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Dynamics of a partially stretched protein molecule studied using an atomic force microscope

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Abstract

The dynamics of a single protein molecule subjected to forced mechanical unfolding was investigated in a millisecond time domain using a custom-made atomic force microscope (AFM) apparatus, which allows simultaneous measurements of an average tensile force applied to a single molecule and its mechanical response with respect to an external oscillation. Our target protein was genetically engineered bovine carbonic anhydrase II (BCA) which is a monomeric globular protein, and it has been shown that the as-expressed BCA from *Escherichia coli* contains two conformational isomers, one with enzymatic activity (type I) and the other without (type II). An interesting feature observed from the dynamic measurements was that when the type I BCA conformer was extended, it often exhibited a clear out-of-phase response against an external oscillation. The type II BCA conformer, however, always exhibited an in-phase response to the external oscillation. This relationship between different types of BCA and their dynamical behaviors was evidently observed around the discontinuous transition point from type I to II.

Keywords: Atomic force microscope; Force spectroscopy; Carbonic anhydrase; Mechanical unfolding; Dynamics; Viscoelasticity

1. Introduction

When a single protein molecule with a native conformation is stretched from two well-defined termini, the molecule is uniaxially deformed first and then exhibits a 'mechanical unfolding'. The experimental techniques called force spectroscopy using an atomic force microscope (AFM) [1] have proven to be suitable methods for investigating the phase transitions of biological macromolecules such as proteins [2–10] and DNA [11] at the single molecular level. In a conventional force spectroscopy experiment, a sample molecule is stretched at a constant velocity, and the applied tensile force is recorded as a function of the extension length. The resulting force–extension (F–E) curves provide new information about the unfolding mechanisms of proteins under mechanical deformation.

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Recently, a modified technique of force spectroscopy has been developed for measuring the dynamics of a single molecule such as proteins [12], synthesized polypeptides [13], synthesized polymers [14] and polysaccharides [15]. In this method, which is sometimes called nanorheology experiments, a sample stage [12,14,15] or a cantilever [13] is vibrated using an external oscillator at a constant amplitude, and the mechanical response of the molecules is monitored through the cantilever deflection as a function of the extension length. This method is analogous to that reported previously [16–18] for measuring the viscoelasticity of sample surfaces.

Dynamic experiments using dimeric bovine carbonic anhydrase II (BCA) [12] recorded an out-of-phase response against an external oscillation with a frequency of less than 1 Hz when the protein molecule was partially extended. It was demonstrated that the anomalous out-of-phase response could be reproduced using a model simulation in which the unfolding and refolding of the protein was set to take place repeatedly during the forced oscillation of the protein. However, little has been done to clarify the relationship between the static and dynamic behaviors of the protein and neither has it been shown whether the transitions of unfolding and refolding originated from intra- or inter-molecular forces.

These questions remained unsolved due to instrumental limitations for dynamic measurement. First, the amplitude of vibration applied to the sample stage was as large as 20 nm [12], so that only a large or tandemly repeated protein could be studied. Second, since simultaneous measurements of an ordinary force–extension curve and dynamic responses to oscillatory input were not possible, detailed correlation between them was not clarified. In order to solve these problems, we designed a new AFM for the investigation of the dynamics of a single protein molecule in the millisecond time domain and succeeded in measuring the mechanical response of a single molecule of genetically engineered BCA [19,20] under continuous vibration of the substrate whose amplitude was smaller and frequency much higher than in our previous report [12].

It has been known that this engineered BCA contains two conformational isomers, one called type I BCA with an enzymatic activity and the other without and called type II BCA [19,20]. Near- and far-UV circular dichroism [20] and X-ray crystallography [21] studies on the engineered BCA showed that the structure of type I BCA is identical to that of the native protein, while type II BCA had a slightly different near-UV spectrum. A distinct structural difference between type I and II BCAs is that the former has a complete knot structure whereas the latter has an incomplete one [20]. Thus, these protein samples are suitable for investigating not only the dynamics of the monomeric protein but also the relationship between the structures and the dynamics. As a result of the present nanorheology experiments, we found that an out-of-phase response was observed even for a monomeric BCA protein, indicating that the out-of-phase response was generated by intra-molecular interactions in a single molecule. Moreover, we found that the dynamic behaviors were different between type I and II BCA. In this paper, we present experimental results and discuss qualitatively the mechanism of the observed out-of-phase response in connection with the folding of BCA protein.

2. Experimental

2.1. Materials

The engineered BCA protein was constructed by addition of a cysteine residue at its N-terminal and replacement of Gln253 with a cysteine, and expressed in *Escherichia coli* and purified as previously reported [20]. Since native BCA contains no cysteine residues, the two genetically inserted cysteines were used for covalently cross-linking the protein to an AFM tip and the surface of a silicon substrate, both of which were treated with 3-aminopropyltriethoxysilane (APTES) to cover their surfaces with amino groups [9]. N-succinimidyl-3-[2-pyridyldithio]-propionate (SPDP) was used as the bifunctional cross-linker between the protein and the amino group of APTES [19,20].

Prior to AFM experiments, a solution containing 2–5 μg/ml mutant BCA in 50 mM Tris–H_2SO_4 buffer at pH 7.5 was incubated on a modified silicon substrate for 1 h at room temperature (~25 °C), and then the substrate was gently rinsed with the same buffer for the removal of non-crosslinked protein. Dynamic measurements were performed in the same buffer solution at room temperature.

Two types of commercially available silicon nitride cantilevers were used to verify the reproducibility of the present experiments. One type (NP-S, Digital Instruments, Santa Barbara, CA) had a nominal spring constant of 0.06 N/m and a resonance frequency of approximately 3 kHz in water and the other (OMCL-TR400PSA-1, Olympus Optical Co., Japan) had 0.08 N/m and ~8 kHz in water, respectively. Using the two kinds of cantilevers, we confirmed that there was no cantilever dependence in the results of our dynamic measurements in the range of 40–60 Hz. The spring constant of the cantilevers was calibrated using the thermal fluctuation method [22,23].

2.2. Dynamic measurements

Fig. 1 shows a schematic illustration of our modified AFM for dynamic measurement based on a commercially available SPM-9500 AFM (Shimadzu Ltd., Tokyo, Japan). The photo detector system including a preamplifier and a laser diode was upgraded and optimized for this study. As a newly designed feature of the AFM instrument, we employed two types of piezoelectric ceramic elements. One was a built-in tube scanner for controlling the substrate position with a homemade high-voltage booster. The other was a thin disk piezo with a diameter of 10 mm and a thickness of less than 2 mm driven by an external oscillator for vibrating the substrate. Employment of the two piezo elements allowed us to change the extension length of the sample protein under continuous oscillation and to prevent cross-talk between extension and oscillation of the sample.

Our procedures for the dynamic measurements were as follows. First, the substrate mounted on the disk piezo was continuously vibrated by the application of sinusoidal voltage at a fixed frequency (40–60 Hz) and with a constant amplitude (~5 nm). Force curve measurements were then carried out changing the substrate position at regular intervals of a few nanometers with a pulling speed of 20–30 nm/s, which may be slow compared with conventional force spectroscopy. Then, a time series of cantilever deflection signals were acquired at each distance together with the reference signal applied to the disk piezo by using an analog-to-digital converter (ADC) having a sampling rate of 1 MHz and a resolution of 14 bits. The tensile force was calculated as a function of the extension length by averaging the time series of the cantilever deflection at a corresponding tip–surface distance. The force measurements were conducted for a few tens of times at once, and all the acquired time series data were temporarily stacked on a computer buffer. Then, only those data leading to a substantial extension of the protein molecule were analyzed. The probability of obtaining such data was in the range of a few percent of the total measurements and it was expected that the force measurements were examined as single molecular events.

3. Results

3.1. Effect of non-contact forces on our AFM

Before investigating the dynamics of the BCA proteins, a preliminary experiment was carried out to clarify the effect of substrate vibration on the cantilever displacement. Fig. 2 shows the time series of cantilever deflection obtained in a buffer solution without protein molecules. When the vibrating substrate with 42 Hz was distanced from the cantilever at ~12 nm from the contact position, the cantilever deflection remained at a constant level not responding to the motion of the substrate (Fig. 2a and b), although not without random noises. This indicated that non-contact interactions between the tip and the surface such as hydrodynamic and/or van der Waals forces do not influence the motion of the cantilever if the tip was at least 6 nm away from the substrate surface at the closest position. In contrast, if the vibrating substrate was in contact with the cantilever, the cantilever displacement precisely followed the reference signal and no phase difference was

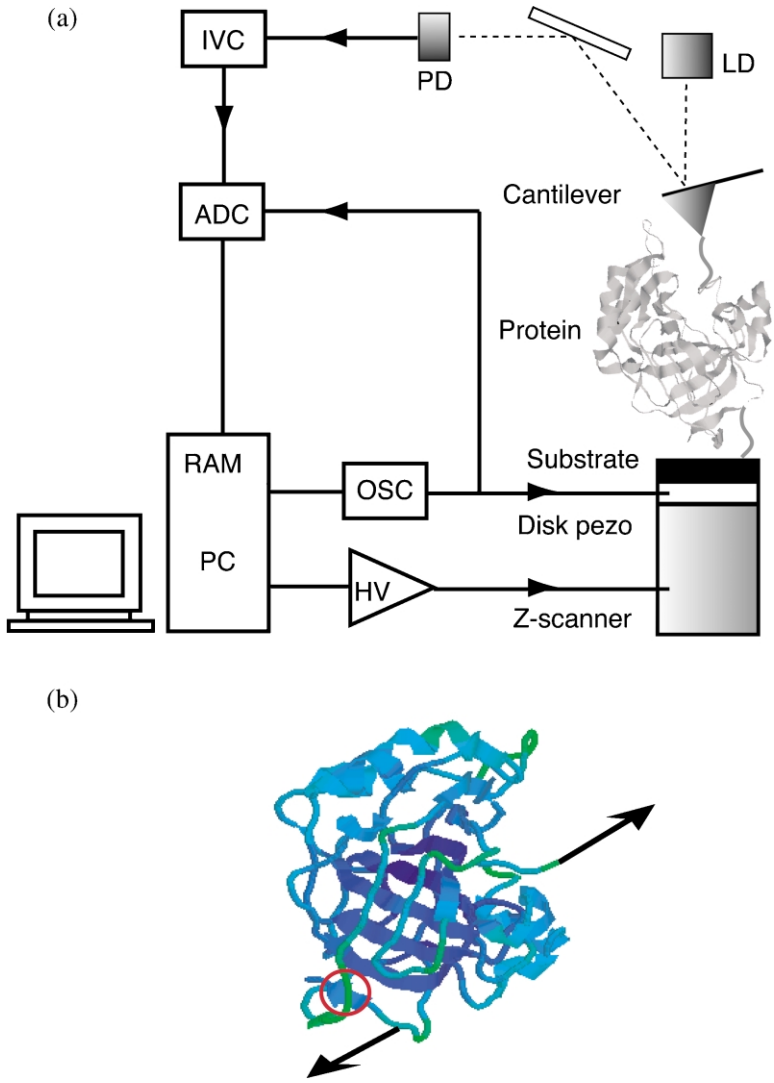

Fig. 1. (A) Schematic illustration of the AFM used for dynamic measurements in this work. A mutant BCA protein was covalently sandwiched between the silicon substrate and the AFM tip through an SPDP cross-linker under physiological conditions. (B) Pulling direction of a single carbonic anhydrase is indicated by arrows, and the knot structure is indicated by an open red circle, using a crystallographic structure of human carbonic anhydrase with a very similar to that of type I BCA.

observed between them (Fig. 2c), indicating that the relaxation of the cantilever deformation is much faster than the timescale of the reference signal.

If the vibration frequency of the substrate was increased to above ~100 Hz, the effect of non-contact force due to the solvent viscosity was apparently observed on the cantilever displacement. Also, the dynamic measurement with a low frequency required a long time, so that measurements often suffered from mechanical drifts of our AFM. Thus, the present dynamic measurements were conducted in the range of 40–60 Hz, and we concluded that the cantilever response measured

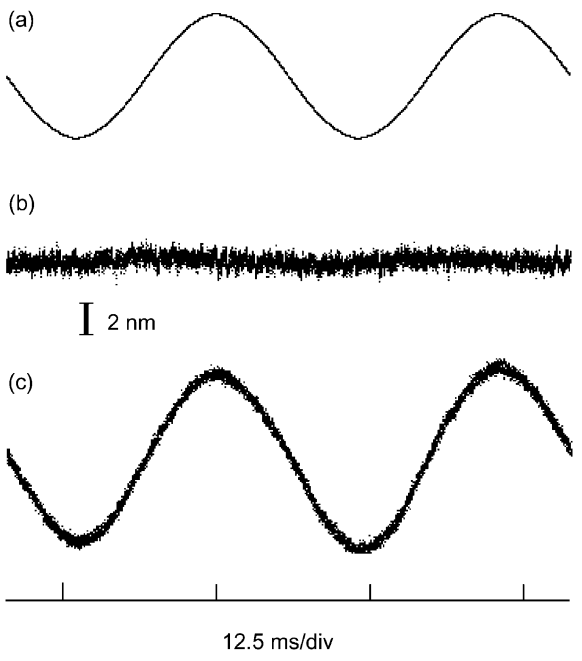

Fig. 2. Cantilever deflection in an aqueous buffer without a protein sample where both the tip of an NP-S cantilever and a silicon substrate were unmodified. (a) Reference signal applied to the disk piezo to vibrate the substrate with a frequency of 42 Hz and an amplitude of 12.5 nm_{p-p}. (b) Time series data of the cantilever deflection corresponding to the reference signal at a position where the substrate was separated from the equilibrium position of the cantilever at 12 nm. (c) Time series of the cantilever deflection corresponding to the reference signal at a position where the substrate was raised 12 nm beyond the point where the cantilever first made contact under the equilibrium state.

in the dynamic experiments described below could be attributed to changes in the dynamics of a protein molecule.

3.2. *F–E* curve and dynamics of BCA

In Fig. 3, two typical force—extension (*F–E*) curves of the mutant BCA protein observed in our AFM are shown. The upper curve had an extension length of ~60 nm before the final rupture of the cross-linking system at a force of ~1.2 nN, while the other had an extension length of ~85 nm, closer to the full extension length of 96 nm as estimated from a model where the length of each amino acid residue was assumed to be 0.37 nm [7]. In our previous experiments [19] using constant-speed force spectroscopy, we assigned force curves having extension lengths of ca. 60–70 nm and 90 nm to the type I and type II conformers, respectively. In our present AFM experiment, it was also confirmed that type I and II BCAs separated on an affinity chromatography column exhibited extension lengths of 50–70 nm and 80–100 nm at the final rupture, respectively, although the former was sometimes extended up to its full length, implying that the structures of some type I BCA molecules cross-linked with the substrate changed to type II during stretching [7]. A distinct difference of the *F–E* curves for type I and II BCAs mentioned above is that type I BCA protein has a mechanically rigid structure which is retained against the force required to break a covalent bond, whereas type II BCA protein has a more loose structure so that the mechanical unfolding is completed with a tensile force less than the force of a covalent bond.

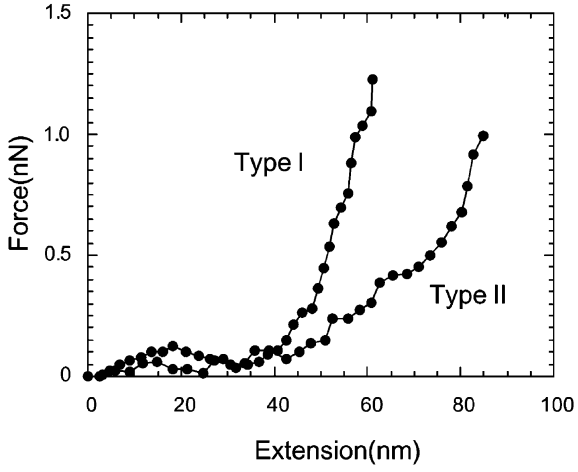

Fig. 3. Typical force–extension (*F–E*) curves of mutant BCA upon the extension process measured with an NP-S cantilever, in which the frequency and amplitude of the substrate vibration were 42 Hz and 8 nm_{p-p}, respectively. Dots represent average values of tensile force estimated from a time series of the cantilever deflection at corresponding distances. The neighboring dots are connected by solid lines.

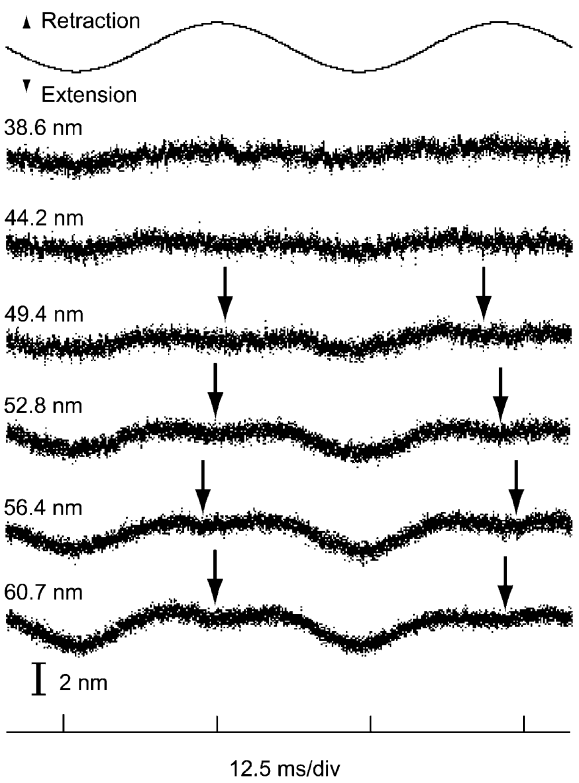

Fig. 4. Time series of the cantilever deflection at several extension lengths in the type I BCA curve shown in Fig. 3. The top sinusoidal curve represents the reference signal applied to the disk piezo. The up and down processes of the reference signal correspond to the retraction and extension of the molecule, i.e. the raising and lowering of the substrate, respectively. Arrows indicate positions where out-of-phase responses were clearly observed.

In Fig. 4, detailed time series data of the cantilever deflection for a type I conformer at several extension lengths are given. Beyond ca. 50 nm in extension, the cantilever amplitude became more pronounced with the extension length. This is because the tensile force increased non-linearly with the increasing extension length as seen in Fig. 3. An interesting feature in Fig. 4 is that the cantilever response upon the retracting process of the molecule is markedly different from the profile of the reference signal. Namely, the contraction of the protein in the climbing period of the substrate led to a plateau response or a substantial inversion of the cantilever deflection with respect to the substrate vibration, as indicated by the arrows on the time series data. We noticed that these profiles of the out-of-phase response were not completely symmetric but the position of the bottom of the concave curves indicated by the arrows shifted in each cycle of retraction. Namely, the dynamical traces of the retracted and extended type I BCA distributed with some variations even when the average extension length of the protein was fixed. As seen in Fig. 3, the tensile force increased monotonically in an extension range of $\sim$40–60 nm. Despite this observation, however, the time series of cantilever deflection in the same extension range exhibited an out-of-phase response, as shown in Fig. 4. Thus, we could not deduce this dynamic response from the $F-E$ curve obtained in the present experiment.

Fig. 5 shows detailed time series data of the cantilever deflection for the type II conformer at several extension lengths. The cantilever amplitude increased with increasing extension length, which is the same feature as in Fig. 4. Unlike the results given in Fig. 4, however, the profile of the tensile force in type II showed in-phase responses against the substrate vibration even under a large extension. Although a slight phase shift was occasionally observed, e.g. at 79.5 nm in extension, the origin of this is still unclear.

It should be noted that the clear-cut out-of-phase responses of the cantilever deflection shown in Fig. 4 were not always observed even for type I BCA in the transition zone to type II, but in-phase time series data were also obtained. The exact reason why the out-of-phase response did not appear unfailingly for the type I conformer is unknown, but we think that it may be due to that the dynamical properties of type I BCA are slightly different for each molecule. In contrast, type II BCA always exhibited in-phase responses as shown in Fig. 5.

3.3. Dynamics of BCA near the transition

Fig. 6 shows an example of successful outcomes, in which extension and retraction processes were recorded for an identical single molecule

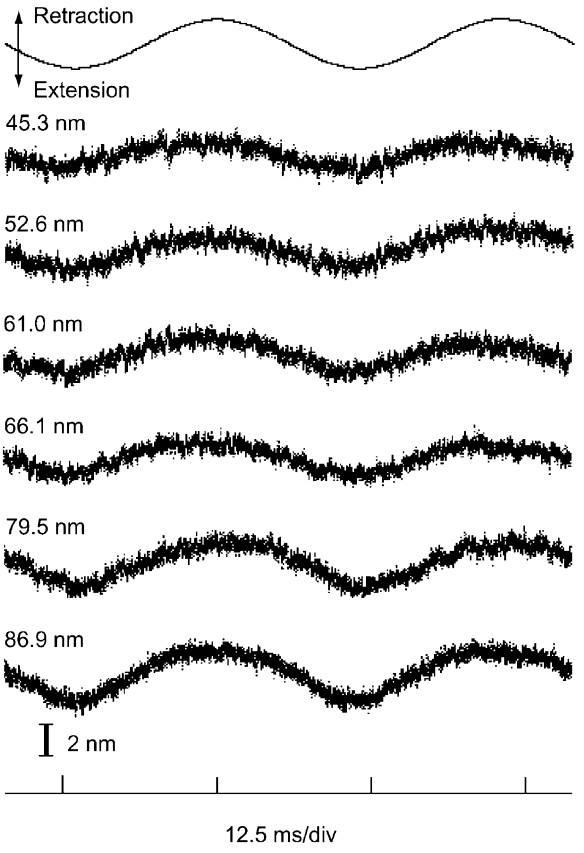

Fig. 5. Time series of the cantilever deflection at several extension lengths in the type II BCA curve shown in Fig. 3.

without breaking any covalent bond in the system. In the first round of the extension process, the tensile force increased non-linearly up to ca. 60 nm. Next, the curve showed a discontinuous break approximately 60 nm at a position marked #2 in the figure and jumped to the position marked as #3. After the breaking of the curve, the molecule was further extended following a different *F–E* curve up to ~90 nm and then the curve was reversed.

By comparing the above results with the curve in Fig. 3, we interpreted the observed jump in the extension process as a sudden transition of type I to a type II-like conformer. Such a discontinuous transition was observed in our previous study [20] using force spectroscopy with a constant-speed extension, but the retraction process was not studied. Interestingly, the dynamic behavior of the protein changed drastically before and after the transition. Before the transition, out-of-phase responses were clearly observed as indicated by the arrows on curves #1 and #2 in Fig. 7, whereas no such responses were observed just after the transition (#3) as well as at a more extended state (#4).

In the retraction process, the tensile force decreased monotonically as the extension length was decreased without back-transition to the type I curve and only in-phase responses were observed at all extensions studied (for instance, see #5 and #6 in Fig. 7). Since the extension lengths of #5 and #6 are the same as those of #1 and #2, respectively, the result strongly indicated that the

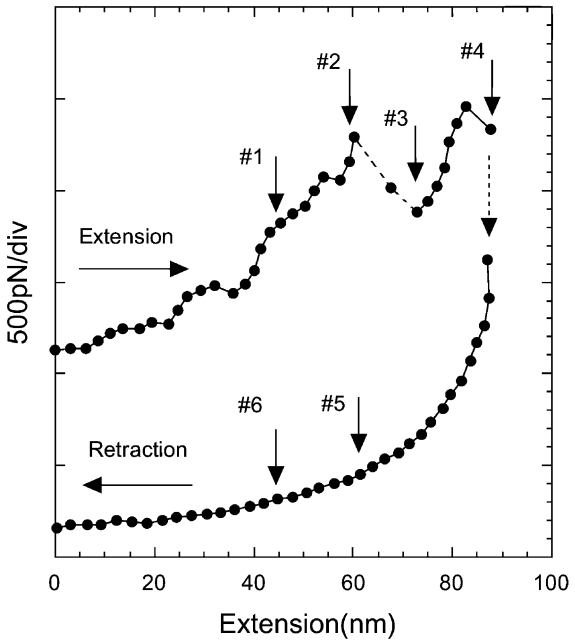

Fig. 6. *F–E* curves upon the extension and retraction processes in a single BCA protein measured with an NP-S cantilever. The frequency and amplitude of the substrate vibration were 58 Hz and 8.5 nm_{p-p}, respectively. A dot between #2 and #3 represents a position where a discontinuous change of the cantilever deflection occurred. The neighboring dots are connected by a solid or dotted line, and the extension and retraction curves are vertically shifted with an arbitrary regular distance.

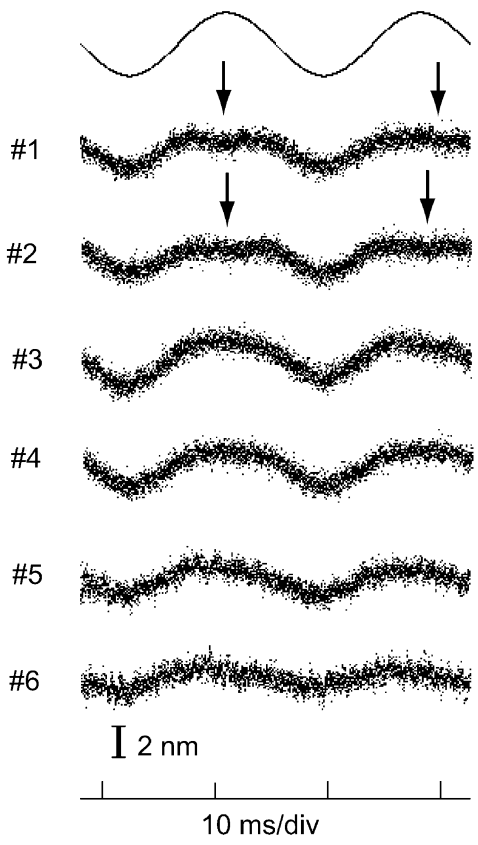

Fig. 7. Time series of the cantilever deflection at several extension lengths shown in Fig. 6. Numbers correspond to those in Fig. 6. Arrows indicate the positions where an out-of-phase response were clearly observed.

out-of-phase response observed for the type I conformer was a unique phenomenon for this particular conformer and not associated with a specific range of chain extension of other conformers or that of the tip–surface distance.

4. Discussion

Since the major feature of this article is the out-of-phase response of type I BCA against a sinusoidal perturbation during static stretching near the transition region from type I to II, we like to present a plausible model to explain the unusual observation. In Fig. 8a, a system consisting of an AFM and a molecule is illustrated schematically, in which the cantilever deflection, ΔD, exhibits an out-of-phase response against the motion of the substrate. When the substrate is lowered, the molecule sandwiched between the substrate and the AFM tip is stretched so that the extension length, L, increases and the cantilever is deformed so that the absolute value of ΔD, |ΔD|, increases due to the tensile force through the stretched molecule, showing an in-phase response to the motion of the substrate. Similarly, as the substrate starts to be raised from A to B, both L and |ΔD| decrease, again giving rise to an in-phase response of the cantilever deflection. However, in further raising of the substrate from B to C, |ΔD| begins to increase although L continues to decrease, showing an out-of-phase response. This means that a contractile force starts working within the protein molecule at this stage and consequently, L starts to decrease not only passively but also actively, i.e. the molecule 'excessively' shrinks. When the substrate turns to be lowered from C to D, |ΔD| starts to decrease despite the stretching of the molecule and |ΔD| changes to increase in further extensions from D to E. Since the response of |ΔD| is attributed to the dynamics of the molecule as shown in Fig. 2, we can say that an out-of-phase response corresponds to the emergence of an extra tensile force when the molecule is relaxed by the upward movement of the substrate, and an extra force originated from intra-molecular interaction of the molecule rather than a force induced externally. This extra force disappears as the once shrunken molecule starts to be stretched again.

In Fig. 8b, the curve P–Q–R represents a quasi-static force curve of the type I conformer, where the extension profile of the force curve has a feature revealing that ΔD monotonically changes with the substrate position, z, satisfying $d(\Delta D)/dz < 0$ [19,20]. As this profile cannot explain the contraction force mentioned above, we assumed transient pathways different from an equilibrium one. Such pathways must have two regions, in one region $d(\Delta D)/dz < 0$ is satisfied corresponding to an in-phase response (the dark gray regions in Fig. 8b) and in the other $d(\Delta D)/dz > 0$ is satisfied corresponding to an out-of-phase response (the light-gray regions in Fig. 8b).

In one of the proposed pathways, i.e. curve u_1–v_1 lying above the quasi-static force curve in the

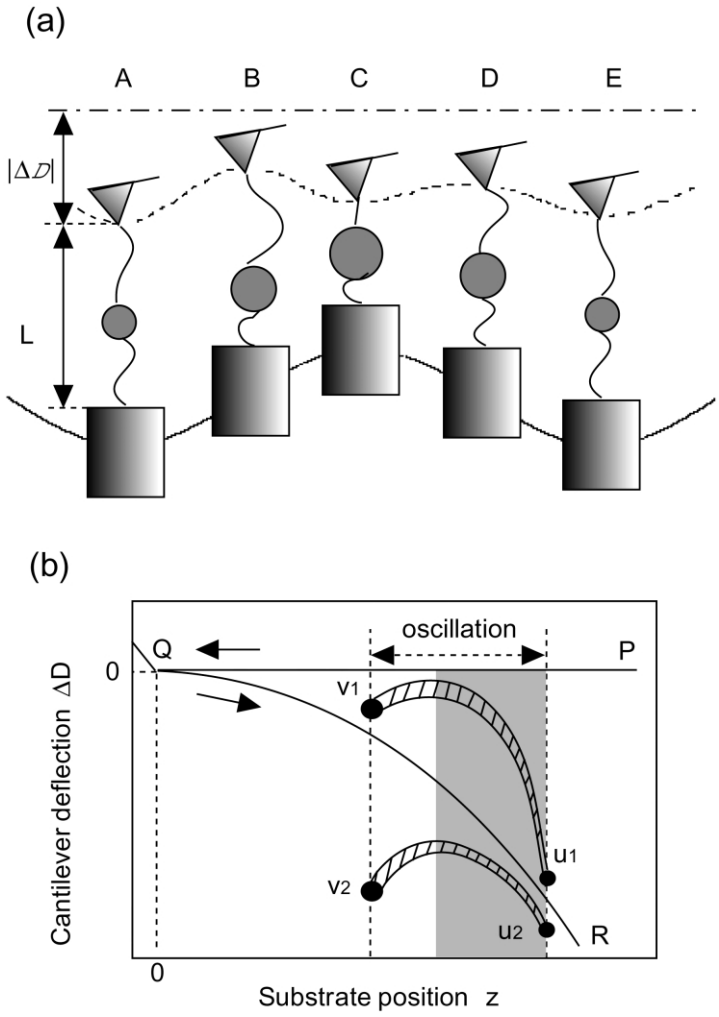

Fig. 8. (a) Schematic illustration of a system consisting of an AFM tip and a substrate with a single BCA protein molecule sandwiched between them exhibiting an out-of-phase response during dynamic measurement. ΔD represents the cantilever deflection, which was directly observable and L represents the extension length of the molecule, which was calculated from ΔD and the position of the substrate. A tentative model of the mechanical unfolding of the molecule is depicted in the figure. The dark circle in the molecule represents the hydrophobic structure of BCA protein, the size of which corresponds to the radius of the circle. The hydrophobic core partially refolds and unfolds following the retracting and extending processes of the piezo scanner, respectively (For details, see Section 4.) (b) Schematic illustration of the quasi-static force curve of BCA stretching together with two possible pathways to show an out-of-phase response of an oscillated BCA molecule. In the quasi-static force curve P–Q–R, the AFM tip approaches the surface from position P to Q, then makes contact at position Q with the sample. The protein molecule is then stretched upon the subsequent separation period of the tip from the sample from position Q to R. The two possible pathways are given, respectively, above and below the quasi-static force curve as curves u_1–v_1 and u_2–v_2. Shaded regions represent a distribution of the folding pathway of the molecule. Dark and light gray regions represent where an in-phase and out-of-phase responses of an oscillated molecule are expected, respectively.

center, when the molecule shrinks toward the position v_1, the value of the tensile force approaches that of the quasi-static force curve showing an out-of-phase response. This means that the molecule approached from a transient state toward an equilibrium state. In other words, the out-of-phase response reflects a delay in refolding of the protein in the retraction process. In the other pathway, i.e. curve u_2-v_2 lying below the quasi-static force curve, when the molecule is retracted to position v_2, where the value of the tensile force is different from that of the quasi-static force curve. This means that the shrunken molecule is likely to attain a new non-equilibrium state, i.e. the shrunken molecule is over-refolded or misfolded.

In both cases, the shrunken molecule at v_1 or v_2 tends to relax just when it starts to be extended, giving rise to an out-of-phase response during the extension process. This may be reasonable if the state at v_1 and/or v_2 is not an equilibrium state of the molecule but a transient one, because a transient state is not energetically stable so that the state (structures of the molecule) may be relaxed immediately when an external perturbation force is applied to extend the molecule.

Previous denaturation experiments of carbonic anhydrases induced by denaturants [24–26] or temperature [27] stated that much of the predominant β-structure forming a hydrophobic core remains almost intact until strong denaturing conditions are applied, whereas the peripheral parts of the structure becomes less ordered under milder denaturing conditions. Furthermore, the stability of the hydrophobic core appears to increase toward the center [24]. Thus, it is natural to expect that even in the mechanical unfolding, the peripheral part of carbonic anhydrase is destabilized first and then the hydrophobic core.

Moreover, Semisotnov et al. [24] argued that the formation of a non-compact tertiary structure of carbonic anhydrase was observed with a relaxation time of 30 ms in its early stage of refolding. Fresgård et al. [25] also observed that the refolding of the interior core formation was very rapid, compared with that of the structure around the C-terminal and the peripheral part and estimated that the formation of the hydrophobic core occurred within 0.1 s. Since the time scale in the present study is in the same order, we believe that the out-of-phase response observed in our present study corresponds to refolding (not misfolding) and unfolding of a partially unfolded core structure as shown in Fig. 8a through the pathway of the curve u_1-v_1 shown in Fig. 8b. A plausible explanation of the mechanism of the dynamic behaviors of BCA protein is that when a part of the denatured chain is refolded as the ordered hydrophobic core by shrinking the molecule (from B to C in Fig. 8a), the amount of segment consisting of unfolded random chain decreases, so that the tensile force due to the entropy elasticity increases. In contrast, when the ordered hydrophobic core is partially unfolded by extending the molecule (from C to D in Fig. 8a), the tensile force decreases because the random chain becomes longer in total.

5. Conclusions

We demonstrated in this paper a method of measuring the dynamic responses of a monomeric BCA protein against sinusoidal perturbations in the millisecond time domain. We found that the native-like conformer with an enzymatic activity, i.e. type I, often exhibited an out-of-phase response in the retraction regime. This shows that the out-of-phase response was generated by the intramolecular interaction in a single molecule. Moreover, such an out-of-phase response of type I BCA proteins was found to disappear just after they underwent a discontinuous transition into type II-like features in tensile force. From our discussion, it is suggested that the refolding of a partially unfolded region of BCA protein, which is probably related to the hydrophobic cluster, leads to the out-of-phase response.

Acknowledgments

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