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Force Measurement and Inhibitor Binding Assay of Monomer and Engineered Dimer of Bovine Carbonic Anhydrase B

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We applied atomic force microscopy (AFM) to study the intramolecular mechanics of the globular protein molecule, bovine carbonic anhydrase B. The immobilized protein on an amino-functionalized silicon wafer was pulled from its N- and C-termini after being covalently cross-linked to the AFM tip, and the relationship between the tensile force applied on the protein and its extension was recorded. The native enzyme (having 261 residues with two Cys added at its ends, and in a theoretical stretching length of 96 nm) was extended only to 13 ± 2 nm under physiological conditions before disruption of the covalent cross-linking system. Contrary to the above observation, an engineered dimer was extended to about 110 nm even in the absence of the denaturant. The difference was ascribed to the presence or presumed absence of a "knot" structure at the C-terminal end of the two forms, respectively. When a specific inhibitor was added to the experimental solution, native monomers (sp activity = 88% of the wild type enzyme) were extended to 28 ± 4 nm, whereas dimers (sp activity = 46%) were extended to about 56 ± 3 nm, suggesting that both monomeric units in the dimer could bind inhibitor molecules, which was further corroborated by a titration experiment using a fluorescent inhibitor. Thus, one of the monomeric units in the engineered dimer was concluded to be enzymatically inactive but capable of binding inhibitors.

Key Words: **carbonic anhydrase B; activity; inhibitor binding; atomic force microscope.**

The atomic force microscope (AFM) was invented in 1986 (1) and it has since then been used as a valuable tool for the atomic resolution imaging of nonconductive biological macromolecules and structures in their adsorbed states to a substrate (2–5). Application of the AFM has been further extended to direct measurements of inter- and intramolecular forces in biological context (6–12). Force measurements between biological samples and an AFM tip can be routinely performed and the results are expressed in the form of force–extension (F–E) curves. From such force profiles, information related to specific intramolecular mechanics has been obtained and used for detailed analyses of the force-induced conformational changes of proteins, DNAs, and other types of biological molecules and structures. The mechanical stretching of tandemly repeated domains in the giant molecule of titin has been extensively studied and the results have been analyzed in great details (6–10). Such analyses of the mechanical unfolding of proteins are now providing a new insight into the structure–function relationship of protein molecules (13–15).

In this paper, we applied a similar methodology of force measurement to study mechanical properties of bovine carbonic anhydrase B (CAB) (it is also called bovine carbonic dehydratase II) under native conditions and in the presence of the specific inhibitor, *p*-aminomethylbenzene sulfonamide, and found an interesting difference in their mechanical responses. In our previous work, we reported (16, 17) about the mechanical distinction between the native and the denatured states of CAB and found that the native CAB could be stretched only to about 15 nm before the covalent cross-linking system was ruptured. On the face of it, this is an unusually tight conformation compared with the published results on other proteins such as titin, spectrin, and bacteriorhodopsin, all of which have been shown to be unfolded with forces less than 0.3 nN (6–9, 18). We referred the cause of this unusually tight folding of CAB to the effect of a presumed 'knot' structure in the C-terminal region. The 'knot' structure has been noted for the human counterpart of CAB as one of its unique features (19, 20), and we have recently confirmed the presence of a similar 'knot'

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structure in bovine CAB as well (21). The crystallographic structures of both human and bovine enzymes show that the residues Arg^{245} to Pro^{249} go over Glu^{26} to Pro^{30}, while Gln^{254} to Phe^{258} go under Ala^{37} to Asp^{41}, and Val^{255} to Pro^{259} go over Pro^{42} to Leu^{44} (the number of the amino acid residues in the engineered CAB is based on that of the wild type CAB), indicating that, if they are pulled from N- and C-termini, the chains would form a single 'knot'. The presence of such a 'knot' would make mechanical unfolding of a protein structure rather difficult, because the structure would be tightened rather than loosened by tensile forces applied to its two ends. We therefore ascribed the unusual tightness of native CAB observed in our previous report to the presumed 'knot' structure. With new direct evidence on the presence of the 'knot', we constructed a tandemly repeated CAB dimer with a short interrupting sequence in between the two monomeric units. The N-terminal upstream monomeric unit, unit I, would not be able to form the 'knot' in its C-terminal region because of the steric hindrance caused by the presence of the downstream unit, unit II, while the latter should be able to form the correct native structure with a 'knot'. We report in this paper that such a dimer of CAB gave, as expected, a total stretching length of about 110 nm, equal to the full extension of the knotless unit I to 98 nm (259 a.a. for CAB + Cys + 4 intervening seq. = 264 a.a. residues) and 13 nm extension of the knotted unit II. Addition of the inhibitor shortened the stretching length of the dimer to 56 nm, suggesting that both unit II and unit I bound the inhibitor, while the specific enzymatic activity of the dimer remained half of that of the monomeric enzyme. It is an interesting example of a protein conformation that is active in inhibitor binding and yet enzymatically inactive.

MATERIALS AND METHODS

Protein expression and purification. CAB monomer and dimer were used as the models in this study. CAB monomer was the same one we used in the previous work (16). Dimeric CAB sequence was based on the wild type monomer, and the connection between two monomeric units was through 4 amino acid residues (Glu-Phe-Lys-Lys) and at each terminus, Cysteine residues were added. The proteins were expressed in BL21(DE3) cells, purified by Ni^{2+} affinity chromatography (Qiagen, Hilden, Germany) under native conditions and kept in 50 mM Tris/5 mM DTT solution at 4°C, as previously described (16). The size and purity of the protein was checked by SDS-PAGE. The protein solution was passed through a HPLC column of Superdex 75 HR 10/30 (Pharmacia, Uppsala, Sweden) to remove DTT right before AFM experiment.

Esterase activity. The enzymatic activity of CAB was determined on a DU7400 spectrophotometer (Beckman Inc., CA) following the method described elsewhere (22). *p*-Nitrophenyl acetate (*p*NPA) was used as a substrate in the experiment. The relative activity of the monomeric and dimeric CAB was compared with that of the commercial CAB (Sigma Chemical Co., MO), which was taken as a standard in the activity assay. CAB reacts with *p*NPA and produces *p*-nitrophenol (*p*NP), whose concentration was detected by the absorption at 348 nm.

Inhibitor binding assay. The fluorescence measurement was performed on a F-3010 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). 5-(dimethylamino)naphthalene-1-sulfonamide (DNSA) was added at a final concentration of 50 μM (23) to three different types of CAB, the commercial CAB, the monomeric and the dimeric CAB at the same concentration of 0.01 mg/mL. The emission bandwidth was 5 nm and the excitation wavelength was 280 nm with a bandwidth of 5 nm.

Substrates and tips. Crystalline silicon wafers (Shin-etsu Silicon Co., Tokyo, Japan) were used as substrates for AFM experiments. Cleaning of the substrates and tips were done according to methods described previously (24). The substrates and tips were then treated with 3-aminopropyltriethoxysilane (APTES) (Aldrich Chem., Milwaukee, WI), as described in our previous paper (16). Then succinimidyl 6-[3′-(2-(pyridyldithio)-propionamido-)hexanoate (LC-SPDP) (Pierce, Rockford, IL) with a contour length of 1.5 nm was served as a cross-linker. Tips were also modified with LC-SPDP. Finally, aliquots of protein solution with a concentration of about 50 μg/ml were applied to the silanized substrates precoated with LC-SPDP. The force measurement was performed in a 50 mM Tris–SO_4^{2+} buffer at pH 7.3.

AFM experiment. Nanoscope III and IIIa multimode AFMs (Digital Instruments, Santa Barbara, CA) were used in force mode as described previously (16). Calibration of the spring constant of individual cantilevers was done in solution by a method based on the measurement of their thermal vibrations as described previously (25). The scan speed used in the experiments was in the range of 100–150 nm/sec. The force curves obtained from experiments were converted to force–extension (F–E) curves, where the ordinate represented the force (F) calculated from the vertical displacement of the cantilever multiplied by its spring constant, and the abscissa corresponded to the vertical extension (E) of the protein molecule sandwiched between the tip and the substrate. In our experiments, covalently immobilized CAB on the substrate was touched with a modified tip with LC-SPDP and stretched from its two ends after occasional disulfide bridge formation between —SH group at one end of the protein and the reactive end of a modified AFM tip. There is no direct evidence for the single molecule stretching, but we believe that our experimental conditions guaranteed that we were dealing with single molecule measurements. First, we kept the successful rate of detecting downward deflections with the final rupture force larger than 1.5 nN (signifying covalent bond breakage) to less than 5% by controlling the concentration of the protein on the modified surface. Most of the remaining curves show perfect overlap with the extension and retraction traces. With such a low rate of cross-linking formation, accidental formation of more than two cross-links, corresponding to multiple chain stretching, should be less than 5% of all the observed force curves. Second, the force curves we obtained were generally constant in their shapes under the given conditions. Force curves got in the reference experiments using un-modified tips did not show downward deflections with the rupture force larger than 0.1 nN, proving that physical adsorption of the protein to the solid surface and tip would not interfere with our experimental results.

RESULTS AND DISCUSSION

Figure 1 shows F–E curves of CAB under a native condition (curve 1), where the protein was extended to 13 ± 2 nm using LC-SPDP as a cross-linker both on the substrate and the AFM tip, instead of one containing a long spacer of polyethylene glycol (PEG cross-linker). In the previous work (16), we used a PEG cross-linker to eliminate unwanted adsorption of protein to the substrate and obtained about 15 nm for the extension of CAB after subtracting that of PEG cross-linker. We

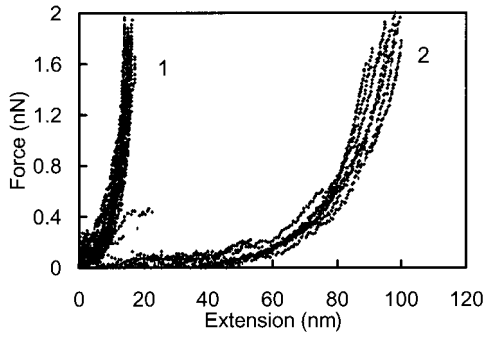


FIG. 1. Unfolding curves of a monomeric CAB in neutral buffer (1) and in 6 M GdmCl solution (2), respectively, using LC-SPDP as a cross-linker. Small peaks within first 5 nm of stretching are probably due to the adsorption of protein to the substrate.

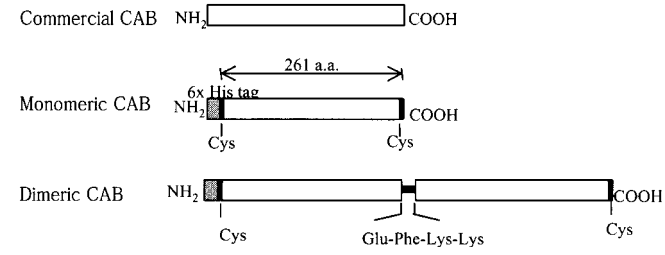
found that there was only a gradual increase of force without any spurious peaks before a steep rise to 1.7 ± 0.2 nN. A small discrepancy between the two extension lengths is due to the uncertainty of subtracting the extension of PEG cross-linker from the total extension in our previous study. We can, therefore, be confident that the initial small peaks in the curve in Fig. 1 were due to nonspecific interaction of the protein with the substrate and the true F–E curve of the protein is represented in the steep rise of the curve before 13 nm in stretching. Since the theoretical length of CAB with 261 amino acid residues is 96 nm, the result of Fig. 1 testifies that the protein resisted unfolding by a tensile force of unusual strength.

As described in the introduction, the observed difficulty of breaking up the native CAB structure beyond 13 nm may be due to the presence of a 'knot' close to its C-terminus (19, 20), whose presence in bovine CAB has recently been verified by X-ray crystallography (21). It is true that when the protein is denatured in 6 M guanidinium chloride, the 'knot' is presumably unraveled and it can be stretched to its full theoretical length (Fig. 1, curve 2). F–E curves of the denatured protein

TABLE 1

Activity of Monomeric and Dimeric CAB

Protein	$V_0/[E]_o$ (min^{-1})	% Activity
Blank	0.0025 ± 0.001	—
Commercial CAB	1.054 ± 0.002	100
Monomeric CAB	0.928 ± 0.001	88
Dimeric CAB	0.485 ± 0.002	46



Note. Commercial CAB was taken as the standard with 100% activity. V_0 is the rate of *p*NP production, in unit of mM/min; $[E]_o$ is the initial concentration of each sample, in unit of mM.

gave a full stretching length of a monomeric protein to about 96 nm as expected, which also confirmed the reliability of length estimate in our experimental system.

To further substantiate the conjecture that the presence of the 'knot' is responsible for the tight folding of CAB, we performed force measurements on the CAB dimer, where the upstream unit I is expected to be devoid of the 'knot' structure. As given in Fig. 2, dimeric CAB was extended to about 110 nm before the covalent cross-linking system was severed with a force of 1.7 nN. The result can be most reasonably interpreted by assuming a full extension of unit I plus the terminal Cys to approximately 96 nm (made possible due to its presumed inability to form a 'knot'), together with the extension of the intervening sequence of 4 residues and a short extension of unit II up to 13 nm as

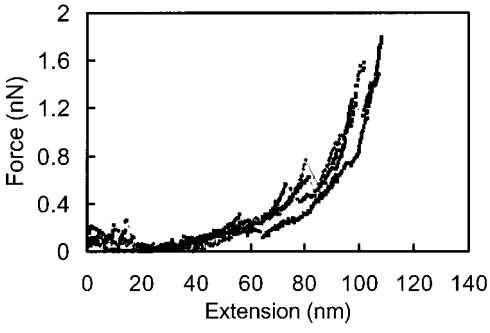


FIG. 2. Collection of the unfolding F–E curves of a dimeric CAB in neutral buffer. Most prevalent stretching curves ends at around 110 nm. The extension part was due to stretching the knotless unit I in a dimer, whereas the unit II could not be extended longer, like a native monomer.

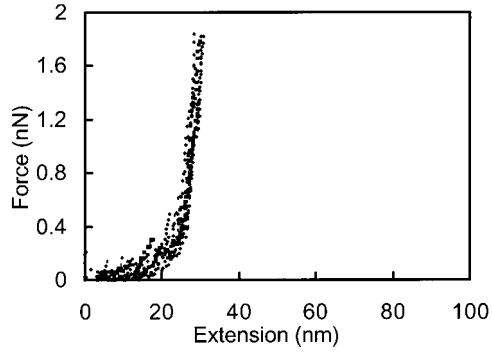


FIG. 3. Representative F–E curves of a monomeric CAB in the presence of 2 mM *p*-aminomethylbenzene sulfonamide, a specific inhibitor of CAB, solution. The significant change, compared with curve 1 in Fig. 1, represents the binding of the inhibitor to its active site dramatically altered the internal mechanics of CAB.

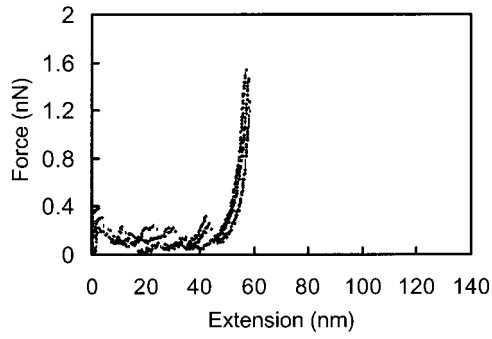


FIG. 4. Typical F–E curves of a dimeric CAB in the presence of 2 mM *p*-aminomethylbenzene sulfonamide solution. The length is twice as long as the extension for a CAB monomer under the same experimental condition, assuming both of the monomeric units in a dimer bound inhibitor molecules.

a native monomer. The sharp rise at the end of the curves in Fig. 2 should then be considered as due to the presence of a tight structure of unit II with a 'knot' at its C-terminal end. The appearance of small peaks around 80 nm is not well understood yet, but we focus on more general features characteristic to the CAB dimer. We proved at least that the knotless half of the dimer could be stretched much longer than the native monomer. Alam *et al.* took a different approach to this problem and stretched a mutant protein, Gln253Cys of CAB monomer, from its N-terminus and Cys253 to avoid the 'knot' formation and have shown that the protein can be extended almost to its full length (26), thus substantiating our conjecture.

The activity of dimeric and monomeric CABs were measured and compared with that of the commercial CAB, which was taken as the standard with 100% activity. As summarized in Table 1, the specific activity of monomeric and dimeric CAB was 88 and 46% of that of the commercial CAB, respectively. Among the three possibilities such as (1) both unit I and II are 50% active, (2) unit I is 100% active and unit II is inactive, (3) unit I is inactive and unit II is 100% active, we think the last one is closest to the actual situation. Unit II is capable of forming the correct conformation, because there is no presumed hindrance for unit II to form the active conformation of the native enzyme whereas, unit I has a difficulty of forming the correct knotted structure at its C-terminus, as described in the introduction.

We then studied the inhibitor binding to monomer and dimer of CAB using the force mode of AFM. The force measurement was carried out in the presence of 2 mM *p*-aminomethylbenzene sulfonamide, a specific inhibitor of CAB, solution. The extension of monomeric CAB was increased to 28 ± 4 nm as shown in Fig. 3. There was a significant change compared with the curves in Fig. 1 (curve 1), attesting that binding of the inhibitor to its active site markedly altered the internal mechanics of CAB. Dimeric CAB was also stretched in the presence of the same inhibitor. The extension of the protein in this case, before the rupture of the covalent system, was 56 ± 3 nm (Fig. 4), significantly shorter than the extension of 110 nm for the original dimer as given in Fig. 2. It was, however, approximately twice as long as the extension of the CAB monomer under the same experimental conditions as shown in Fig. 3, which is reasonable if we assume that both of the monomeric units in a dimer bound inhibitor molecules (27, 28). Similar data were obtained using 5-(dimethylamino)naphthalene-1-sulfonamide, DNSA, as an inhibitor.

Since the above result was an unexpected one, we did inhibitor-binding experiment, using a specific fluorescent inhibitor, DNSA, and, to our surprise, found that both of the monomeric units in the dimer bound the inhibitor. DNSA is known to bind near the active center of the enzyme and is a particularly good acceptor for the resonance energy transfer from tryptophan residues within the native enzyme molecule (23, 27). The excitation at 280 nm results in a fluorescence emission of DNSA at λ_{max} = 460 nm. The experimental results (Table 2) showed that the increase of fluorescence intensity at 460 nm was almost same for the commercial, monomeric and dimeric CAB on the weight basis, which indicated that both of the monomeric units in dimer were active in DNSA binding.

We interpreted the above results as indicating that unit II has a complete active site capable of catalyzing the hydrolysis of *p*NPA, as well as binding DNSA, whereas the active site in unit I was incomplete being only active in DNSA binding. It is possible that DNSA

TABLE 2

Changes in Fluorescent Spectrum Induced by DNSA Binding

Protein	Indensity before adding DNSA at 341 nm	Indensity after adding DNSA at 341 nm	Indensity before adding DNSA at 460 nm	Indensity after adding DNSA at 460 nm
Commercial CAB	14.97 ± 0.02	9.91 ± 0.02	0.02 ± 0.01	11.81 ± 0.03
Monomeric CAB	15.27 ± 0.01	10.16 ± 0.02	0.01 ± 0.02	12.57 ± 0.02
Dimeric CAB	12.34 ± 0.02	8.25 ± 0.01	0.02 ± 0.01	13.90 ± 0.02

Note. The weight concentrations of three samples used are identical (0.1 mg/mL).

is capable of inducing the formation of an active site from a more or less disordered conformation of unit I.

CONCLUSION

By using a CAB dimer, we obtained an extension of the polypeptide chain reaching 110 nm, whereas a native monomer was extended only to 13 nm. We believe this extension corresponds to that of unit I, which is presumably not capable of forming a 'knot' structure at its C-terminal region. Though the mechanical behavior of unit I is very much different from that of the native enzyme, it was shown to be active in inhibitor binding, suggesting that it has native like structure. It is, however, not active as an enzyme. A direct proof of extension of unit I should come from our future study using different kinds of mutant proteins. Although it has been proved that bovine CAB has a similar 'knot' structure as the human counterpart, we have yet to prove that the modified protein with Cys residues at its two termini still has a similar structure or not. With precautions such as given above, we interpret the data as an indication of a strong effect of a 'knot' on the mechanical unfolding process, at least in the case of CAB. It will be interesting to see if the same or similar effect can be detected for other types of carbonic anhydrases.

Two new and intriguing observations emerged from this study were (1) unparallel nature of the enzymatic activity of CAB dimer and its inhibitor binding. Namely, the specific activity of CAB dimer was only half of that of a monomer and yet the dimer was fully active in inhibitor binding; (2) inhibitor binding to CAB monomer increased its extension under the tensile force from 13 nm to 28 nm, suggesting an increase of readily stretchable parts of the enzyme upon inhibitor binding. The first point is conceivable, if we assume that for the enzymatic activity, a more precisely constructed active site is required than for inhibitor binding. It is also likely that the specific inhibitor used in this study was better in inducing appropriate structure for its binding from a more or less disordered unit than *p*NPA used for activity measurement. *p*NPA is after all not a physiological substrate of CAB. The second point may be explained from the fact noted by M. T. Alam and A. Ikai that the thermal factor in the crystal structure of human carbonic anhydrase II (PDB data) shows that, after binding of an inhibitor, the central part of the enzyme becomes less labile while the thermal factor of the surrounding peripheral parts becomes larger. It is, therefore, possible that the AFM measurement is actually detecting the change in thermal factor of the peripheral parts of the molecule as an increase in the stretchable parts. We will continue the crystallography of CAB to verify these conjectures.

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