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Atomic Force Microscopy Study of Conformational Change of Immobilized Calmodulin

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Abstract

Maintaining the biological functionality of immobilized proteins is the key to the success of numerous protein-based biomedical devices. To that end, we studied conformational change of calmodulin (CaM) immobilized on chemical patterns. 1-cysteine mutated calmodulin was immobilized on a mercapto-terminated surface through the cysteine-Hg-mercapto coupling. Utilizing Atomic Force Microscope (AFM), the average height of the immobilized calmodulin was determined to be 1.87 ± 0.19 nm. After incubation in EGTA solution, the average height of protein changed to 2.26 ± 0.21 nm, indicating conformational change of CaM to Apo-CaM. The immobilized CaM also demonstrated conformational change upon the reaction with known calmodulin antagonist chlorpromazine (CPZ). After incubation in CPZ solution, the average height of CPZ-bound CaM increased to 2.32 ± 0.20 nm, demonstrating the immobilized CaM still has the similar response as in bulk solution. These results show that immobilization of calmodulin on a solid support does not interfere with the ability of the protein to bind calcium and calmodulin antagonists. Our results demonstrate the feasibility of employing AFM to probe and understand protein conformational changes.

Keywords

calmodulin; protein conformation change; AFM

Introduction

Certain proteins undergo conformational changes when they bind to their target ligands. Characterization of such conformational changes is the first step to understand a protein's conformation-function relationship.

Calmodulin can change its conformation upon binding to ligands. Calmodulin is involved in the regulation of a number of cellular signaling pathways that control many processes such as movement, growth and proliferation.¹⁻³ Calmodulin is a small (148 amino acid residues), acidic (pI = 4.3), and heat resistant protein. In solution, the calcium bound calmodulin (CaM) has a typical size of $6.7 \times 4 \times 2.5$ nm³, with a shape of a dumbbell.^{4, 5} Two termini of the dumbbell, the globular C-terminal domain and globular N-terminal domain, are connected by a “handle” of 8-turn central α -helix.^{6, 7} There are two pairs of calcium-binding EF hands on each domain, respectively. The dumbbell conformation of CaM exposes a

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hydrophobic binding pocket, which is used for binding ligands and activation of a series of kinases.⁷⁻⁹ When calcium ions are removed from CaM's EF hands, calmodulin molecule undergoes conformation change. The dumbbell-shaped conformation changes to the bound conformation Apo-Calmodulin (Apo-CaM), in which the hydrophobic binding pocket is inaccessible to external ligands. The dumbbell-shaped CaM also binds to a range of antagonists. One of the representatives is chlorpromazine (CPZ), a member of phenothiazine family of antipsychotic drugs that bind to the hydrophobic pocket of CaM.¹⁰ The CPZ binding induces CaM conformation to change from the extended dumbbell shape to a compact bound conformation with an approximate size of $5.7 \times 3 \times 3 \text{ nm}^3$, as measured from the Apo-CaM crystal structure 1CFC.¹¹ Immobilized calmodulin has the potentials to be used in high-performance bio-assay chips and high-throughput screening assay for identifying inhibitors of calmodulin-dependent kinase. However, immobilized proteins often behave differently from their solution state counterparts. In the case of calmodulin, although the conformational change of calmodulin has been extensively studied in solution state, how calmodulin undergoes conformational change in the immobilized state has not been fully understood yet. Broadly speaking, how the functions of immobilized protein in biomedical devices¹²⁻¹⁴ deviate from the solution state needs to be investigated individually. Therefore, the development of tools that can image and quantify the conformational change of immobilized proteins at the molecular level is important for the application of protein based bio-devices.

This work utilizes Atomic Force Microscopy (AFM) to characterize the conformational change of calmodulin immobilized on a chemically patterned surface. AFM is a technique used to precisely measure the tip-surface interactions and reveal the surface structures at nanometer level.¹⁵ AFM provides structural and functional information and is a non-destructive technique capable of imaging biological molecules, which make it ideal for studying the conformational change of immobilized proteins. Our AFM study revealed that the dumbbell-shaped calmodulin was anchored on the surface with its longest axis parallel to the surface. Upon conformation change, the two termini of the dumbbell contract, making the immobilized calmodulin higher.

Materials, instrumentation and methods

Materials

[Chemicals for preparing surface] OTS (octadecyltrichlorosilane, 97%) and mercaptoundecyltrimethoxysilane (MUTMS) were purchased from Gelest. Chloroform (HPLC grade) was purchased from Mallinckrodt Baker. Toluene (HPLC grade) was from EMD. Silicon (100) wafers were purchased from KC electronics (Nitrogen doped, $5 \Omega \cdot \text{cm}$ resistivity).¹⁶

[Chemicals for protein expression, purification, and reaction] Ethylenedis(oxyethylenenitrilo)tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), isopropyl- β -D-thiogalactopyranoside (IPTG), calcium chloride (CaCl_2) and chlorpromazine hydrochloride (CPZ) were purchased from SigmaAldrich Corp. (St. Louis, MO). Luria-Bertani (LB) broth, used for growing cell culture, was obtained from Difco Laboratories (Detroit, MI). CaM was purified using chitin beads from New England Biolabs (Ipswich, MA). 2-Anilinonaphthalene-6-sulfonic acid (2,6-ANS) used for fluorescence experiment and SDS-PAGE used to confirm the expressed protein were obtained from Invitrogen Corporation (Carlsbad, CA). CaM was also confirmed by Western blot analysis utilizing rabbit-raised monoclonal calmodulin primary antibody (EP799Y) and goat-raised antibody against rabbit immunoglobulin G conjugated to alkaline phosphatase (IgG-AP) that were purchased from Novus Biologicals (Littleton, CO). Immobilized tris(2-carboxyethyl)

phosphine hydrochloride (TCEP) disulfide reducing gel was purchased from Pierce (Rockford, IL). Reverse osmosis water (Milli-Q Water Purification System, Millipore, Bedford, MA) was used to prepare all solutions.

Instrumentation

The fabrication and characterization of the chemical pattern were performed with an Agilent PicoPlus AFM in an environmental chamber. The software used for image processing was WSxM.¹⁷ The sample pattern was cleaned by a carbon dioxide snow cleaning system from Applied Surface Technologies. The characterizations of the patterns were conducted in AC mode with MikroMasch NSC-14 tips. Protein patterns were imaged in air with 75%-100% RH humidity (at 25°C). The imaging set point was maintained at 99% of the tip free oscillation amplitude in air. Under this setup, the tip taps the protein surface under minimal force. The AFM tip is grounded, while the Si wafer sample is floating. Since the tip touches the protein surface in the humid environment, possible electrostatic charge from the sample is dissipated after the tip touches the sample. Hence the height measurement is not affected by protein's electrostatic charge. Nikon Eclipse 55C microscope and QuantaMaster 40 UV/VIS spectrofluorometer (Photon Technology International, NJ) were utilized for fluorescence imaging and measurement.¹⁶ The fluorescence images were acquired using UV-2A filter and analyzed using Nikon NIS elements BR software.

Methods

[Protein purification] Purification and expression of genetically engineered calmodulin with cysteine on N-terminus is based on instructional manual by New England Biolabs.¹⁸ In order to prevent dimer formation, the cysteine-mutated CaM was reduced with immobilized tris(2-carboxyethyl) phosphine hydrochloride (TCEP) disulfide reducing gel according to instructions provided by Pierce (Rockford, IL). The protein was kept in buffer containing 20 mM HEPES, 100 mM KCl and 10 mM CaCl₂.

The viability of the expressed 1-cysteine mutated CaM was demonstrated by the conformational change of CaM upon its reaction with a known calmodulin antagonist CPZ, which has been a well-established procedure.¹⁹ Upon binding to CPZ, the dumbbell-shaped CaM changes to the bound conformation, with the hydrophobic binding pocket folded inside the protein molecule. 2,6-ANS is a fluorescent probe for hydrophobicity¹⁹⁻²¹ that emits fluorescence only in a hydrophobic environment while not exerting it in aqueous solution. Therefore, when bound in hydrophobic binding pocket of the dumbbell conformation CaM, 2,6-ANS molecule emits fluorescence. When CPZ is added to the solution, the conformational change of CaM causes the release of the bound 2,6-ANS from CaM to the aqueous solution. Hence the fluorescence intensity from 2,6-ANS decreases. Therefore, by monitoring the fluorescence intensity of the 2,6-ANS labeled CaM as a function of the amount of CPZ added to the solution, we can confirm the conformational change of calmodulin, which is an indication of viability.¹⁰ During the experiment, the protein was labeled with 1:1 ratio of 2,6-ANS over night at room temperature followed by dialysis against the same buffer. The labeled CaM (10⁻⁶ M final concentration) was mixed with 10⁻⁸ M, 10⁻⁷ M and 10⁻⁶ M final concentrations solutions of CPZ. The solutions were excited at 310 nm and the emission spectra in the range from 400 nm to 500 nm were obtained against ANS-free CaM solutions the same type of buffer and CPZ concentrations.¹⁹ The results show that the increase of CPZ concentration led to a decreased fluorescence intensity at the characteristic ANS emission peak position (430 nm). The fluorescence intensity drop indicates that our expressed CaM is properly folded and is capable of changing its conformation in solution state. In addition, the identity and integrity of our purified CaM are also independently confirmed by the Western blot analysis utilizing rabbit-raised

monoclonal calmodulin primary antibody and goat-raised antibody against rabbit immunoglobulin G conjugated to alkaline phosphatase (IgG-AP).

[Surface preparation and patterning] CaM was immobilized on the chemical patterns created on the OTS film. The OTS film was prepared according to the following procedures. The silicon wafer was boiled for 20 min in the piranha solution (1 part of 30% hydrogen peroxide solution in 3 parts of 98% sulfuric acid) at 125 °C followed by rinsing in DI water and drying in a carbon dioxide (CO₂) jet, which generated a clean silicon wafer surface. Then the wafer was incubated in a 5 mM OTS toluene solution for overnight. A monolayer of closely packed OTS film on the clean silicon surface formed through the self-assembly process of OTS silane molecules. The wafer with OTS film was then rinsed with toluene and stabilized in a sealed vial with 100% relative humidity at 40 °C. This series of steps was repeated in 3 cycles with the final step of rinsing and drying in a stream of N₂.²²

The carboxylic acid-terminated chemical templates (OTS_{Spd}, partially degraded OTS) on the OTS film were fabricated using scanning probe deep oxidation lithography in a 100% relative humidity environment, as shown in Figure 1a. Contact mode AFM was utilized with Pt–Ti coated conducting AFM tip for pattern fabrication. The bias of 10 V was applied to the silicon wafer sample. The OTS film under the tip was electrochemically oxidized, producing the partially degraded, carboxylic acid-terminated surface (OTS_{Spd}). The detailed description of pattern fabrication and characterization have been published elsewhere.^{16, 22}

The wafer with OTS_{Spd} pattern was incubated in 10% hydrochloric acid for 10 min and then cleaned with a CO₂ snow jet. As a result of these procedures, possible electrostatic charges and contaminants were completely removed.

To convert the carboxylic acid-terminated OTS_{Spd} surface pattern to mercapto-terminated pattern, the OTS_{Spd} pattern was incubated in the 5 mM 11-mercaptoundecyltrimetoxysilane (MUTMS) toluene solution for 30 min. The MUTMS forms an additional layer of silane on top of the OTS_{Spd} pattern, as illustrated in Figure 1b. Further control experiments show that the OTS film, OTS_{Spd} pattern and OTS_{Spd}MUTMS bi-layer silane pattern did change their heights upon incubation in buffer (20 mM HEPES, 2 mM EGTA, pH 8) or CPZ (20 mM HEPES, 25 mM CPZ, pH 8) solutions or during the immobilization process..

[Protein immobilization and assay] The dumbbell-shaped, genetically engineered CaM with cysteine on the N-terminus was immobilized on a mercapto-terminated surface pattern through cysteine-mercapto coupling reaction, as shown in Figure 1c. Mercapto-terminated surface pattern was soaked for 1 h in 10⁻⁷ M CaM buffer solution (20 mM HEPES, 0.5 mM CaCl₂, pH 8) at room temperature. Then the sample was swabbed with a piece of ChemWipe paper. Results from our group showed that with a ~5N force, the non-specifically adsorbed protein on OTS surface could be completely removed, whereas the specifically adsorbed protein remained on the surface template was intact.²²

[CaM conformational changes] The immobilized CaM was induced to undergo two types of conformational change. In the first test, the immobilized CaM was incubated with in EGTA containing buffer (20 mM HEPES, 2 mM EGTA, pH 8) at room temperature for 1 h to remove the calcium ion from CaM, inducing CaM (Figure 1c) changed to Apo-CaM conformation (Figure 1d).

In the second test, the immobilized CaM (Figure 1c) was soaked for 1 h in CPZ containing buffer (20 mM HEPES, 25 mM CPZ, pH 8) at room temperature, leading the CaM to change to the bound conformation (Figure 1e).

Results

Our experimental scheme for protein immobilization and reactions is illustrated in Figures 1. Scanning probe deep oxidation lithography was used to fabricate OTSpd patterns on a hydrophobic, methyl-terminated OTS film in a 100% relative humidity environment (Fig. 1a). In a humid environment, when a conducting AFM probe contacts OTS surface, a water meniscus forms around the AFM tip. Once 10V bias is applied to the OTS surface, the water is electrolyzed. The active oxygen species generated during water electrolysis oxidize the OTS surface under the tip, generating a carboxylic acid-terminated surface that is 1.02 ± 0.07 nm below the OTS surface.¹⁶ This surface is called OTSpd, since it is the partially degraded OTS. A stationary conducting AFM probe generated a disk-shaped OTSpd while a slow moving tip generated line patterns.

After the OTSpd pattern was fabricated, the carboxylic acid-terminated OTSpd surface was converted to mercapto-terminated surface using the constructive lithography, as shown in Fig. 1b.²³⁻²⁵ The OTSpd surface is a hydrophilic surface whereas the OTS background is hydrophobic. Once this sample was incubated in the toluene solution of MUTMS, the MUTMS molecules reacted with the trace amount of water in the solution, forming silanols in the first step. Then the silanols cross-linked and selectively anchored on the hydrophilic OTSpd surface, adding an additional mercapto-terminated silane layer. Next, the MUTMS pattern was used to immobilize cysteine-mutated CaM through the Hg^{2+} coupling (Fig. 1c). AFM characterizations showed that the MUTMS pattern was 1.08 ± 0.18 nm above OTS background. After this immobilization procedure, the height of patterns changed to 2.95 ± 0.06 nm above the OTS background, indicating new materials were immobilized on the chemical pattern. In order to confirm that the immobilized material was cysteine-mutated CaM, the immobilized CaM was labeled with fluorescent probe 2,6-ANS. Next, we characterized this ANS-labeled CaM pattern using both AFM and fluorescence microscopy. The immobilization of CaM on the chemical pattern was confirmed by comparing the AFM and fluorescence results, as shown in Figure. 2. In the AFM topography image in Fig. 2a, the immobilized material that was higher than the OTS was rendered as dark spot. Fig. 2b shows the fluorescence image of the same region on the sample. The image was rendered in the blue-green color scale, in which the blue represents the no fluorescence whereas the green represents fluorescence signal emitting from the ANS-labeled calmodulin. The AFM topography image in Fig. 2a was overlaid on the optical fluorescence image in Fig. 2b. The combined image in Fig. 2c demonstrates that the AFM topography and the corresponding fluorescence image match exactly. Therefore, the materials immobilized on the chemical pattern were calmodulin. Thus, Figure 2 indicates that our immobilization method can anchor cysteine-mutated calmodulin to the SH-terminated surface through the Hg^{2+} coupling.

The viability of immobilized CaM was confirmed by the fluorescence microscopy. We fabricated an immobilized CaM array on the OTS film, which has the structure shown in Figure 1c. The immobilized CaM pattern was then labeled with 2,6-ANS. After rinsing with 10 mM Tris buffer and DI water, we acquired the fluorescence image of the immobilized CaM array under the ultrapure nitrogen environment. Next, the sample was incubated in 10 mM EGTA solution for 15 min, followed by rinsing in DI water for three times. Since EGTA removed Ca^{2+} from the immobilized CaM, the active immobilized CaM would change its conformation from the dumbbell shape (Fig. 1c) to the bound form ApoCaM (Fig. 1d) and released the 2,6-ANS, leading to the decrease of the fluorescence intensity.

Figure 3 shows the fluorescence images of an immobilized CaM pattern before (Fig. 3a) and after (Fig. 3b) the EGTA treatment, which were acquired using the same setting and rendered in the same X-Y scale and intensity scale. Control experiments show that under the

ultrapure nitrogen environment, the photo-bleaching effect is negligible. The fluorescence intensity of the immobilized CaM (Fig. 3a) is 59 counts whereas the intensity in Fig. 3b drops to 33 counts after EGTA treatment. The decrease in fluorescence intensity after the EGTA treatment indicates that the immobilized CaM can change its conformation, confirming the activity of the immobilized protein.

In summary, the complimentary AFM and fluorescence studies demonstrate that the 1-cysteine mutated CaM can be immobilized on chemical patterns through the cysteine-Hg-S linking. The immobilized CaM maintains its activity and can undergo conformational changes.

Next, the calmodulin immobilization process, its subsequent reactions with EGTA, and CPZ were characterized using AFM. Figure 4 shows a series of representative AFM topography images of MUTMS pattern, immobilized CaM on MUTMS pattern, the Apo-CaM pattern, and the CPZ-bound CaM pattern, respectively. The disk in Fig. 4a1 is the MUTMS silane layer self-assembled on top of the OTSpd pattern. Fig. 4a2 is the corresponding height histogram. The peak-fitting program in MicroCal Origin was used to fit the height histogram into two Gaussian-shaped peaks. The distance between these two peaks is the height of the disk pattern in Fig. 4a1, which is 1.09 nm. Since the OTSpd pattern is 1.02 ± 0.07 nm below the OTS background, the actual thickness of the MUTMS silane layer can be calculated as 2.11 nm, which is about the same as the full length of the MUTMS silane molecules. This film thickness indicates that the MUTMS silane molecules stand perpendicularly on the OTSpd pattern.

Figure 4b1 is a representative image of the monolayer calmodulin immobilized on the chemical pattern. The topography images show that the immobilized calmodulin existed as a monolayer on the chemical patterns in most cases. No crystalline lattice structure was observed inside the calmodulin layer. The corresponding histogram in Fig 4b2 indicates that the calmodulin disk is 3.06 nm above OTS background for this image.

Figure 4c1 shows a representative immobilized calmodulin layer that was incubated in EGTA solution for 1 hour and rinsed with 5 mM Tris buffer. The EGTA solution removed the bound Ca^{2+} from the immobilized calmodulin, causing the calmodulin changed its conformation from the dumbbell shape to the bound conformation Apo-CaM.

The histogram in Figure 4c2 indicates that the Apo-CaM disk is 3.33 nm above OTS background. Similarly, Figure 4d1 is the calmodulin pattern treated with CPZ. The histogram reveals that the height of the CPZ-CaM disk in Fig. 4d1 is 3.34 nm above OTS background.

The images and heights of patterns shown in Figure 4 are only the representative values. MUTMS, CaM, Apo-CaM and CPZ-CaM were also characterized for different samples to obtain better statistical results. Using the same approach described above, the heights of these patterns were obtained on different samples. The final results are summarized in Table 1.

From Table 1, the calculated height of the immobilized CaM is 1.87 ± 0.19 nm, whereas the height of immobilized Apo-CaM is 2.26 ± 0.21 nm. Incubating immobilized CaM pattern in EGTA solution removed the Ca^{2+} bound to calmodulin, which caused the calmodulin conformation changed from the dumbbell shape to elongated globular shape. This conformational change corresponded to a 0.39 ± 0.13 nm increase in thickness of the immobilized calmodulin layer.

CPZ is a known antagonist of calcium bound calmodulin. CPZ binds to calmodulin and can induce calmodulin conformational change as well. The result in Table 1 reveals that the CPZ binding causes the height of calmodulin layer to increase from 1.87 ± 0.19 nm to 2.32 ± 0.20 nm.

In summary, the immobilized CaM changed its height upon the reactions of EGTA and CPZ. Although the measured height of Apo-CaM and CPZ-CaM are similar to the 2.5 nm dimension of CaM in the solution state ($6.7 \times 4 \times 2.5$ nm³), this similarity is just a coincidence. Control experiments and fluorescence microscopy have confirmed the identities immobilized Apo-CaM and CPZ-CaM. The observed height change reflected the conformation change of the immobilized CaM.

Discussion

The immobilized CaM was anchored on the surface through the Hg-Cysteine coupling. The height of 1.87 ± 0.19 nm is computed from the apparent height difference between the MUTMS pattern and the immobilized CaM on MUTMS pattern. Thus, this value refers to the height of the Hg-bound CaM.

The fluorescence microscopy study confirmed that the immobilized CaM could change to Apo-CaM. The complementary AFM characterization reveals that this conformational change corresponds to a height change from 1.87 ± 0.19 nm to 2.26 ± 0.21 nm.

[Analysis of the hydration on the exposed protein surface] The CaM to Apo-CaM conformational change also altered the amino acid residues composition of exposed protein surface, which affects the water molecules distribution on the protein surface. Different surface hydration states might lead to different measured protein dimensions. We employed the AC mode AFM phase signal to quantify the hydration state of CaM. The phase signal reflects the energy dissipation during the tapping of an oscillation tip. In our imaging condition, *i.e.* humid air environment, the hydrophilicity determines the energy dissipation. For a more hydrophilic surface, more water molecules adsorb on the surface. Therefore, when a tip taps on such surface, more energy is dissipated to overcome the surface tension and capillary forces between the tip and the surface. Therefore, a hydrophilic surface yields a high phase signal (larger phase lag angle). Theoretically, the phase signal φ (in term of phase lag) is expressed by²⁶:

$$\sin \varphi = \frac{A}{A_0} - \frac{Q}{\pi k A A_0} E \quad (1)$$

where A and A_0 are the AFM tip's set oscillating amplitude and free oscillating amplitude, Q and k are the quality factor and the spring constant of the tip, and E is the energy dissipation, which reflects the surface hydrophilicity. The equation shows that the phase signal is also a function of the imaging parameters (A and A_0) and the tip conditions (Q and k). Thus, the value of the phase signal can not be directly used to assess the surface hydrophilicity (E in the equation). We use the chemical patterns to overcome this difficulty. We fabricated chemical patterns on surface and deliberately imaged the protein surface and different chemical pattern in the same image. Here, the chemical patterns serve as an internal reference to quantify the surface hydrophilicity.

As shown in Figure 5, under 100% RH humidity (at 25°C), we imaged a CaM and an Apo-CaM disk pattern with the same tip in the AC mode, respectively. The protein films only partially covered the MUTMS disks. The images in Figure 5 show the surfaces of OTS, MUTMS and protein in the same time. In order to characterize the hydrophilicity of the

protein surface, we measured the average phase signals of the OTS surface, MUTMS surface, CaM surface and Apo-CaM surface in the corresponding phase channel images of Fig. 5a and 5b. These values are listed in Table 2.

We use the surface hydrophilicity factor H to quantify the hydrophilicity of the protein surface. The hydrophilicity factor H is defined as:

$$H \equiv \frac{\varphi_{\text{protein}} - \varphi_{\text{OTS}}}{\varphi_{\text{MUTMS}} - \varphi_{\text{OTS}}}.$$

When φ is smaller than 10° , $\varphi \approx \sin \varphi$.

Thus, based on equation (1),

$$H \equiv \frac{\sin \varphi_{\text{protein}} - \sin \varphi_{\text{OTS}}}{\sin \varphi_{\text{MUTMS}} - \sin \varphi_{\text{OTS}}} = \frac{\left(\frac{A}{A_0} - \frac{Q}{\pi k A A_0} E_{\text{protein}}\right) - \left(\frac{A}{A_0} - \frac{Q}{\pi k A A_0} E_{\text{OTS}}\right)}{\left(\frac{A}{A_0} - \frac{Q}{\pi k A A_0} E_{\text{MUTMS}}\right) - \left(\frac{A}{A_0} - \frac{Q}{\pi k A A_0} E_{\text{OTS}}\right)} = \frac{E_{\text{protein}} - E_{\text{OTS}}}{E_{\text{MUTMS}} - E_{\text{OTS}}}$$

Therefore, the H factor of a protein surface is independent of tip status and imaging parameters. It is only a function of the energy dissipation of OTS, MUTMS and protein surface. Here, since the surfaces of OTS (methyl group-terminated) and MUTMS (-SH group terminated) are stable, well ordered, and have the same roughness and packing density, $E_{\text{MUTMS}} - E_{\text{OTS}}$ can be used as a standard. Hence, using the known chemical patterns as standard, we can measure the phase signals over unknown surfaces (such as protein surfaces) and calculate the H factor, which is an indication of the protein hydrophilicity (E_{protein}). Using the measured values in Table 2, we get $H_{\text{CaM}} = 1.16 \pm 0.02$ and $H_{\text{Apo-CaM}} = 1.10 \pm 0.06$. Furthermore, the relative change can be calculated as:

$$R = \frac{H_{\text{CaM}}}{H_{\text{Apo-CaM}}} = \frac{E_{\text{CaM}} - E_{\text{OTS}}}{E_{\text{Apo-CaM}} - E_{\text{OTS}}} = 1.05 \pm 0.06.$$

The R value reveals that the surface hydrophilicities of immobilized CaM and Apo-CaM do not have statistically significant difference. Therefore, the observed height change for the CaM/Apo-CaM transition (1.87 ± 0.19 nm to 2.26 ± 0.21 nm) is not from the difference in water adsorption on the protein surfaces.

[Possible orientations of the adsorbed protein] The solution state CaM has a dimension of $6.7 \times 4 \times 2.5$ nm³ (measured from the calmodulin structure 2V02), whereas the solution state Apo-CaM has a dimension of $5.7 \times 3 \times 3$ nm³ (measured from the calmodulin structure 1CFC).^{11, 27} If the CaM stands upright on the surface (the “handle” of the dumbbell is vertical), then the immobilized CaM to immobilized Apo-CaM conformation change would lead to a decrease in height. In addition, the observed thickness of the immobilized protein film would be around 6 nm. Nevertheless, this orientation is inconsistent with the AFM height measurements in this study. The observed protein heights for CaM (1.87 ± 0.19 nm) and Apo-CaM (2.26 ± 0.21 nm) are significantly different from the expected height for the standing-up orientation. Furthermore, AFM shows that the CaM to Apo-CaM conformational change corresponds to an increase in height. Therefore, instead of the standing-up orientation, the laying-down configuration would be the possible adsorption orientation for CaM where the “handle” of the dumbbell is parallel to the surface. When the dumbbell-shaped immobilized CaM changes its conformation to the bound shaped Apo-CaM or CPZ-CaM, the respective crystal structures reveal that the “handle” of the dumbbell

disappears and the two globular domains come together, making the protein molecules shorter in long axis direction and bigger in the other dimension. In the laying-down orientation, such conformational change corresponds to an increase in the apparent heights of the protein, which is consistent with our observation.

Our observed AFM images show that the immobilized protein film is not a 2-D crystal. The observed heights of protein molecules have a statistical distribution, as shown in Figure 4. Hence, within the film, other orientations are also likely to occur. Our data only indicate that the laying-down orientation is the most probable adsorption orientation under our immobilization conditions.

Previously, Ando group immobilized biotinylated calmodulin on a crystalline streptavidin surface.²⁸ The height of the immobilized Apo-CaM was reported as 1.9 ± 0.3 nm, which is lower than our result of 2.26 ± 0.21 nm. However, the height of CaM was reported as 2.6 ± 0.4 nm, which is higher than our result (1.87 ± 0.19 nm). More importantly, our study shows that immobilized CaM is lower than Apo-CaM, whereas Ando group's result is the opposite. We notice that Ando group's result was measured from the single CaM molecule on the surface and was obtained in the buffer solution. In contrast, our result reflected the height of the CaM film, which was obtained in humid air. In the case of Ando group, a single CaM was immobilized on the surface. The CaM molecule had vast empty space around. In solution, such CaM molecule can rotate, wobble, or stand up freely. The observed height would be an average height corresponding to these possible orientations. In contrast, in our case, CaM existed as a film. Each immobilized CaM molecule is embraced by neighboring immobilized CaM molecules. Furthermore, the immobilized CaM was imaged in humid air environment, which maintains the protein surface fully hydrated and limits the motions of the flexible parts within the protein molecules. It is well known that protein imaged in humid air usually has less mobility and flexibility, which leading to a higher image resolution and better measurement accuracy.^{29, 30} In our case, since motions (such as standing-up or wobbling) of the immobilized CaM molecules were suppressed, most CaM molecules still remained as the flat laying orientation during imaging, which might be the reason for the difference in the observed CaM height.

The protein immobilization method requires a terminal cysteine group to anchor it to the SH-terminated surface. Most protein can be prepared through the established procedure to engineer a terminal cysteine group. Therefore, our method would be helpful research and assay works evolving engineered proteins.

Conclusion

Our results show that immobilization of CaM on a solid support does not interfere with the ability of the protein to bind calcium attaining its active dumbbell shaped conformation. Moreover, immobilized CaM retains its activity that is proved by its conformational change when binding CPZ. We found that the dumbbell-shaped CaM adsorbed on the surface with its "handle" parallel to the surface. Upon conformational change to the bound shaped Apo-CaM or CPZ-bound CaM, the apparent height of CaM molecule increases.

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References

1. Means AR, Dedman JR. *Nature*. 1980; 285:73. [PubMed: 6990273]
2. Klee CB, Crouch TH, Richman PG. *Annu. Rev. Biochem.* 1980; 49:489. [PubMed: 6250447]
3. Cheung WY. *Science*. 1980; 207:19. [PubMed: 6243188]
4. Babu YS, Bugg CE, Cook WJ. *J. Mol. Biol.* 1988; 204:191. [PubMed: 3145979]
5. Babu YS, Sack JS, Greenhough TJ, Bugg CE, Means AR, Cook WJ. *Nature*. 1985; 315:37. [PubMed: 3990807]
6. Jurado LA, Chockalingam PS, Jarrett HW. *Physio. Rev.* 1999; 79:661.
7. Chin D, Means AR. *Trends in Cell Biology*. 2000; 10:322. [PubMed: 10884684]
8. Swindells MB, Ikura M. *Nat. Struc. Bio.* 1996; 3:501.
9. Osawa M, Swindells MB, Tanikawa J, Tanaka T, Mase T, Furuya T, Ikura M. *J. Mol. Biol.* 1998; 276:165. [PubMed: 9514729]
10. Crivici A, Ikura M. *Ann. Rev. Biophys. Biomolecular Struc.* 1995; 24:85.
11. Kuboniwa H, Tjandra N, Grzesiek S, Ren H, Klee CB, Bax A. *Nat. Struc. Bio.* 1995; 2:768.
12. Lin PC, Weinrich D, Waldmann H. *Macromolecular Chem. Phys.* 2010; 211:136.
13. Labib M, Hedstrom M, Amin M, Mattiasson B. *Analytica Chimica Acta*. 2010; 659:194. [PubMed: 20103124]
14. Eguilaz M, Agui L, Yanez-Sedeno P, Pingarron JM. *J. Electroanalytical Chem.* 2010; 644:30.
15. Sugimoto Y, Pou P, Abe M, Jelinek P, Perez R, Morita S, Custance O. *Nature*. 2007; 446:64. [PubMed: 17330040]
16. Cai YG. *Langmuir*. 2009; 25:5594. [PubMed: 19432492]
17. Horcas I, Fernandez R, Gomez-Rodriguez JM, Colchero J, Gomez-Herrero J, Baro AM. *Rev. Sci. Instrum.* 2007; 78:013705. [PubMed: 17503926]
18. [May 2, 2011] Procedure: Purification and expression of genetically engineered calmodulin with cysteine on n-terminus..
http://wolfson.huji.ac.il/purification/PDF/Expression_Systems/NEB_Impact_TWAIN_Manual.pdf
19. Sharma B, Deo SK, Bachas LG, Daunert S. *Bioconjugate Chem.* 2005; 16:1257.
20. Schonbrunn E, Eschenburg S, Luger K, Kabsch W, Amrhein N. *Proc. Natl. Acad. Sci. U.S.A.* 2000; 97:6345. [PubMed: 10823915]
21. Laporte DC, Wierman BM, Storm DR. *Biochemistry*. 1980; 19:3814. [PubMed: 6250577]
22. Gao P, Cai Y. *Ultramicroscopy*. 2009; 109:1023. [PubMed: 19345502]
23. Maoz R, Frydman E, Cohen SR, Sagiv J. *Adv. Mater.* 2000; 12:725.
24. Maoz R, Frydman E, Cohen SR, Sagiv J. *Adv. Mater.* 2000; 12:424.
25. Zeira A, Chowdhury D, Hoeppe S, Liu S, Berson J, Cohen SR, Maoz R, Sagiv J. *Langmuir*. 2009; 25:13984. [PubMed: 19835384]
26. Anczykowski B, Gotsmann B, Fuchs H, Cleveland JP, Elings VB. *Appl. Surf. Sci.* 1999; 140:376.
27. Kursula P, Majava V. *Acta Crystallographica Section F-Structural Biology and Crystallization Communications*. 2007; 63:653.
28. Yamamoto D, Nagura N, Omote S, Taniguchi M, Ando T. *Biophys. J.* 2009; 97:2358. [PubMed: 19843468]
29. Chen CH, Clegg DO, Hansma HG. *Biochemistry*. 1998; 37:8262. [PubMed: 9609723]
30. Yermolenko IS, Lishko VK, Ugarova TP, Magonov SN. *Biomacromolecules*. 2011; 12:370. [PubMed: 21192636]

Figure 1.

The experimental scheme. a) OTS_{pd} pattern fabrication. b) Converting the OTS_{pd} pattern into mercapto-terminated group by assembling an additional layer of MUTMS silane on top of the OTS_{pd} pattern. c) Immobilization of CaM on the MUTMS pattern through cysteine-Hg-SH coupling. d) Reacting with EGTA, the Ca²⁺ bound to CaM was removed, which induced the immobilized CaM to undergo conformational changes from the dump-bell shape to bound conformation ApoCaM. e) The immobilized CaM in c) changed its conformation upon incubation in CPZ solution.

Figure 2.

Image comparison of pattern with immobilized 2,6-ANS-labelled CaM probed with AFM and fluorescent microscope: a) AFM image (topography, AC mode), b) Fluorescent microscope image, c) AFM-fluorescence combined image.

Figure 3.

The fluorescence image of an immobilized ANS- labeled-CaM array before (a) and after (b) EGTA incubation. The two fluorescence images were acquired using the same exposure time (135 sec) and rendered in the same X-Y and intensity scale. After EGTA incubation, the immobilized CaM changed its conformation from the dumbbell-shape to the bound shaped Apo-CaM. The fluorescence intensity of the array dropped from 59 counts to 33 counts.

Figure 4.

Representative AFM topography images of chemical pattern and protein patterns on the chemical pattern template. The four topography images are rendered in the same 10 nm height scale. a1) The MUTMS silane pattern self-assembled on the OTSpd pattern. The disk pattern has an -SH terminal functional group. b1) The Ca^{2+} bound dumbbell shape calmodulin (CaM) immobilized on the MUTMS pattern through the cys-Hg-S coupling. c1) The apo-calmodulin (Apo-CaM) pattern. d1) The calmodulin pattern treated with CPZ (CPZ-CaM). a2, b2, c2 and d2 are the histograms corresponding to a1, b1, c1, and d1. The distance between the two peaks in the histogram specifies the height of the disk pattern in the image.

Figure 5. Partially covered protein patterns that reveal different surfaces. AC mode topography images. a) A partially covered immobilized CaM layer on the MUTMS pattern. b) A partially covered immobilized Apo-CaM layer on the MUTMS pattern.

Table 1

The Height of Surface Patterns

	Apparent Height above OTS	N
MUTMS	1.08 ± 0.18 nm	30
CaM	2.95 ± 0.06 nm	18
Apo-CaM	3.34 ± 0.11 nm	15
CPZ-CaM	3.41 ± 0.09 nm	15

Table 2

The Phase signals and hydrophilicity factors (H) of the protein surfaces in Figure 5.

	Sample a in Figure 5 (CaM)			Sample b in Figure 5 (Apo-CaM)		
	Phase Signal		H	Phase Signal		H
	mV	Degree		mV	Degree	
OTS	0 ± 10	0.00 ± 0.09	-	4 ± 7	0.00 ± 0.06	-
MUTMS	431 ± 4	3.88 ± 0.04	-	127 ± 8	1.14 ± 0.07	-
CaM	498 ± 9	4.48 ± 0.08	1.16 ± 0.02	-	-	-
Apo-CaM	-	-	-	139 ± 8	1.25 ± 0.07	1.10 ± 0.06