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Scanning Force Microscopy of the Interaction Events between a Single Molecule of Heavy Meromyosin and Actin

Hideo Nakajima, Yuki Kunioka, Katsushi Nakano, Kohtaro Shimizu, Masaru Seto, and Toshio Ando[1]

Department of Physics, Faculty of Science, Kanazawa University Kakuma-machi, Kanazawa 920-11, Japan

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How close an approach is necessary for two interactive protein molecules to recognize each other before association? How strong a force field is exerted between two proteins at the recognition distance? How extensive are the association interfaces? How strong a force is necessary to pull the associated proteins apart? By means of atomic force microscopy at a truly single molecule level, these fundamental and intriguing questions were answered with the muscle proteins actin and myosin. © 1997 Academic Press

Biomolecular interaction has hitherto been described by biochemical kinetics in terms of dissociation constant, dissociation and association rate constants, etc. From these constants, we cannot obtain a physical view of biomolecular interaction, such as the force field exerted between biomolecules, the origins of forces supporting the initial recognition process and the final stable complex formation, the time required from the initial recognition to the complex formation, the flexibility of the association interfaces, the maximum length to which the bond can be kept, etc. These physical descriptions are very important for understanding the mechanism by which biomolecular interaction brings about a new function that cannot be achieved with a single biomolecule. However, because of the lack of appropriate methodologies, we have not been able to attain these descriptions. The atomic force microscope (AFM) developed as a high resolution imager of surface structure(2) has recently been shown to have great potential for studying the physical processes, as mentioned above, of biomolecular interaction. Unbinding forces between avidin-biotin(3, 4), complementary strands of DNA(5), cell adherence proteoglycans(6), and antibody-antigen(7) were recently measured by AFM. In these studies, however, the number of molecules involved in the measured force has not been determined directly, moreover binding processes have not been observed at a single molecular level. So, physical description about biomolecular interaction has still been limited. In our present work we studied the actin-heavy meromyosin (HMM) interaction using a homemade AFM integrated with an epifluorescence microscope. Utilizing the two microscopes' integration we first developed a method to capture a truly single molecule of HMM at an AFM probe tip and observed the interaction force between the captured HMM and actin fixed on the surface. We could observe not only the unbinding event but also the binding event from which knowledge of the inter-proteins force field was obtained for the first time at a single molecular level. Analysis of the unbinding event yielded the unitary unbinding force and the effective rupture length (the maximum distance at which the binding is kept). The unbinding force was an order of magnitude smaller than other protein systems(7, 8) , while the effective rupture length was an order of magnitude larger than other biomolecular systems(4, 7, 8). It has been a mystery how myosin moves from one actin to the next without detaching from actin. For this issue a hand-over-hand mechanism by the two heads of myosin (not only myosin but also other double-headed motor proteins) has been hypothesized(9). However, there exist several types of single-headed myosins in cells(10). From the unique nature of the actin-myosin bond described above, we postulated single head hand-over-hand movement by sequential unbinding and binding of flexible bonds having long reaches.

MATERIALS AND METHODS

Sample preparation. Actin, myosin and HMM were prepared from rabbit skeletal muscle as described in (11). The distal end of

[1] To whom correspondence should be addressed. Fax: 0762-64-5739, e-mail: tando@kenroku.ipc.kanazawa-u.ac.jp.

Abbreviations: AFM, atomic force microscope; HMM, heavy meromyosin.

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the tail part of HMM is specifically modified with biotincadaverine by guinea-pig transglutaminase-catalized amino exchange reaction(11). UltraAvidin-coated fluorescent polyacrylamide nanobeads (acrylamide chains are partially cross-linked with bis-acrylamide; the fluorophore, Cy3(Amersham)) were prepared as described in (11). To ensure the formation of one-to-one complex of HMM-bead biotinylated HMM was mixed with ten times molar excess amount of UltraAvidin-coated nanobeads, followed by purification by actin-affinity chromatography. Actin bundles were prepared in two ways; (1) F-actin partially labelled with rhodamine green phalloidin was incubated for 2 hr in a solution containing 20 mM $MgCl_2$ and F-buffer (25 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 25 mM imidazole-KOH (pH 7.6)). (2) G-actin and α-actinin were mixed in 30:1 molar ratio and dialyzed against F-buffer plus 2 mM ATP overnight. Bundles were fixed onto a coverslip coated with positively charged acrylamide beads, followed by washing with F-buffer. A solution of HMM-bead (50 pM, 50 μl) was put on the coverslip and incubated for 10 min. Unattached HMM-bead was washed out with F-buffer that now additionally contained reagents for the elimination of oxygen(12).

Functionalization of cantilever tip. A cantilever tip was functionalized as follows. One percent anhydrous methanolic solution of 3-aminopropyldimethylmethoxysilane was reacted with an equimolar amount of succinimidyl N[N′-(D-biotinyl)-6-aminohexanoyl]-6′-aminohexanoate at 4°C overnight. A droplet of the biotinylated silane (10 to 20 μl) was put on a coverslip, extended over the surface (4.8 cm^2) and dried to some extent. The coverslip was mounted on a sample stage of an AFM apparatus and then a cantilever tip (Microcantilever TR-400, Olympus) was put closer to the coverslip. After engagement they were incubated for 30 sec and then pulled apart. The cantilever thus functionalized was dried in an electric desiccator.

Determination of spring constant. The spring constant of biotinylated cantilevers was determined by measuring the thermal fluctuations in air at a sampling rate of 200 kHz (13).

Force-distance measurement. The interaction force curves were measured in approach-retract cycles at 0.167 Hz and 100 nm amplitude. The sample stage was displaced stepwise (0.2 nm step per 1 msec) and halted for 5 msec during which the position sensor outputs were sampled 1000 times and their mean value was taken as a force record. The sample was in F-buffer plus oxygen-elimination reagents at 25°C. When ATP was present in the medium any adhesive force was never observed. Therefore, even when there was nonspecific binding between the probe tip (or the nanobead) and actin bundles (or the substratum), the adhesive force would be very small (less than our detection limit, 3 pN).

RESULTS AND DISCUSSION

In our previous paper(11) we have shown electron micrographs of the complexes of the biotinylated HMM and UltraAvidin-coated fluorescent polyacrylamide beads (ca. 30 nm in diameter). They revealed that HMM was tagged with the bead at the distal end of the tail part. In most cases a HMM molecule was linked to a single bead, while in a few cases a HMM molecule was linked to two beads. We could not find a bead that had more than two HMM molecules. From the condition in which HMM-bead was prepared we can estimate the probability of finding a bead with more than two HMM molecules to be 0.05 at the most. However, in reality it should be less than this estimate because of the presence, in the prepurified sample, of denatured HMM that cannot dissociate from actin in the presence of ATP. According to our experience such denatured HMM exists around 50 % in HMM samples stored in liquid nitrogen. A bead with more than two HMM molecules had more chance to be removed by actin affinity chromatography. Therefore, when a sample of the purified HMM-bead was illuminated under a fluorescence microscope each of the individual fluorescent spots almost certainly belongs to a single molecule of HMM.

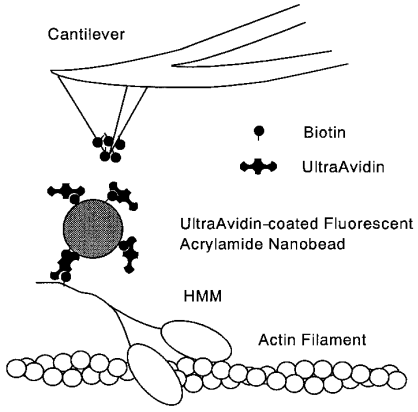

FIG. 1. Strategy to link a single molecule of HMM to a probe tip. This strategy is based on the strong affinity of biotin for avidin and on our previous finding that the distal end of the tail part of HMM is specifically modified with biotincadaverine by guinea-pig transglutaminase-catalized amino exchange reaction(11). Since avidin has a tendency to bind to protein non-specifically(30), UltraAvidin was used instead.

A strategy for linking a single molecule of HMM to a cantilever tip is sketched in Fig.1. The AFM apparatus used here was mounted on an epi-fluorescence video microscope. A small number of the HMM-bead complexes were attached to actin bundles that had been fixed onto a coverslip. The average bead-to-bead distance was about 10 μm. A cantilever tip was functionalized by biotinylated monosilane. The position of the functionalized tip was fine-tuned, within optical resolution, close to one of the fluorescent spots on the actin bundles, then the sample stage was scanned slowly within a small area (1 μm^2) surrounding the optically pinpointed position and finally lowered to separate the tip from the surface. By focusing an objective lens on the actin bundles and then on the apex of the probe tip, we found that the light-emitting spot was transferred from the actin bundles to the tip.

The HMM-linked tip was brought right over a fluorescent actin bundle. Then interaction force curves were measured in approach-retract cycles. A typical interaction force curve obtained is shown in Fig.2. Characteristic positions were marked with letters. From qualitative and quantitative points of view more three sets of experiments with different HMM molecules gave quite similar force curves. The steep jump-in feature (from B to C) in the approaching regime was rarely observed (the probability, 0.03), while the steep pull-off feature (from H to I) in the retracting regime was often observed (the probabil-

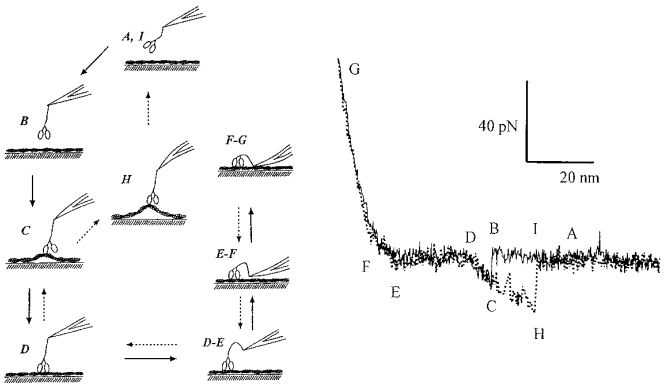

FIG. 2. Typical interaction force curves in an approach-retract cycle and the interaction events of a single HMM and actin inferred from the curves. The approaching (from A to G) and retracting (from G to I) regimes were drawn in a solid line and a dotted line, respectively.

ity, 0.6). The stage positions marked with letters changed little during many approach-retract cycles. Interaction events inferred from the force curves are sketched in Fig.2. When the probe tip jumped in contact, the deflection of the cantilever was on average only 0.66 nm (the corresponding force, 13.2 pN). However, a rather long further approach (about 6.3 nm) was necessary for this deflection to vanish completely. This means that when the probe tip was pulled down at C a portion of an actin filament was probably pulled up. The spring constant (k_a) of a structure that held the actin filament is therefore 13.2/(6.3 − 0.66)=2.34 pN/nm, 8.5 times softer than the cantilever (k_c = 20 pN/nm). Just before jump-in (B) forces were balanced and the gradient of force (df/dz) exerted between the two proteins is approximately equal to $\tilde{k}$ ($\equiv k_a k_c/(k_a+k_c)$=2.09 pN/nm). When the gradient exceeds this value jump-in takes place. Just before jump-in, there was no sign of deflection of the cantilever. Our detection limit of force is 3 pN, therefore the detection limit of the cantilever deflection is 0.15 nm. Provided the cantilever has deflected downward just before jump-in by this amplitude an actin filament must have deflected by 1.28 (= 3/2.34) nm. Therefore, just before jump-in the surface of HMM head was remote from the surface of actin more than 4.87 (6.3 − 0.15 − 1.28) nm. This long-range force is very likely electrostatic taking into account the length as well as observations made by other studies(14, 15, 16), although we will discuss later other possibilities. This inference can be tested by assuming Debye-H*ü*ckel potential and then calculating the actin-HMM head distance at B. This distance should be larger than 4.87 nm and smaller than 6.3 nm. Otherwise our assumption would be wrong. The Debye-H*ü*ckel potential is given by

$$\psi(z) = -A\frac{exp(-z/\lambda)}{z}, \qquad (1)$$

where A is a coefficient, λ is the Debye radius. Here, we have to note that this expression of potential should be limited to the case where the two proteins are distant more than λ. When distance between the two proteins is around λ or less the potential will become a complex function of the two protein's detailed geometry. In our solution condition the ionic strength was ca. 0.05, which gives λ=1.36 nm. The ratio of force $f(\equiv -d\psi/dz)$ to force gradient f' ($\equiv df/dz$) can be given, to a good approximation, by

$$f/f' = -\lambda(1 - \lambda/z) \qquad (2)$$

under the condition of $\lambda/z < 1$. Now, let's find out how far the tip surface of HMM head was apart from the surface of an actin filament when it started jumping into contact. Just after jump-in the sum of deflections of an actin filament and the cantilever is equal to 6.3 nm. In the case of just before jump-in the sum of three distances (deflection of an actin filament, z_j^a, deflection of the cantilever, z_j^c, the distance between tip surfaces of HMM head and actin, z_j) is almost the same as the length, 6.3 nm. This is because just before and after jump-in, the positions of the sample stage are the same or different only by the step displacement distance, 0.2 nm. Ignoring the small difference, 0.2 nm, we obtain

$$z_j = 6.3 - z_j^a - z_j^c. \qquad (3)$$

From a relation, $k_a z_j^a = k_c z_j^c$, eq.(3) becomes

$$z_j = 6.3 - \left(1 + \frac{k_c}{k_a}\right) z_j^c. \qquad (4)$$

Eq.(4) can be rewritten as eq.(5) using $\tilde{k}$ instead of k^a.

$$z_j = 6.3 - \frac{k_c}{\tilde{k}} z_j^c. \qquad (5)$$

Therefore the ratio of $f(z_j)$ to $f'(z_j)$ is given by

$$\left(\frac{f}{f'}\right)_{z=z_j} = (-k_c z_j^c)/\tilde{k} = z_j - 6.3. \qquad (6)$$

Combining eqs. (2) and (6), and solving a resulting quadratic equation, we obtained z_j = 5.29 nm. As expected the calculated value of z_j was between 4.87 and 6.3 nm. Then by putting this value for z_j and the values for k_c and $\tilde{k}$ into eq.(5) we obtained z_j^c =0.106 nm, and thus $f(z_j) = -k_c z_j^c = -2.12$ pN.

How close a distance is required for the two proteins' mutual recognition that thereafter leads to attracted approach and contact? For this let's define a recognition distance (z_r) as the distance where the potential energy equals $-\frac{1}{2}k_B T$, where k_B and T are Boltzmann's constant and the absolute temperature, respectively. To

estimate the recognition distance from the Debye-Hückel potential we have to determine a value of the coefficient (A) first. It was determined to be 591.94 $pN \cdot nm^2$ from $f(z_j) = -(d\psi/dz)_{z=z_j} = -2.12$ pN. With this value of A eq. (1) yields $z_r = 5.4$ nm, quite close to the jump-in distance, z_j. At this recognition distance f and f' are -1.9 pN and 1.82 pN/nm, respectively. We can also estimate how many effective electric charges (N_m and N_a) on a myosin head and actin are involved in the attractive force before contact. The value of A and the dielectric constant of water yield $N_m \times N_a = 16.3$. This value was approximately the same as that expected from the number of negative charges on the actin-binding site of myosin and the number of positive charges on the myosin-binding site of actin(15, 16). This approximate coincidence also suggests that only one head of the HMM was involved in the jump-in event.

In the discussion above we considered only electrostatic force as the long-range attractive force. Although hydrophobic interaction is still poorly understood, we discuss here the possibility of this type of interaction. It has been known that hydrophobic force also can be long-range force, as first experimentally shown by Israelachvili and Pashley(17) with the use of a surface-force apparatus(18) for surfactant monolayers adsorbed on mica surfaces in aqueous solution. This work and other similar studies(19, 20) have shown that the hydrophobic interaction decays exponentially with distance with a decay length of 1∼3 nm below 10∼15 nm. In these studies the interactive interfaces contain only hydrophobic groups. In the rigor actomyosin interfaces there are hydrophobic patches both on actin and on myosin head. These patches are however flanked on charged regions(21). As stated in (17) and documented in (22, 23), when both hydrophilic and hydrophobic groups are present at interfaces, the hydrophobic interaction tends to be neutralized. This is probably because the local structure of water molecules is dominated by their interaction with nearby hydrophilic groups(17). Therefore, it is unlikely that the long-range attractive force we observed originates from hydrophobic interaction. As to the long-range attractive force we may also have to consider thermal bending motion of an actin filament. An actin filament held to surface had an apparent spring constant of 2.34 pN/nm, therefore the mean amplitude of the thermal bending is 1.33 nm. Although this thermal bending might affect the deduced range of the attractive force, it is too small to overthrown the presence of long-range force between the two proteins.

Between the points D and E (distance, 25 nm) in both the approach and retract regimes, there was no deflection of the cantilever, reflecting the presence of a long and flexible connection. This entity is apparently the HMM's tail part (43 nm in length and 1 nm in width).

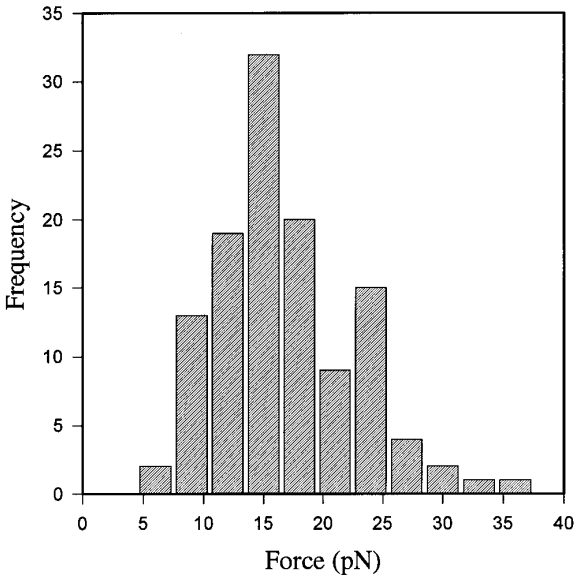

FIG. 3. A histogram of the unbinding force measured in 200 consecutive approach-retract cycles. In this specific experiment the rupture forces appeared in 118 cases of the 200 continuous cycles (60%). Apparently this histogram had two peaks. Curve fitting with the least-squares method of the histogram to a sum of two gaussians yielded the lower centered force of 14.8 pN with the standard deviation of 4.0 pN and the higher centered force of 24.7 pN with the standard deviation of 1.4 pN.

In the retracting part of the interaction force curves the cantilever began to be pulled down at a stage position almost the same as that where the zero-deflection had been reached after jump-in in the approaching regime. The pull-off event (from H to I) never took place in two steps, but always took place in one step. On the other hand, the magnitude of the pull-off forces observed in many consecutive approach-retract cycles distributed around two distinct force regions centered at 14.8 pN and 24.7 pN (Fig.3). HMM has a double-headed structure and each head has an ability to bind to actin. The distribution of the unbinding force having two peaks suggests that single-head binding often occurred while two-head binding occurred with much less probability. This is probably because an actin filament is not freely mobile and therefore has less chance to bind to both heads of HMM. Even when both of the two heads bound to actin, they would detach from actin almost simultaneously. The reason is that at the moment when one head is detached from actin as a given magnitude of force is applied, another head that has been until then bound to actin suddenly feels a nearly twofold increase in force, and therefore is pulled off immediately from actin. We can conclude that the unitary unbinding force (f_u) of a single head-actin pair is around 14.8 pN, an order of magnitude smaller than other interprotein forces(6, 7). The rigor bond can be ruptured also by pulling an actin filament along its

length, as has been done using optical tweezers by Nishizaka et al(24). In their case the unbinding force was 9.2 ± 4.4 pN.

The lifetime (τ) of the single rigor bond with force of 14.8 pN can be estimated to be 11.9 ms from $2\sigma/(dF/dt)$, where σ is a standard deviation of the unitary unbinding force and (dF/dt) is vertical scan velocity times cantilever spring constant(7). The reported values of the lifetime (τ_0) of the single rigor bond without force ranges from 5 s to 100 s(25, 26). Therefore the lifetime is reduced 300-5600 times by the force of 14.8 pN. As reported(1, 7, 24, 27) , an effective rupture length (l_r) can be estimated by the relation $\tau = \tau_0 \times exp(-l_r f_u / k_B T)$, which yields l_r =1.7∼2.5 nm. This is unusually long compared to values, 0.23 nm for antigen-antibody(7), 0.15∼0.3 nm for streptavidin-biotin(4) and 0.05∼0.3 nm for actin-α-actinin(8), but similar to a value (2∼3 nm) for actin-HMM reported by Nishizaka et al. (wherein actin was pulled along its length)(24). Actin-myosin interfaces contain several bonds between flexible loop structures(21, 28, 29). When pull force is applied to actin-myosin interfaces either in perpendicular or parallel to the interfaces, bonds between stiffer structures will be ruptured first and later bonds between flexible structures will be ruptured. This sequential rupture mechanism accounts for the weak unbinding force as well as the long rupture length. Although our experiment was made without ATP, this type of sequential rupture of elemental bonds may take place also in the presence of ATP. In the mutual sliding of actin and myosin, myosin moves from one actin to the next, during which they have to keep in contact with each other. The long rupture length and a possible flexible long reach for binding facilitate myosin to keep in contact with either actin promoter during the dislocation. Therefore, hand-over-hand movement may be possible even with single-headed myosin, although it has been attributed to the double-headed structure.

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