin−antibody interaction than studies using conventional bulk measurement such as surface plasmon resonance.



Molecular recognition such as receptor−ligand and antigen−antibody interactions plays a crucial part in biological systems. The interaction is characterized by kinetic parameters, such as association and dissociation rates, and an energy landscape consisting of potential energy barriers along reaction coordinates. Examination of the temperature dependence of the rate constants for the interaction can provide the activation energy that corresponds to the height of the energy barrier in the energy landscape. Several techniques have been employed to determine the rates of the antibody−antigen interaction, including surface plasmon resonance (SPR) and stopped-flow techniques.[1−5] In these techniques, data are obtained by bulk measurements.

In the past decade, dynamic force spectroscopy (DFS), which consists of measurements of the unbinding forces (intermolecular forces) between a molecule of interest and a complementary molecule at the single-molecule level using an atomic force microscope or optical tweezers, and analyses of the resulting force spectra (the unbinding force versus loading rate) have been employed to investigate the dissociation rate at zero force, which is thought to correspond to the dissociation rate obtained by bulk measurements. In addition, DFS can provide information about the position of the energy barrier in the reaction coordinate for the interaction, which cannot be obtained from bulk measurements.[6−9] Until now, several DFS measurements of the interaction between biomolecules such as avidin/streptavidin−biotin[10−18] and antibody−antigen systems[19−27] have been performed to investigate the dissociation rate at zero force and position of the energy barrier.

Furthermore, some recent studies have shown that examination of the temperature dependence of the parameters using DFS can provide the values of the activation energy and energy landscape roughness.[28−30] On the other hand, in bulk measurements and conventional DFS measurement, the surface of the energy landscape is idealized to be smooth, but the real surface is considered to be rough and rugged. The roughness obtained from DFS corresponds to that of the real surface. Therefore, DFS analyses at several temperatures offers information about not only the temperature effect on the intermolecular force between the molecule of interest and complementary molecule but also the various parameters of the energy landscape as well as the kinetics for the interaction. In DFS measurements of the molecular interaction, the activation energy and energy landscape roughness for the interaction

Received: August 8, 2011
Revised: December 1, 2011
Published: December 1, 2011

dx.doi.org/10.1021/bi201245k | *Biochemistry* 2012, 51, 32−42

between the streptavidin−biotin[14] and GTPase Ran-importin−β systems[31] have been reported. However, there have been no reports on the DFS measurements of the activation energy and roughness for antibody−antigen interaction. Measurements of the unbinding forces between antibody and antigen at different temperatures by DFS may provide more information than those carried out at room temperature.

Several methods used for the measurement of the unbinding force between antibody and antigen at room temperature by atomic force microscopy (AFM) have limitations. This is because some studies have reported that the unbinding forces are often obscured by nonspecific adhesive forces between the atomic force microscope probe (cantilever) and the surface of the substrate.[32,33] To precisely measure the unbinding force by AFM, one should reduce the nonspecific adhesive force. Recently, we demonstrated that use of a nonreactive protein and detergent, which have been widely used to reduce background signals originating from nonspecific interactions in standard immunoassays such as the enzyme-linked immunosorbent assay (ELISA) and kinetic measurements such as SPR, are useful for reducing the nonspecific adhesive force.[34]

In the present study, we developed an experimental condition for reducing the nonspecific adhesive force in the measurement of the unbinding force between β-lactoglobulin (one of the major allergens in bovine milk) and anti-β-lactoglobulin antibody using AFM, and we examined the temperature effect on the unbinding force. Moreover, we determined the parameters of the kinetic and energy landscape for the β-lactoglobulin−antibody interaction from the force spectra obtained by AFM measurements.

EXPERIMENTAL PROCEDURES

Chemicals. β-Lactoglobulin from bovine milk and ferritin (type I saline solution) were purchased from Sigma-Aldrich (St. Louis, MO). Antibovine β-lactoglobulin IgG polyclonal antibody was purchased from Shima Laboratories Company Limited (Tokyo, Japan). EDC and NHS were purchased from Pierce (Rockford, IL). Tween 20 (Nacalai Tesque, Kyoto, Japan) was used as the detergent, and bovine serum albumin (BSA; Chon Fraction V powder, Iwai Chemical, Tokyo, Japan) was used as the nonreactive protein for reducing the nonspecific adhesive force. The blocking solution contained 0.1% (v/v) Tween 20 and 0.1% (w/v) BSA in PBS.

Preparation of Cantilevers. Anti-β-lactoglobulin antibodies were immobilized to gold-coated cantilevers (OMCL-TR400PB-1, Olympus, Tokyo, Japan) via self-assembled monolayers (SAMs) by activating 7-carboxyl-1-heptanethiol with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) using a method similar to that described in our previous article.[34]

First, the cantilever was washed in piranha solution (H_2SO_4/30% H_2O_2, 70%/30% (v/v)), and then SAMs of 7-carboxy-1-heptanethiol were prepared by immersing the cantilever in a solution of 0.5 mg/mL 7-carboxy-1-heptanethiol in ethanol for 16 h. (*CAUTION: the piranha solution has very strong oxidizing power and should be used with extreme caution.*) Carboxylic acid terminal groups of 7-carboxy-1-heptanethiol of the SAMs cantilever were activated by 10 mg/mL EDC and 5 mg/mL NHS in 0.1 M MES buffer (pH 6.0) for 30 min. The activated cantilever was incubated in 0.01 mg/mL anti-β-lactoglobulin rabbit polyclonal IgG in 0.1 M MES buffer (pH 6.0) for 4 h to form cross-links between the cantilever and antibodies. The cantilever was then immersed in 20 mM glycine in 0.1 M MES buffer (pH 6.5) for 30 min to block unreacted sites and rinsed with phosphate-buffered saline (PBS).

Evidence of Specific Interaction. To ascertain if the antibodies immobilized on the cantilever have a specific binding ability to the antigen on the substrate, three experiments were carried out, as described below.

(a) The surface of the cantilever-immobilized β-lactoglobulin antibodies in the blocking solution (see the Chemicals subsection) was observed under a fluorescence microscope after reacting with 1.0 μM β-lactoglobulin (antigen) or ferritin (nonantigen) labeled with fluorescent dye (Cy3-reactive dye; GE Healthcare, Piscataway, NJ) in the blocking solution for 1 h. The fluorescent proteins were prepared according to the manufacturer's protocol.

(b) AFM measurements of the unbinding force between β-lactoglobulin antibodies immobilized on the cantilever and β-lactoglobulin or ferritin immobilized on the substrate (see the Preparation of Substrate subsection) were carried out at 25 °C in PBS alone or in the blocking solution (see the DFS subsection).

(c) AFM measurements of the unbinding force between the antibodies immobilized on the cantilever and β-lactoglobulin immobilized on the substrate were carried out in the blocking solution to which β-lactoglobulin (1.0 μM) is added.

In experiments (b) and (c), all the detected unbinding events were grouped according to retraction velocity and histograms were generated.

Preparation of Substrate. β-Lactoglobulin or ferritin was covalently immobilized on a substrate using a similar method to that described in our previous paper.[34] First, freshly cleaved mica was silanized with the vapor of (3-aminopropyl)-triethoxysilane in nitrogen gas for 12 h. The silanized mica was immersed in 0.5% (v/v) glutaraldehyde for 1 h. Then the mica was washed with water and PBS. Next, β-lactoglobulin (antigen) or ferritin (nonantigen) solution in PBS was dropped onto the mica and incubated for 20 min. Next, the protein-immobilized mica was rinsed with PBS to remove unbound protein molecules.

β-Lactoglobulin and ferritin solution (in PBS) of two concentrations were used: 1 μM and 10 nM. For the immunoassay of the antibody immobilized on the cantilever, a higher concentration solution was used to increase the probability of unbinding events between the antibodies immobilized on the cantilever and β-lactoglobulin or ferritin immobilized on the substrate, which originate from not only a single-molecule but also a multiple-molecule interaction. For the evaluation of the effect of temperature on the β-lactoglobulin−anti-β-lactoglobulin antibody interaction using AFM, a lower concentration solution was used to increase the probability of the unbinding events that mainly originate from the single-molecule interaction.

DFS. The unbinding forces between antibodies and proteins were estimated on the basis of force−distance curves, which were measured using a commercial atomic force microscope (Nano wizard, JPK Instruments, Berlin, Germany). To obtain the unbinding forces at various loading rates, force−distance curves were recorded at different approach/retraction velocities of the cantilever, ranging from 50 nm/s to 10 μm/s. AFM measurements were carried out in PBS or blocking solution at 25, 35, and 45 ± 0.1 °C. For measurements obtained at fast retraction rate (≥2 μm/s), the value of the measured force was corrected for hydrodynamic drag forces acting on the cantilever. The hydrodynamic force was estimated using the

dx.doi.org/10.1021/bi201245k | *Biochemistry* 2012, 51, 32−42

method described by Janovjak et al.[35] The temperature of the specimens was controlled using a commercial temperature controller for AFM (BioCell, JPK Instruments, Berlin, Germany). The spring constant of each cantilever at each temperature was determined by thermal fluctuation analysis.[36] The spring constants of the cantilevers were 0.017−0.035 N/m as determined by the thermal fluctuation analysis. The typical result of the analysis is shown in Figure S1 of the Supporting Information.

Data Analyses of AFM Measurements of the Temperature Effect on Parameters of the Kinetics and Energy Landscape. The unbinding force that originates in the breaking of the bond between the antibody and β-lactoglobulin was determined on the basis of the maximum downward deflection of the cantilever during the retraction phase in the force−distance curve (Figure 1). The loading rate (r) was estimated using eq 1:

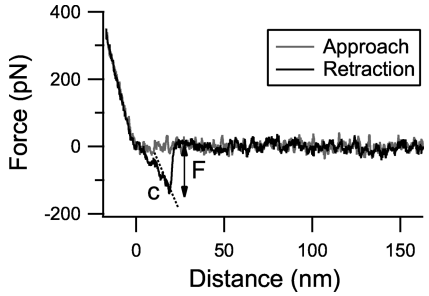


Figure 1. Representative example of the force−distance curve acquired at the retraction velocity of 1.0 μm/s and at 25 °C. F and c are the slope of the force−distance curve and the unbinding force, respectively. The loading rate and unbinding force were determined from the curves as described in the text (see Data Analysis of the Measurements of the Temperature Effect on the Parameters of Kinetics and Energy Landscape by AFM in the Experimental Procedures section).

$$r = cv \tag{1}$$

where c and v are the slope of the force−distance curve and the retraction velocity of the cantilever, respectively. The slope c was estimated by linearly fitting the slope of the force−distance curve at the prior rupture point.[24] All the detected rupture events were grouped according to loading rates, and histograms were generated. About 200−1000 rupture events were used to generate each histogram. The most probable unbinding forces (F) were determined by fitting the histogram with a double-Gaussian function using a least-squares method. The first peaks were assigned as a one-paired unbinding force on the basis of the following considerations: An adhesion frequency in the measurements of the temperature effect on the unbinding force was ca. 5%. According to Poisson statistics, this frequency (5%) ensures a >97% probability that the adhesion event is mediated by a single molecule.[10,37] Also, β-lactoglobulin exists as a homodimer under the present experimental conditions.[56] Therefore, most unbinding forces are considered to be originated from interaction between one molecule of β-lactoglobulin dimer and the antibody or interaction between two molecules of β-lactoglobulin dimer and the antibody. Furthermore, the values of most probable unbinding forces obtained from fitting the double-Gaussian function to the histogram were tested by a nonparametric test based on the kernel density estimation function (see Figures S4 and S5 in the Supporting Information).

Finally, these most probable unbinding forces, which originated from a one-pair molecule event, were plotted against the loading rate.

The dissociation rate at zero force (k_{off}) and the position (Δx) of the energy barrier in the energy landscape were determined by fitting the force−loading rate curve with the following Bell model:

$$F = \frac{k_B T}{\Delta x} \ln\left(\frac{r \Delta x}{k_{off} k_B T}\right) \tag{2}$$

where k_B and T are the Boltzmann constant and the absolute temperature, respectively.[24]

The energy landscape surface roughness (ε) of the β-lactoglobulin−antibody interaction was calculated using Nevo's modification of the Hyeon and Thirumalai approximation, as follows:

$$\varepsilon^2 \approx \frac{\Delta x(T_1) k_B T_1 \Delta x(T_2) k_B T_2}{\Delta x(T_2) k_B T_2 - \Delta x(T_1) k_B T_1} \left[\Delta F_0 \left(\frac{1}{\Delta x(T_1)} - \frac{1}{\Delta x(T_2)}\right) + \frac{k_B T_1}{\Delta x(T_1)} \ln\frac{r(T_1)\Delta x(T_1)}{k_{off}(T_1) k_B T_1} - \frac{k_B T_2}{\Delta x(T_2)} \ln\frac{r(T_2)\Delta x(T_2)}{k_{off}(T_2) k_B T_2}\right] \tag{3}$$

where ΔF_0 is the height of each potential (the activation energy) and $r(T_1)$ and $r(T_2)$ are the loading rates at two different temperatures (T_1 and T_2, respectively), which cause the same unbinding force.[31] ΔF_0 was determined by fitting the k_{off} versus absolute temperature curve with the Arrhenius law (i.e., $k_{off} = A \exp(-\Delta F_0/k_B T)$, where A is a constant).

All data are reported as mean ± standard error.

RESULTS

Evidence of Specific Interactions. First, the binding ability of the antibodies immobilized on the cantilever to the antigen protein was investigated by observing the binding efficiency of the fluorescence-labeled β-lactoglobulin (antigen) or ferritin (nonantigen) to the surface of the cantilever. Figure 2

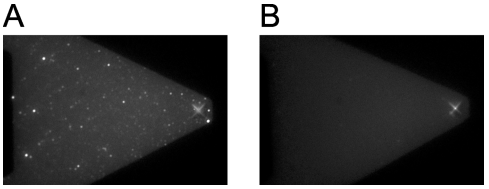


Figure 2. Fluorescent images of the surface of antibodies immobilized on the cantilever. (A) and (B) show the surfaces reacted with Cy3-labeled β-lactoglobulin and Cy3-labeled ferritin, respectively.

shows a fluorescence image of the surface of the cantilever that reacted with the fluorescent β-lactoglobulin (Figure 2A) or ferritin (Figure 2B). Fluorescent spots were observed on the surface of the cantilever reacted with the β-lactoglobulin labeled with the fluorescent dye. In contrast, no such spots were observed on the surface reacted with the fluorescent ferritin. These results indicated that the antibodies immobilized on the cantilever specifically bind to β-lactoglobulin.

AFM measurements of the unbinding force between the antibodies immobilized on the cantilever and the proteins

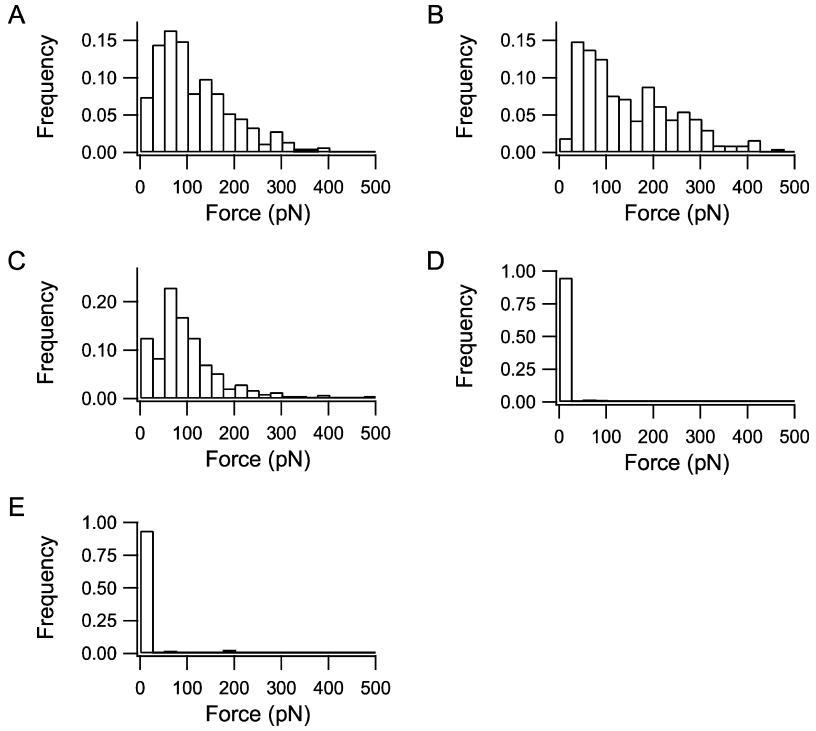


Figure 3. Distribution of unbinding forces measured under different conditions at the retraction velocity of 1.0 μm/s and at 25 °C. (A) and (B) show the distribution of forces between β-lactoglobulin antibody immobilized on the cantilever and β-lactoglobulin (antigen) immobilized on the mica and between the cantilever and ferritin (nonantigen) immobilized on the mica in PBS alone, respectively. (C) and (D) show the distribution of forces between the cantilever and β-lactoglobulin on mica and between the cantilever and ferritin immobilized on mica in PBS with 0.1% BSA and Tween 20, respectively. (E) shows the distribution of forces between the cantilever and β-lactoglobulin on mica in PBS with 0.1% BSA, Tween 20, and free β-lactoglobulin. All histograms were normalized to the total counts of the force curve measurements at each experimental condition.

immobilized on the substrate were carried out. The results of the force measurements recorded at the retraction velocity of 1.0 μm/s at 25 °C are summarized in Figure 3. In the absence of Tween 20 and BSA, the distribution of the unbinding forces between the anti-β-lactoglobulin antibody immobilized on the cantilever and β-lactoglobulin (antigen) immobilized on the substrate (Figure 3A) was similar to that of the forces between the antibodies immobilized on the cantilever and ferritin (nonantigen) immobilized on the substrate (Figure 3B). These results indicated that, in the absence of the nonreactive protein and detergent, most forces between the antibodies immobilized on the cantilever and protein on the substrate probably originate from nonspecific interactions.

In our previous report,[34] we showed that use of a detergent and nonreactive protein is effective in reducing the nonspecific interaction in AFM measurement of the unbinding force between antibody and antigen. Therefore, next, the force measurements were carried out in blocking solution (i.e., in the presence of BSA (nonreactive protein) and Tween 20 (detergent)). The distribution of the unbinding forces between the antibodies immobilized on the cantilever and β-lactoglobulin immobilized on the substrate in the presence of BSA and Tween 20 (Figure 3C) was almost identical to distribution of the unbinding forces in the absence of BSA and Tween 20 (Figure 3A). In contrast, the unbinding forces between the antibodies on the cantilever and ferritin on the substrate in the presence of BSA and Tween 20 (Figure 3D) decreased more significantly than those in the absence of BSA and Tween 20 (Figure 3B). Figure S2 shows a typical force−distance curve between β-lactoglobulin antibody immobilized on the cantilever and ferritin immobilized on the mica in the presence of 0.1% BSA and Tween 20. The values of most unbinding forces between the antibodies on the cantilever and ferritin on the substrate in the presence of BSA and Tween 20 were below the detection limit (<10 pN). Furthermore, to confirm that the specific antibody−antigen interaction was causing the observations in the unbinding force measurements when Tween 20 and BSA were present in the experimental solution, the unbinding force measurements were carried out in the blocking solution to which free β-lactoglobulin was added. Figure S3 shows a typical force−distance curve between β-lactoglobulin antibody immobilized on the cantilever and β-lactoglobulin immobilized on the mica the presence of 0.1% BSA, Tween 20, and free β-lactoglobulin. Most of the unbinding forces between the anti-β-lactoglobulin antibodies on the cantilever and the β-lactoglobulin on the substrate were not observed within our detection limit (Figure 3E). In addition, similar results were observed for the measurements at different retraction velocities and temperatures (data not shown).

These results indicate that the unbinding forces between the anti-β-lactoglobulin immobilized on the cantilever and the β-lactoglobulin immobilized on the substrate in the presence of Tween 20 and BSA without excess β-lactoglobulin originated from the specific antibody−antigen interaction.

AFM Measurements of the Temperature Effect on the Energy Landscape. To investigate the effect of temperature on the unbinding force between anti-β-lactoglobulin antibody and β-lactoglobulin, as well as the parameters of kinetics and energy landscape for the interaction, force measurements were carried out at different temperatures in the presence of BSA and Tween 20. Figure 4 shows representative histograms from comparable loading rates at 25 and 45 °C. Also,

dx.doi.org/10.1021/bi201245k | Biochemistry 2012, 51, 32−42

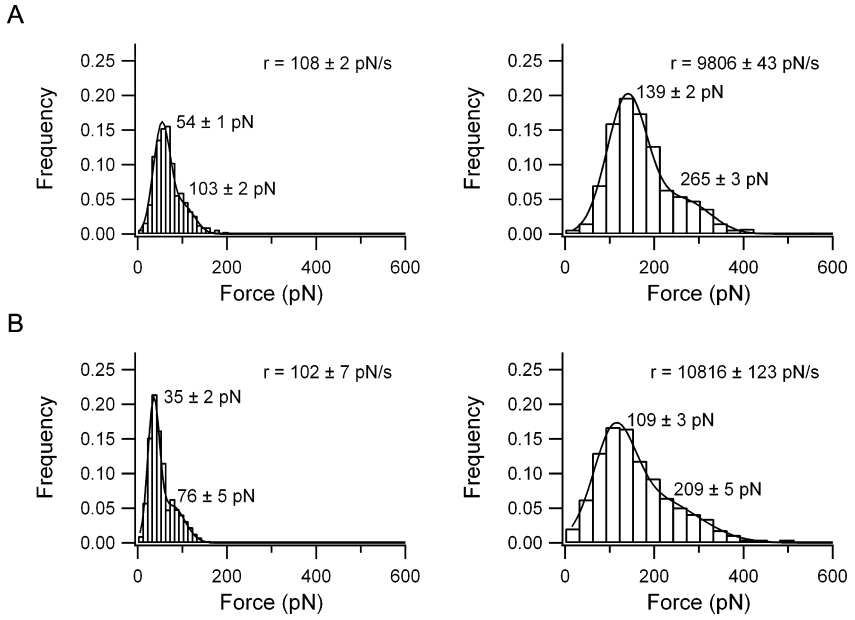


Figure 4. Representative distributions of the unbinding forces between anti-β-lactoglobulin antibody immobilized on the cantilever and β-lactoglobulin immobilized on the mica measured at different loading rates and at different temperatures. (A) Distribution of forces measured at 25 °C. (B) Distribution of forces measured at 45 °C. All histograms were normalized to the total count of detection events that originated from the specific interaction between anti-β-lactoglobulin antibody and β-lactoglobulin. The solid lines at each histogram are the corresponding double-Gaussian function fittings used to extract the most probable unbinding force.

Figures S4 and S5 show typical density plots of the loading rates and the unbinding forces obtained from the kernel density estimation function analysis. As can be seen from these figures, the density plots obtained from the analysis were similar to the distribution obtained from the histograms. Furthermore, the value of the most probable unbinding forces obtained the analysis were almost same as those obtained from fitting the double-Gaussian function to the histograms. Similar results were observed in the comparisons between the histogram and the density plot from the kernel density estimation function analysis at different loading rates and temperatures (data not shown).

The most probable unbinding forces, which were estimated by fitting the double-Gaussian function to each histogram of the unbinding forces, were plotted as a function of loading rates in Figure 5. At comparable loading rates, the unbinding forces decreased as temperature increased (Figures 4 and 5). At each temperature, the unbinding force exhibited two linear regimes (Figure 5). To obtain the dissociation rate at zero force and the position of the energy barrier for the antibody–β-lactoglobulin interaction, each of the two regimes, shown in Figure 5, were fitted by the Bell model (eq 2). The parameters of the interaction obtained from the Bell model at each temperature are summarized in Table 1. The dissociation rate and position of the energy barrier increased as temperature increased.

Energy landscape surface roughness of the antibody–β-lactoglobulin interaction was determined as follows. First, the activation energy (the height of the energy barrier) of the outer and inner transition states was determined using the Arrhenius law. The heights of the inner and outer barriers obtained from the Arrhenius law were 49.7 k_BT and 14.5 k_BT, respectively. Next, the energy landscape surface roughness was calculated using eq 3. Table 2 summarizes the energy landscape surface roughness that we computed at five unbinding forces. The values of the energy landscape roughness were approximately 10–20% of the height of the energy barrier.

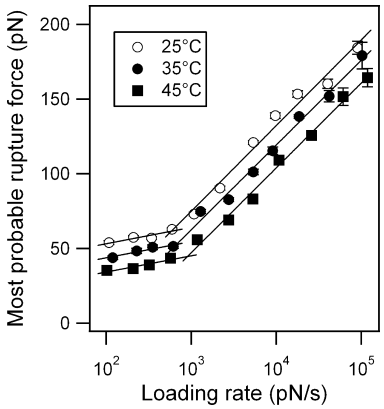


Figure 5. Dynamic force spectra (most probable unbinding force versus loading rate) for the anti-β-lactoglobulin antibody–β-lactoglobulin interaction. The first peaks in the distribution of the unbinding force measured at each loading rate and temperature was used as the most probable force (see Data Analysis of the Measurements of the Temperature Effect on Parameters of the Kinetics and Energy Landscape by AFM in the Experimental Procedures section).

Schematic Diagram of the Energy Landscape for the β-Lactoglobulin–Antibody Interaction. Using the parameters obtained from the present study, we can sketch a one-dimensional energy landscape for the dissociation process of the β-lactoglobulin–antibody complex (Figure 6). In Figure 6, the amplitude of each roughness is assumed to be a Gaussian distribution of barrier heights with standard deviation ε. Figure 6 shows how the energy landscape can be modified if the roughness is considered.

DISCUSSION

We investigated the effect of temperature on the β-lactoglobulin–anti-β-lactoglobulin antibody interaction using AFM. Such a

dx.doi.org/10.1021/bi201245k | Biochemistry 2012, 51, 32–42

Table 1. Parameters of the Anti-β-lactoglobulin Antibody−β-Lactoglobulin Interaction As Obtained from the Bell Model[a]

	low loading rate		high loading rate	
T (°C)	x_1 (nm)	k_{off}^{1} (s^{-1})	x_2 (nm)	k_{off}^{2} (s^{-1})
25	0.828 ± 0.065	(4.48 ± 3.78) × 10^{-4}	0.165 ± 0.004	1.99 ± 0.22
35	0.916 ± 0.086	(1.74 ± 1.55) × 10^{-3}	0.173 ± 0.003	3.21 ± 0.24
45	0.969 ± 0.09	(1.02 ± 0.75) × 10^{-2}	0.186 ± 0.004	4.94 ± 0.28

[a] x_i, potential position; k_{off}^{i}, dissociation rate at zero force.

Table 2. Energy Landscape Roughness (ε) Determined by Eq 3

		ε (k_BT) for T_1, T_2 (°C)		
barrier	F (pN)	25, 35	25, 45	35, 45
outer	43			5.91
	53	6.23		
inner	70	2.93	3.17	3.35
	120	2.93	3.17	3.35
	170	2.93	3.17	3.35

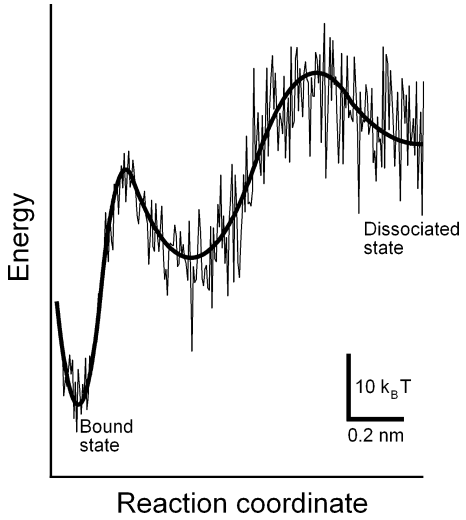


Figure 6. Diagram of a smooth (thin line) and rough (thick line) energy landscape for the anti-β-lactoglobulin antibody−β-lactoglobulin interaction at 25 °C assuming Gaussian-distributed roughness amplitudes.

study can provide information about not only the intermolecular force (unbinding force) between a molecule of interest immobilized on the cantilever and a complementary molecule immobilized on the substrate but also the parameters of the energy landscape and kinetics for the dissociation process of the complex of the molecule of interest and complementary molecule. Our study attempts to evaluate for the first time the effect of temperature on the antigen−antibody interaction using AFM. Here, for this investigation, we performed three procedures. First, we developed methods for reducing nonspecific signals in the AFM measurements of the β-lactoglobulin−antibody interaction because several studies using AFM reported that the force between antibody and antigen are often obscured by nonspecific signals.[32−34] Second, we measured the unbinding forces between β-lactoglobulin and the antibody at different temperatures using these methods. Lastly, we determined the parameters of kinetics and energy landscape for the dissociation process of the β-lactoglobulin−antibody complex from the resulting force data.

Evidence of Specific Interactions. Some studies have reported that in the measurements of the unbinding force between antigen and antibody molecules using AFM forces are often obscured by the lack of mobility of the molecule on the cantilever and nonspecific adhesive forces between the antibody immobilized on the cantilever and the surface of the substrate.[32−34] Several attempts have been made to overcome the difficulty of molecular mobility,[19,38−41] but few studies have reported on how to reduce nonspecific adhesive forces. We have previously shown that use of a detergent and nonreactive protein is useful for reducing nonspecific adhesive forces in the measurement of the unbinding force between ferritin and polyclonal antiferritin IgG antibody using AFM.[34] In the present study, in the absence of BSA (nonreactive protein) and Tween 20 (detergent), the distribution of unbinding forces between the anti-β-lactoglobulin IgG antibody immobilized on the cantilever and ferritin (nonantigen) immobilized on the substrate was similar to that between the antibody immobilized on the cantilever and β-lactoglobulin (antigen) immobilized on the substrate. This finding indicated that the forces in the absence of both nonreactive protein and detergent contained nonspecific adhesive force. Conversely, in the presence of BSA and Tween 20, distinct changes in the distributions of the forces between β-lactoglobulin and the ferritin-coated substrate were observed. Moreover, in the presence of free β-lactoglobulin as well as Tween 20 and BSA, the force decreased (Figure 3C−E). Therefore, we could readily measure the forces originating from the specific interaction between β-lactoglobulin and anti-β-lactoglobulin antibody using the experimental solution containing the detergent and nonreactive protein.

Detergents and nonreactive proteins have been widely used to reduce the background signal originating from nonspecific interactions in conventional immunoassays such as ELISA[42,43] and kinetic measurements such as SPR.[44−48] Nonreactive proteins have also been used to reduce nonspecific signals in recent single-molecule experiments of motor proteins, such as the *in vitro* motility assay[49−51] and force measurements using optical tweezers.[52,53] Our previous and present studies demonstrates that a similar strategy for the immunoassay and single-molecule experiments (i.e., use of a detergent and nonreactive protein) are useful for reducing the nonspecific adhesive forces in the AFM measurement of the unbinding force between antigen and antibody.

Temperature Effects on the Unbinding Force between β-Lactoglobulin and Anti-β-lactoglobulin Antibody. The unbinding forces between β-lactoglobulin and antibody obtained from the present study at 25 °C were in the same range as reports of AFM measurements of other antibody−antigen systems at room temperature.[19−27]

In the previous measurements of the antibody−antigen system using AFM, several linker molecules have been used to tether the molecule of the interest to the surface of the AFM tip and/or the substrate in the proper orientation for maximum complementary molecule binding. For example,

dx.doi.org/10.1021/bi201245k | *Biochemistry* 2012, 51, 32−42

Hinterdorfer et al. used poly(ethylene glycol) (ca. 8 nm in length) as the linker molecule.[19] In contrast, compared with these previous measurements using the linker molecule for the proper orientation, the length of the linker molecule used in our present study (ca. 1 nm) is short. Nevertheless, the unbinding force between β-lactoglobulin and the antibody at 25 °C was in the same range as reports of other antibody−antigen systems at room temperature. The similarity suggests that the linker molecule used in our present study is sufficient for the antigen binding.

As the temperature was increased, the unbinding forces decreased over the entire range of loading rates (Figure 5). There are two possibilities to explain this result: (i) the unbinding forces between β-lactoglobulin and antibody include forces that originate from temperature-dependent interactions, such as hydrogen bonding, and (ii) the secondary and/or tertiary structures of β-lactoglobulin is destroyed by heating. As a result, the denatured β-lactoglobulin reduced the immunoactivity of the anti-β-lactoglobulin antibody. The denaturing process of β-lactoglobulin induced by heating has been investigated using various physicochemical techniques such as circular dichroism analyses,[54−56] differential scanning calorimetry,[57−59] and fluorescence measurements.[60−62] According to these studies, thermal denaturation of β-lactoglobulin at neutral pH occurs through different steps characterized by well-defined temperature thresholds as follows. First, at ca. 50 °C, the native β-lactoglobulin transitions into the R state. The conformation of the R state differs from that of the native state in a few side chains and accessibility of a free thiol group, but there are no major differences in the secondary and tertiary structure between the native and R states. Next, the R state transits into the molten globule state at ca.70 °C. Finally, at ca.130 °C, the molten globule state transitions into the unfolded state. Also, Kaminogawa et al. demonstrated that the binding constants between β-lactoglobulin and five anti-β-lactoglobulin monoclonal antibodies at temperatures ranging from room temperature to 97 °C correlated with the denaturation process of β-lactoglobulin induced by heating and that the binding constant at temperatures ranging from room temperature to 60 °C was almost identical.[63] These studies showed that the major secondary and tertiary structural changes of β-lactoglobulin do not occur at the temperature range used in the present study (25−45 °C). Therefore, we conclude that probably (i) is the case. This hypothesis may be supported by the results from X-ray crystal structure analysis of the IgE Fab fragment in complex with β-lactoglobulin, as determined by Niemi et al.[64] They reported that there are two salt bridges and 13 hydrogen bonds between β-lactoglobulin and the IgE Fab fragment. Furthermore, they compared the epitopes of the IgE Fab fragment−β-lactoglobulin system obtained from the X-ray crystal analysis with those of IgG antibody−β-lactoglobulin system obtained from studies using decapeptides synthesized according to the known amino acid sequence of β-lactoglobulin,[65] PEPSCAN assay with 151 overlapping dodecapeptides,[66] and a competitive immunoassay with 52 monoclonal antibodies.[67] From the results of these comparisons, they speculated that the epitopes of the IgG−β-lactoglobulin system may have overlapping areas with minor antigenic sites, even though the epitopes of the IgE Fab fragment−β-lactoglobulin system are not located in the most antigenic area accessible for IgG. Therefore, there may have been some hydrogen bonds between β-lactoglobulin and IgG antibody, which is used in the present study.

Also, previous studies using these peptides have shown that epitopes of the IgG polyclonal antibody−β-lactoglobulin system are mainly located in the same region as those of IgG monoclonal antibody and IgE polyclonal antibody−β-lactoglobulin system.[65−67] Thus, similar results to our present study may be observed in the IgG monoclonal antibody and the IgE polyclonal antibody−β-lactoglobulin system.

Temperature Effects on the Parameters of the Energy Landscape and Kinetics. At each temperature, two regimes were observed in the force spectra (Figure 5). These findings indicate that the dissociation process of the β-lactoglobulin−antibody complex passes at least two energy barriers from the bound state to dissociated state. The two regimes have been observed in other antibody−antigen systems.[21,22,24,25,27] To confirm whether there are at least the two energy barriers for the dissociation process of the β-lactoglobulin−antibody complex, the force spectra were fitted by the Dudko−Hummer model as follows:

$$F = \frac{\Delta G}{\nu x}\left\{1 - \left[\frac{1}{\beta \Delta G}\ln\left(\frac{k_{off} e^{\beta \Delta G}}{\beta x r}\right)\right]^{\nu}\right\}$$

where ΔG, k_{off}, and Δx are the activation energy, the dissociation rate at zero force, and the position of the energy barrier, respectively. ν is a scaling factor, and β is $(k_B T)^{-1}$.[68] In the fitting of the Dudko−Hummer model to the force spectra, two microscopic models were used: linear cubic theory ($\nu = 2/3$) and the cusp theory ($\nu = 1/2$). Until now, the Bell model has been mainly used to determine the parameters of the kinetics and the energy landscape from the force spectra obtained from AFM measurements. Recently, not only the Bell model but also the Dudko−Hummer model has been used to determine the parameters.[68,69] In the Dudko−Hummer model, it is assumed that there is a single energy barrier in the reaction coordinate. Figure S6, Table S1, and Table S2 in the Supporting Information show results of Dudko−Hummer model fitting. As can be seen in Figure S6, at low loading rate region, the values of the most probable rupture force calculated by the Dudko−Hummer model are significantly different from the experimental data. The differences at low loading rate region indicate that the Dudko−Hummer model cannot be applied to our present experimental results. Also, similar differences at the low loading rate region are observed in results of fitting of Bell model for a single energy barrier (the result not shown). Therefore, the differences probably arise from the assumption that there is a single energy barrier in the reaction coordinate. From these considerations, we concluded that in dissociation process of the β-lactoglobulin−antibody complex the two energy barrier model is more adequate than a single energy barrier model.

Table 1 shows the parameters of the energy landscape and kinetics for the interaction obtained from the Bell model (eq 2) fitting of each regime shown in Figure 5. The position of each energy barrier slightly increased as temperature increased, suggesting that the size of the complex of β-lactoglobulin and the antibody increases as temperature increases. As mentioned above, the major secondary and tertiary structural changes of β-lactoglobulin are considered not to occur in the temperature range used in the present study. Therefore, the temperature effect on the position may reflect moderate swelling of the tertiary structure of the complex. This is probably due to

dx.doi.org/10.1021/bi201245k | Biochemistry 2012, 51, 32−42

changes in the distance of the hydrogen bond in the complex induced by heat.

The temperature dependences of the position of the barrier have been observed in previous DFS measurements of the dissociation process between the molecular complex and protein-unfolding process. Rico and Moy showed that the position of the inner barrier for the dissociation process of the streptavidin−biotin interaction changed from 0.09 to 0.15 nm, whereas the position of the outer barrier for the interaction did not change considerably (<22%) as temperature increased from 17 to 37 °C.[14] Nevo et al. reported that the position of the energy barrier for the dissociation process of the GTPase Ran−importin-β interaction decreased from 0.44 to 0.21 nm as temperature increased from 7 to 32 °C.[31] That is, the increase/decrease and the magnitude of the temperature effect on the position of the energy barrier for the dissociation process of the β-lactoglobulin−antibody interaction were different from those obtained in previous studies. The differences between the values from previous studies and the present study may reflect differences in the origin of the intermolecular forces between molecular complexes. This hypothesis may be supported by two findings from previous studies. First, according to X-ray crystal structure analysis, the bound complex of streptavidin−biotin is formed by a network of ≥7 hydrogen bonds. In addition, hydrophobic and van der Waals contributions from at least three tryptophan contacts stabilize the molecular complex of streptavidin and biotin.[14,70−72] That is, the origins of intermolecular forces between β-lactoglobulin and antibody are different from those between streptavidin and biotin. Second, in general, the origin of the intermolecular forces between antibody and antigen and that of between the intermolecular forces ligand and receptor is due to noncovalent interaction (e.g., hydrogen bonding and hydrophobic interaction). Such interactions also are the origin of the force for stabilizing the tertiary structure of proteins. In protein-unfolding studies by DFS, the increase/decrease and magnitude of temperature effects on the position of the energy barrier for the protein-unfolding process are different among protein species. The differences may be mainly due to the relative importance of hydrophobic interactions to hydrogen bonds in the formation of the transition state of each protein.[73] Thus, the differences in the effects of temperature on the position of the energy barrier between the present and previous studies may reflect differences in the composition of noncovalent interactions.

The dissociation rates obtained from the present study at low loading rate at 25 °C were in the same range as seen in previous bulk measurements of the dissociation rate (k_{dis}) for the β-lactoglobulin−IgE Fab fragment interaction (k_{dis} = (1.61 ± 0.2) × 10^{-3} and (8.05 ± 0.2) × 10^{-3} s^{-1})[74] and another antibody−protein antigen interaction at room temperature using SPR (k_{dis} = 10^{-4}−10^{-2} s^{-1}).[4] In addition, the dissociation rates at zero force increased as temperature increased (Table 1). A similar temperature effect on the dissociation rate has been observed in bulk measurements.[44,49] These results suggest that the dissociation rates at zero force for the complex of β-lactoglobulin and antibody obtained from DFS reflect the same event observed in bulk measurements. This is in accordance with reports of the AFM measurement of other molecular interactions.[24]

The values of the height of the inner and outer potential barriers obtained from the Arrhenius law were 49.7 k_BT and 14.5 k_BT. The values of the heights obtained from the present study are similar to the values of the activation energy of the dissociation process of complexes of other antigen−antibody systems obtained from previous bulk measurements.[1,44,46,47] In addition, in the presence of inner and outer potential barriers in the reaction coordinates, the difference in their heights ($\Delta\Delta G$) is given by

$$\Delta\Delta G = k_BT \ln(k_{off}{}^1/k_{off}{}^2) \quad (4)$$

where $k_{off}{}^i$ is the dissociation rate at zero force of each transition state.[9,14] The value of the difference between our results at 25 °C using eq 4 was 8.4 k_BT, which is smaller than that obtained from the Arrhenius law. The differences between the values obtained from the Arrhenius law and eq 4 may contribute to the fitting of the Arrhenius law to fewer data points.

Energy Landscape Roughness. In general, the real surface of the energy landscape is considered to be rough and rugged.[29−31] Until now, the roughness value has been estimated using only computer simulation and theory. Recently, several authors reported methods for direct measurements of roughness using DFS.[28−31] In the present study, we determined the roughness of the β-lactoglobulin−antibody interaction on the basis of the method by Nevo et al.[31] The roughness for the inner and outer barriers of the dissociation process of the β-lactoglobulin−antibody complex was ca. 6.1 k_BT and 3.2 k_BT, respectively (Table 2). Similar values have been reported in the AFM measurements of other molecular interactions, such as the unbinding forces of streptavidin−biotin (4.9−9.8 k_BT)[14] and GTPase Ran−importin-β (5.7 k_BT) complexes.[31] In addition, similar values have been observed in AFM measurements of the unfolding process of proteins, such as the unfolding of the transmembrane helices of bateriorhodopsin (4−6 k_BT)[75] and that of titin (4.3 k_BT).[73] The similarity in the roughness values between the present and previous studies suggests a common origin of roughness. Rico and Moy speculated that the origin of roughness of the streptavidin−biotin interaction was due to competition between solvent water molecules with hydrogen bonds that stabilized the complex and to the flexibility of the "3−4" loop of streptavidin, which may induce the multiple conformational substates in the streptavidin−biotin complex.[14] As mentioned above, several hydrogen bonds are thought to be in the β-lactoglobulin−antibody interface. Thus, the roughness of the β-lactoglobulin−antibody interaction obtained in the present study may originate from the competition of solvent water molecules with hydrogen bonds that stabilize the β-lactoglobulin−antibody complex.

CONCLUSIONS

We investigated the effect of temperature on the interaction between β-lactoglobulin and the IgG polyclonal antibody using AFM. The unbinding forces between β-lactoglobulin and the anti-β-lactoglobulin antibody decreased as temperature increased. The dissociation rates at zero force and the barrier positions of the dissociation process of the β-lactoglobulin−antibody complex were determined by fitting the Bell model to the force spectra. The dissociation rates and barrier positions increased as temperature increased. Furthermore, we determined the height of the potential barrier in the reaction coordinate and energy landscape roughness of the β-lactoglobulin−antibody interaction.

The present study showed that AFM measurements of the unbinding forces at different temperatures can provide more information than measurements of reaction rates at different temperatures using conventional bulk measurements such as SPR.

dx.doi.org/10.1021/bi201245k | Biochemistry 2012, 51, 32−42

■ ASSOCIATED CONTENT

Supporting Information

Example of thermal fluctuation analysis of the cantilever (Figure S1), typical force−distance curves (Figures S2 and S3), typical density plots of the loading rates and the unbinding forces (Figures S4 and S5) obtained from the kernel density function estimation method, results of Dudko−Hummer model fitting to the dynamic force spectra (Figure S6), and parameters of the anti-β-lactoglobulin antibody−β-lactoglobulin interaction as obtained from the Dudko−Hummer model (Tables S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-29-838-8054. Fax: +81-29-838-7181. E-mail: ssugi@affrc.go.jp.

Funding

This work was supported by the Food Nanotechnology project, MAFF (Japan).

■ ABBREVIATIONS

AFM, atomic force microscopy; BSA, bovine serum albumin; DFS, dynamic force spectroscopy; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; MES, 2-morpholinoetanesulfonic acid; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; SAMs, self-assembled monolayers; SPR, surface plasmon resonance.

■ REFERENCES

(1) Absolom, D. R., and van Oss, C. J. (1986) The nature of the antigen-antibody bond and the factors affecting its association and dissociation. *CRC Crit. Rev. Immunol. 6*, 1−46.

(2) Homola, J. (2003) Present and future of surface plasmon resonance biosensors. *Anal. Bioanal. Chem. 377*, 528−539.

(3) Piliarik, M., Vaisocherova, H., and Homola, J. (2009) Surface plasmon resonance biosensing. *Methods Mol. Biol. 503*, 65−88.

(4) Lin, S., Lee, A. S., Lin, C. C., and Lee, C. K. (2006) Determination of Binding Constant and Stoichiometry for Antibody-Antigen Interaction with Surface Plasmon Resonance. *Curr. Proteomics 3*, 271−282.

(5) McDonnell, J. M. (2001) Surface plasmon resonance: towards an understanding of the mechanisms of biological molecular recognition. *Curr. Opin. Chem. Biol. 5*, 572−577.

(6) Evans, E., and Ritchie, K. (1997) Dynamic strength of molecular adhesion bonds. *Biophys. J. 72*, 1541−1555.

(7) Evans, E. (1998) Energy landscapes of biomolecular adhesion and receptor anchoring at interfaces explored with dynamic force spectroscopy. *Faraday Discuss. 111*, 1−16.

(8) Evans, E. (2001) Probing the relation between force--lifetime--and chemistry in single molecular bonds. *Annu. Rev. Biophys. Biomol. Struct. 30*, 105−128.

(9) Lee, C. K., Wang, Y. M., Huang, L. S., and Lin, S. (2007) Atomic force microscopy: determination of unbinding force, off rate and energy barrier for protein-ligand interaction. *Micron 38*, 446−461.

(10) Merkel, R., Nassoy, P., Leung, A., Ritchie, K., and Evans, E. (1999) Energy landscapes of receptor-ligand bonds explored with dynamic force spectroscopy. *Nature 397*, 50−53.

(11) Yuan, C., Chen, A., Kolb, P., and Moy, V. T. (2000) Energy landscape of streptavidin-biotin complexes measured by atomic force microscopy. *Biochemistry 39*, 10219−10223.

(12) Lo, Y. S., Zhu, Y. J., and Beep, T. P. Jr. (2001) Loading-Rate Dependence of Individual Ligand-|Receptor Bond-Rupture Forces Studied by Atomic Force Microscopy. *Langmuir 17*, 3741−3748.

(13) de, Odrowaz Piramowicz, M., Czuba, P., Targosz, M., Burda, K., and Szymonski, M. (2006) Dynamic force measurements of avidin-biotin and streptavidin-biotin interactions using AFM. *Acta Biochim. Pol. 53*, 93−100.

(14) Rico, F., and Moy, V. T. (2007) Energy landscape roughness of the streptavidin-biotin interaction. *J. Mol. Recognit. 20*, 495−501.

(15) Sekiguchi, H., Ikai, A., Arakawa, H., and Sugiyama, S. (2006) AFM analysis of interaction forces between bio-molecules using ligand-functionalized polymers. *e-J. Surf. Sci. Nanotechnol. 4*, 149−154.

(16) Taninaka, A., Takeuchi, O., and Shigekawa, H. (2009) Site-Selective Anatomy of Step-by-Step Reactions in Ligand? Receptor Bonding Processes Using Dynamic Force Spectroscopy. *Appl. Phys. Express 2*, 085002−085004.

(17) Taninaka, A., Takeuchi, O., and Shigekawa, H. (2010) Hidden variety of biotin-streptavidin/avidin local interactions revealed by site-selective dynamic force spectroscopy. *Phys. Chem. Chem. Phys. 12*, 12578−12583.

(18) Taninaka, A., Takeuchi, O., and Shigekawa, H. (2010) Reconsideration of dynamic force spectroscopy analysis of streptavidin-biotin interactions. *Int. J. Mol. Sci. 11*, 2134−2151.

(19) Hinterdorfer, P., Baumgartner, W., Gruber, H. J., Schilcher, K., and Schindler, H. (1996) Detection and localization of individual antibody-antigen recognition events by atomic force microscopy. *Proc. Natl. Acad. Sci. U. S. A. 93*, 3477−3481.

(20) Schwesinger, F., Ros, R., Strunz, T., Anselmetti, D., Guntherodt, H. J., Honegger, A., Jermutus, L., Tiefenauer, L., and Pluckthun, A. (2000) Unbinding forces of single antibody-antigen complexes correlate with their thermal dissociation rates. *Proc. Natl. Acad. Sci. U. S. A. 97*, 9972−9977.

(21) Dettmann, W., Grandbois, M., Andre, S., Benoit, M., Wehle, A. K., Kaltner, H., Gabius, H. J., and Gaub, H. E. (2000) Differences in zero-force and force-driven kinetics of ligand dissociation from beta-galactoside-specific proteins (plant and animal lectins, immunoglobulin G) monitored by plasmon resonance and dynamic single molecule force microscopy. *Arch. Biochem. Biophys. 383*, 157−170.

(22) Berquand, A., Xia, N., Castner, D. G., Clare, B. H., Abbott, N. L., Dupres, V., Adriaensen, Y., and Dufrene, Y. F. (2005) Antigen binding forces of single antilysozyme Fv fragments explored by atomic force microscopy. *Langmuir 21*, 5517−5523.

(23) Kienberger, F., Kada, G., Mueller, H., and Hinterdorfer, P. (2005) Single molecule studies of antibody-antigen interaction strength versus intra-molecular antigen stability. *J. Mol. Biol. 347*, 597−606.

(24) Neuert, G., Albrecht, C., Pamir, E., and Gaub, H. E. (2006) Dynamic force spectroscopy of the digoxigenin-antibody complex. *FEBS Lett. 580*, 505−509.

(25) Odorico, M., Teulon, J. M., Bessou, T., Vidaud, C., Bellanger, L., Chen, S. W., Quemeneur, E., Parot, P., and Pellequer, J. L. (2007) Energy landscape of chelated uranyl: antibody interactions by dynamic force spectroscopy. *Biophys. J. 93*, 645−654.

(26) Teulon, J. M., Odorico, M., Chen, S. W., Parot, P., and Pellequer, J. L. (2007) On molecular recognition of an uranyl chelate by monoclonal antibodies. *J. Mol. Recognit. 20*, 508−515.

(27) Morfill, J., Neumann, J., Blank, K., Steinbach, U., Puchner, E. M., Gottschalk, K. E., and Gaub, H. E. (2008) Force-based analysis of multidimensional energy landscapes: application of dynamic force spectroscopy and steered molecular dynamics simulations to an antibody fragment-peptide complex. *J. Mol. Biol. 381*, 1253−1266.

(28) Hyeon, C., and Thirumalai, D. (2003) Can energy landscape roughness of proteins and RNA be measured by using mechanical unfolding experiments? *Proc. Natl. Acad. Sci. U. S. A. 100*, 10249−10253.

(29) Hyeon, C., and Thirumalai, D. (2007) Measuring the energy landscape roughness and the transition state location of biomolecules using single molecule mechanical unfolding experiments. *J. Phys: Condens. Matter 19*, 113101.

(30) Kapon, R., Nevo, R., and Reich, Z. (2008) Protein energy landscape roughness. *Biochem. Soc. Trans. 36*, 1404−1408.

(31) Nevo, R., Brumfeld, V., Kapon, R., Hinterdorfer, P., and Reich, Z. (2005) Direct measurement of protein energy landscape roughness. *EMBO Rep. 6*, 482−486.
(32) Willemsen, O. H., Snel, M. M., Cambi, A., Greve, J., De, Grooth, B. G., and Figdor, C. G. (2000) Biomolecular interactions measured by atomic force microscopy. *Biophys. J. 79*, 3267−3281.
(33) Brogan, K. L., Shin, J. H., and Schoenfisch, M. H. (2004) Influence of surfactants and antibody immobilization strategy on reducing nonspecific protein interactions for molecular recognition force microscopy. *Langmuir 20*, 9729−9735.
(34) Wakayama, J., Sekiguchi, H., Akanuma, S., Ohtani, T., and Sugiyama, S. (2008) Methods for reducing nonspecific interaction in antibody-antigen assay via atomic force microscopy. *Anal. Biochem. 380*, 51−58.
(35) Janovjak, H., Struckmeier, J., and Müller, D. J. (2005) Hydrodynamic effects in fast AFM single-molecule force measurements. *Eur. Biophys. J. 34*, 91−96.
(36) Hutter, J. L., and Bechhoefer, J. (1993) Calibration of atomic force microscope tips. *Rev. Sci. Instrum. 64*, 1143970.
(37) Tees, D. F., Waugh, R. E., and Hammer, D. A. (2001) A microcantilever device to assess the effect of force on the lifetime of selectin-carbohydrate bonds. *Biophys. J. 80*, 668−682.
(38) Dammer, U., Hegner, M., Anselmetti, D., Wagner, P., Dreier, M., Huber, W., and Guntherodt, H. J. (1996) Specific antigen/antibody interactions measured by force microscopy. *Biophys. J. 70*, 2437−2441.
(39) Willemsen, O. H., Snel, M. M., van, der Werf, K. O., de, Grooth, B. G., Greve, J., Hinterdorfer, P., Gruber, H. J., Schindler, H., van, Kooyk, Y., and Figdor, C. G. (1998) Simultaneous height and adhesion imaging of antibody-antigen interactions by atomic force microscopy. *Biophys. J. 75*, 2220−2228.
(40) Idiris, A., Kidoaki, S., Usui, K., Maki, T., Suzuki, H., Ito, M., Aoki, M., Hayashizaki, Y., and Matsuda, T. (2005) Force measurement for antigen-antibody interaction by atomic force microscopy using a photograft-polymer spacer. *Biomacromolecules 6*, 2776−2784.
(41) Ros, R., Schwesinger, F., Anselmetti, D., Kubon, M., Schafer, R., Pluckthun, A., and Tiefenauer, L. (1998) Antigen binding forces of individually addressed single-chain Fv antibody molecules. *Proc. Natl. Acad. Sci. U. S. A. 95*, 7402−7405.
(42) Kenna, J. G., Major, G. N., and Williams, R. S. (1985) Methods for reducing non-specific antibody binding in enzyme-linked immunosorbent assays. *J. Immunol. Methods 85*, 409−419.
(43) Steinitz, M. (2000) Quantitation of the blocking effect of tween 20 and bovine serum albumin in ELISA microwells. *Anal. Biochem. 282*, 232−238.
(44) Roos, H., Karlsson, R., Nilshans, H., and Persson, A. (1998) Thermodynamic analysis of protein interactions with biosensor technology. *J. Mol. Recognit. 11*, 204−210.
(45) Schuck, P., Millar, D. B., and Kortt, A. A. (1998) Determination of binding constants by equilibrium titration with circulating sample in a surface plasmon resonance biosensor. *Anal. Biochem. 265*, 79−91.
(46) Zeder-Lutz, G., Zuber, E., Witz, J., and Van, Regenmortel, M. H. (1997) Thermodynamic analysis of antigen-antibody binding using biosensor measurements at different temperatures. *Anal. Biochem. 246*, 123−132.
(47) Lipschultz, C. A., Yee, A., Mohan, S., Li, Y., and Smith-Gill, S. J. (2002) Temperature differentially affects encounter and docking thermodynamics of antibody--antigen association. *J. Mol. Recognit. 15*, 44−52.
(48) Kim, M. G., Shin, Y. B., Jung, J. M., Ro, H. S., and Chung, B. H. (2005) Enhanced sensitivity of surface plasmon resonance (SPR) immunoassays using a peroxidase-catalyzed precipitation reaction and its application to a protein microarray. *J. Immunol. Methods 297*, 125−132.
(49) Harada, Y., Noguchi, A., Kishino, A., and Yanagida, T. (1987) Sliding movement of single actin filaments on one-headed myosin filaments. *Nature 326*, 805−808.
(50) Warshaw, D. M., Desrosiers, J. M., Work, S. S., and Trybus, K. M. (1990) Smooth muscle myosin cross-bridge interactions modulate actin filament sliding velocity in vitro. *J. Cell. Biol. 111*, 453−463.
(51) Ali, M. Y., Uemura, S., Adachi, K., Itoh, H., Kinosita, K. Jr., and Ishiwata, S. (2002) Myosin V is a left-handed spiral motor on the right-handed actin helix. *Nat. Struct. Biol. 9*, 464−467.
(52) Tominaga, M., Kojima, H., Yokota, E., Orii, H., Nakamori, R., Katayama, E., Anson, M., Shimmen, T., and Oiwa, K. (2003) Higher plant myosin XI moves processively on actin with 35 nm steps at high velocity. *EMBO J. 22*, 1263−1272.
(53) Ishijima, A., Kojima, H., Funatsu, T., Tokunaga, M., Higuchi, H., Tanaka, H., and Yanagida, T. (1998) Simultaneous observation of individual ATPase and mechanical events by a single myosin molecule during interaction with actin. *Cell 92*, 161−171.
(54) Sawyer, W. H., Norton, R. S., Nichol, L. W., and McKenzie, G. H. (1971) Thermodenaturation of bovine β-lactoglobulin. Kinetics and the introduction of β-structure. *Biochim. Biophys. Acta 243*, 19−30.
(55) Iametti, S., De, Gregori, B., Vecchio, G., and Bonomi, F. (1996) Modifications occur at different structural levels during the heat denaturation of beta-lactoglobulin. *Eur. J. Biochem. 237*, 106−112.
(56) Qi, X. L., Holt, C., McNulty, D., Clarke, D. T., Brownlow, S., and Jones, G. R. (1997) Effect of temperature on the secondary structure of beta-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis. *Biochem. J. 324*, 341−346.
(57) de Wit, J. N., and Swinkels, G. A. (1980) A differential scanning calorimetric study of the thermal denaturation of bovine beta-lactoglobulin. Thermal behaviour at temperatures up to 100 degrees C. *Biochim. Biophys. Acta 624*, 40−50.
(58) Relkin, P., Eynard, L., and Launay, B. (1992) Thermodynamic parameters of beta-lactoglobulin and alpha-lactalbumin. A DSC study of denaturation by heating. *Thermochim. Acta 204*, 111−121.
(59) Qi, X. L., Brownlow, S., Holt, C., and Sellers, P. (1995) Thermal denaturation of beta-lactoglobulin: effect of protein concentration at pH 6.75 and 8.05. *Biochim. Biophys. Acta 1248*, 43−49.
(60) Mills, O. E. (1976) Effect of temperature on tryptophan fluorescence of beta-lactoglobulin B. *Biochim. Biophys. Acta 434*, 324−332.
(61) Cairoli, S., Iametti, S., and Bonomi, F. (1994) Reversible and irreversible modifications of beta-lactoglobulin upon exposure to heat. *J. Protein Chem. 13*, 347−354.
(62) Manderson, G. A., Hardman, M. J., and Creamer, L. K. (1999) Effect of heat treatment on bovine beta-lactoglobulin A, B, and C explored using thiol availability and fluorescence. *J. Agric. Food Chem. 47*, 3617−3627.
(63) Kaminogawa, S., Shimizu, M., Ametani, A., Hattori, M., Ando, O., Hachimura, S., Nakamura, Y., Totsuka, M., and Yamauchi, K. (1989) Monoclonal antibodies as probes for monitoring the denaturation process of bovine beta-lactoglobulin. *Biochim. Biophys. Acta 998*, 50−56.
(64) Niemi, M., Jylha, S., Laukkanen, M. L., Soderlund, H., Makinen-Kiljunen, S., Kallio, J. M., Hakulinen, N., Haahtela, T., Takkinen, K., and Rouvinen, J. (2007) Molecular interactions between a recombinant IgE antibody and the beta-lactoglobulin allergen. *Structure 15*, 1413−1421.
(65) Järvinen, K. M., Chatchatee, P., Bardina, L., Beyer, K., and Sampson, H. A. (2001) IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *Int. Arch. Allergy. Immunol. 126*, 111−118.
(66) Williams, S. C., Badley, R. A., Davis, P. J., Puijk, W. C., and Meloen, R. H. (1998) Identification of epitopes within beta lactoglobulin recognised by polyclonal antibodies using phage display and PEPSCAN. *J. Immunol. Methods 213*, 1−17.
(67) Clement, G., Boquet, D., Frobert, Y., Bernard, H., Negroni, L., Chatel, J. M., Adel-Patient, K., Creminon, C., Wal, J. M., and Grassi, J. (2002) Epitopic characterization of native bovine beta-lactoglobulin. *J. Immunol. Methods 266*, 67−78.

(68) Dudko, O. K., Hummer, G., and Szabo, A. (2006) Intrinsic rates and activation free energies from single-molecule pulling experiments. *Phys. Rev. Lett. 96*, 108101.
(69) Kamper, S. G., Porter-Peden, L., Blankespoor, R., Sinniah, K., Zhou, D., Abell, C., and Rayment, T. (2007) Investigating the specific interactions between carbonic anhydrase and a sulfonamide inhibitor by single-molecule force spectroscopy. *Langmuir 23*, 12561−12565.
(70) Weber, P. C., Ohlendorf, D. H., Wendoloski, J. J., and Salemme, F. R. (1989) Structural origins of high-affinity biotin binding to streptavidin. *Science 243*, 85−88.
(71) Chilkoti, A., and Stayton, P. (1995) Molecular Origins of the Slow Streptavidin-Biotin Dissociation Kinetics. *J. Am. Chem. Soc. 117*, 10622−10628.
(72) Freitag, S., Le, Trong, I., Chilkoti, A., Klumb, L. A., Stayton, P. S., and Stenkamp, R. E. (1998) Structural studies of binding site tryptophan mutants in the high-affinity streptavidin-biotin complex. *J. Mol. Biol. 279*, 211−221.
(73) Taniguchi, Y., Brockwell, D. J., and Kawakami, M. (2008) The effect of temperature on mechanical resistance of the native and intermediate states of I27. *Biophys. J. 95*, 5296−5305.
(74) Jylha, S., Makinen-Kiljunen, S., Haahtela, T., Soderlund, H., Takkinen, K., and Laukkanen, M. L. (2009) Selection of recombinant IgE antibodies binding the beta-lactoglobulin allergen in a conformation-dependent manner. *J. Immunol. Methods 350*, 63−70.
(75) Janovjak, H., Knaus, H., and Muller, D. J. (2007) Transmembrane helices have rough energy surfaces. *J. Am. Chem. Soc. 129*, 246−247.