

The importance of being knotted: effects of the C-terminal knot structure on enzymatic and mechanical properties of bovine carbonic anhydrase II¹

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Abstract In order to better understand the contribution of the knotted folding pattern to the enzymatic and mechanical properties of carbonic anhydrases, we replaced Gln-253 of bovine carbonic anhydrase II with Cys, which allowed us to measure the mechanical strength of the protein against tensile deformation by avoiding knot tightening. The expressed protein, to our surprise, turned out to contain two conformational isomers, one capable of binding an enzymatic inhibitor and the other not, which led to their separation through affinity chromatography. In near- and far-UV circular dichroism and fluorescence spectra, the separated conformers were very similar to each other and to the wild-type enzyme, indicating that they both had native-like conformations. We describe new evidence which supports the notion that the difference between the two conformers is likely to be related to the completeness of the C-terminal knot formation. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Atomic force microscope; Carbonic anhydrase II; Mechanical unfolding; Conformer; Knot structure

1. Introduction

A comprehensive computational search of protein structure databases together with our current knowledge of protein folding indicates that few proteins have a knot topology in their folding pattern [1,2]. Human carbonic anhydrase I and II (collectively HCA) belong to them having similar 3D structures with root-mean-square deviations of 1.0 Å for back bone atoms [3–6]. The importance of the unique C-terminal folding pattern of HCA having a trefoil knot was implicated in a series of refolding studies done by Carlsson and his colleagues [4,7]. They showed that human carbonic anhydrase II (HCAII) could be refolded from the denatured state with sev-

eral distinct kinetic steps, the slowest among them having a half-time of 9 min. Using various labeling techniques, they showed that refolding of the C-terminal region constituted one of the slow reactions and was almost synchronous with the recovery of the enzymatic activity. They also showed that proteolytic truncation of three consecutive amino acid residues from the C-terminus of HCAI under denaturing conditions reduced the extent of recovery of refolded protein to only 25% of the starting material and the refolded protein showed decreased stability [8]. These observations on HCA indicate that the C-terminal sequence involved in the knot topology contains vital information for the correct folding of the enzyme leading to the formation of an active site. In addition to the role of knot formation in the enzymatic activity of carbonic anhydrases, we became interested in studying the effect of such a knot structure on the mechanical response of the protein to a tensile force applied to stretch it from two defined sites. Mechanical stretching of protein molecules using the atomic force microscope (AFM) was initiated by Mitsui et al. [9] and has been extensively used to measure the force to break globular units in tandemly repeated proteins such as titin and fibronectin [10–12]. It seemed to us that the presence of a knot very near to the C-terminus of carbonic anhydrases should have a significant effect on the mechanical properties of the protein, and this would be a good test of the usefulness of the AFM for the study of protein nanomechanics. For this purpose, we chose the bovine homolog (BCAII) of HCAII and its recombinant mutants as experimental materials because the protein does not have cysteine residues and refolding studies have been done on it as extensively as on HCA [13,14]. BCAII has 259 amino acid residues with 77% sequence homology to HCAII [15], and not surprisingly it has recently been shown that BCAII has a very similar 3D structure to those of HCA including the C-terminal knot [16]. Wang and Ikai reported that BCAII resisted forced unfolding on the AFM to a remarkable degree, extending a mere 13 ± 2 nm (out of the theoretical full length of 96 nm for a protein having 259 amino acid residues) against a tensile force of 1.7 ± 0.2 nN which was strong enough to break covalent cross-linkers used to tether the molecule to the substrate [17–19].

To study the effect of the knot on the mechanical response of BCAII more precisely, we constructed a mutant protein having a cysteine residue at the N-terminus and another at the 253rd position replacing Gln-253 which is located close to the C-terminus as mechanical stretching from this position and the N-terminus should avoid knot tightening (Fig. 1). The expressed and purified protein sample satisfied our ex-

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Abbreviations: AFM, atomic force microscope; F–E, force–extension; BCAII, bovine carbonic anhydrase II; HCAII, human carbonic anhydrase II; CD, circular dichroism

pectations of knot avoidance as it was stretched longer than 70 nm before the breakdown of the covalent crosslinking system, but it turned out to contain two conformational isomers, type I and II as we designated them, the former capable of binding enzymatic inhibitors while the latter not. The two separated isomers were found to have very similar optical properties but their force vs. extension (F–E) behavior in mechanical stretching experiments is clearly different. We discuss the significance of our new findings in relation to lines of evidence strongly supporting the notion that type I has a knotted C-terminal conformation while type II is most likely unknotted. The mutant protein used in this study or closely related ones will be a good choice for further investigation of the effect of a knot on BCAII folding and activity.

2. Materials and methods

2.1. Protein engineering

Two Cys residues were introduced at designated sites of the bovine carbonic anhydrase II (BCAII) by using a standard technique [20]. *Escherichia coli* strain BL21 (DE3) (Novagen, Madison, WI, USA) was used to overexpress the mutant gene cloned in the pRSETB vector (Invitrogen, San Diego, CA, USA) following a procedure reported earlier [21]. The expressed His₆-tagged mutant protein was preliminarily purified by metal chelate affinity chromatography using a Ni-NTA agarose column (Qiagen, Hilden, Germany) and the purity of the protein preparation was >97% as determined by SDS-PAGE. The expressed protein contained 36 extra amino acid residues including an enterokinase recognition sequence and six histidine residues at N-terminal. The presence of the long extra peptide did not interfere with the activity of the enzyme. The protein was kept in 50 mM Tris–SO₄ (pH 7.5) buffer containing 5 mM DTT at 4°C and the reducing reagent together with remaining impurities were removed with Superdex 75 HR 10/30 (Amersham Pharmacia Biotech, Uppsala, Sweden) column immediately before measurements of various properties of the protein. The enzymatic activity was determined by using the esterase reaction as described previously [22].

2.2. Affinity chromatography

The affinity matrix (1 ml) used for separation of the two conformers was prepared by coupling *p*-aminomethylbenzenesulfonamide with activated agarose gel with amine reactive *N*-hydroxysuccinimide (HiTrap NHS activated column, Amersham Pharmacia Biotech) according to manufacturer's manual. The purified enzyme was applied to the column and eluted with 50 mM Tris–SO₄ (pH 7.5) containing 0.4 M NaN₃ [23]. The releasing agent (0.4 M NaN₃) in the elution was removed with Sephadex G-25 desalting column (Amersham Pharmacia Biotech). In this experiment, active enzyme was not detected in the flow-through fraction.

2.3. Circular dichroism (CD) measurements

CD measurements were carried out on a Jasco J-720WI spectropolarimeter (Tokyo, Japan) with constant N₂ flushing. Far- and near-UV CD spectra were obtained using two types of quartz cells respectively having 1 and 10 mm optical path-lengths, with a sample concentration of 15 μM for both measurements. All the spectra given in this paper were averages of five to eight repeated scans each at 0.5 nm intervals and were corrected for the base line. The resulting spectra were smoothed using the minimum filter included in the instrumental software. A spectral contribution from the extra N-terminal peptide was subtracted as a random coil-type spectrum of the corresponding number of amino acid residues and the resultant spectrum was analyzed for the estimation of the secondary structure by CONTIN, a computer program that implemented the ridge regression [24]. Ellipticity is reported as the mean residue molar ellipticity, $[\theta]$ in degree cm² dmol⁻¹, calculated according to the following equation.

$$[\theta] = [\theta]_{\text{obs}} \cdot \text{mrw} / 10 \times l \times c,$$

where $[\theta]_{\text{obs}}$ is the observed ellipticity in degrees, mrw is the mean residue molecular weight, c is the protein concentration in g/ml and l is the optical path-length in cm.

2.4. Force-induced mechanical measurements

The engineered protein was immobilized on a silicon surface made reactive towards sulfhydryl groups by grafting the bifunctional crosslinker, SPDP (*N*-succinimidyl-3-[2-pyridylthio]propionate; Pierce, Rockford, IL, USA) or NHS-PEG3400-MAL (Shearwater Polymers, Huntsville, AL, USA) as described [21]. To ensure extension of a single molecule at one time, the protein density on the substrate was adjusted so that less than 10% of all force measurements gave positive indications of protein stretching. To reduce the protein density on the surface, a mono-functional crosslinker NHS-PEG2000 (Shearwater Polymers) was simultaneously engrafted on the silicon surface as a diluent. A similarly functionalized cantilever (NP-S-type Si₃N₄ tip; Digital Instruments, Santa Barbara, CA, USA) with a calibrated spring constant determined by thermal fluctuation analysis [25] was maneuvered (100–150 nm/s) toward the immobilized proteins on the sample stage using a Nanoscope IIIa multimode AFM (Digital Instruments) until it touched the sample with a maximum threshold force of 1.8 nN and a contact time of 0.5 s. During the retraction of the sample stage, occasional downward deflections of the AFM cantilever were observed. Most such deflections were terminated with a final rupture force of ≤2 nN corresponding to the breakage of a covalent bond after a piezo displacement relative to the tip position corresponding to the stretching of a protein molecule up to 70–100 nm. Each experiment was repeated several times to obtain approximately 1000 such cycles and only force curves with the above characteristics were collected for further F–E analysis following a procedure reported earlier [26]. The experimental setup was carefully verified either by experiments with blanks where all the possible combinations of modified and unmodified substrates and tips were tested, or using spacers of different lengths and the results confirmed that the force curves collected for further analysis corresponded uniquely to protein stretching events. All AFM experiments were performed in a liquid environment containing 50 mM Tris–SO₄ (pH 7.5) at room temperature (22–26°C) unless otherwise stated.

2.5. Refolding of type II conformer

Denaturation and reactivation of the type II conformer were carried out according to Persson et al. [27], where the reactivation buffer (50 mM Tris–SO₄, pH 7.5) contained 10 mM KCl, 10 mM MgSO₄, 1 mM Mg–ATP and GroEL as a chaperone in a two-fold molar excess of type II. During refolding, a fraction (approx. 10–20%) of the protein was found aggregated and/or dimerized and, therefore, the activity of the refolded protein was measured after HPLC separation of such fractions together with GroEL and extra salts.

2.6. Carboxypeptidase Y (CP-Y) digestion of the conformers

Digestion of sample proteins was initiated by mixing each protein substrate and CP-Y (Sigma, St. Louis, MO, USA) at a 5:1 molar ratio in 0.1 M ammonium acetate buffer, pH 6.0, at 25°C. At appropriate intervals, aliquots were withdrawn from the reaction mixture and subjected to MALDI TOF mass spectroscopic analyses (AXIMA-CFR, Shimadzu, Tokyo, Japan) following the procedure reported earlier to determine the extent of digestion [28].

3. Results and discussion

The purified protein (BCAII_{Gln253Cys}) showed a single narrow band on SDS-PAGE. The far-UV CD and fluorescence spectra of the engineered protein were almost indistinguishable from those of the wild-type enzyme. However, the enzymatic activity was about 49% of that of a commercial wild-type BCAII as shown in Table 1. An addition of Zn(II) did not increase the activity significantly. The result suggested two possibilities, (i) the particular mutation employed in this experiment reduced the activity of all the molecules to about one half of that of the wild-type enzyme, or (ii) the sample was composed of two different conformers with dissimilar enzymatic properties. Since our preliminary mechanical unfolding experiment using the engineered BCAII (BCAII_{Gln253Cys}) revealed the co-existence of two types of F–E curves [29], one interacting with an enzymatic inhibitor

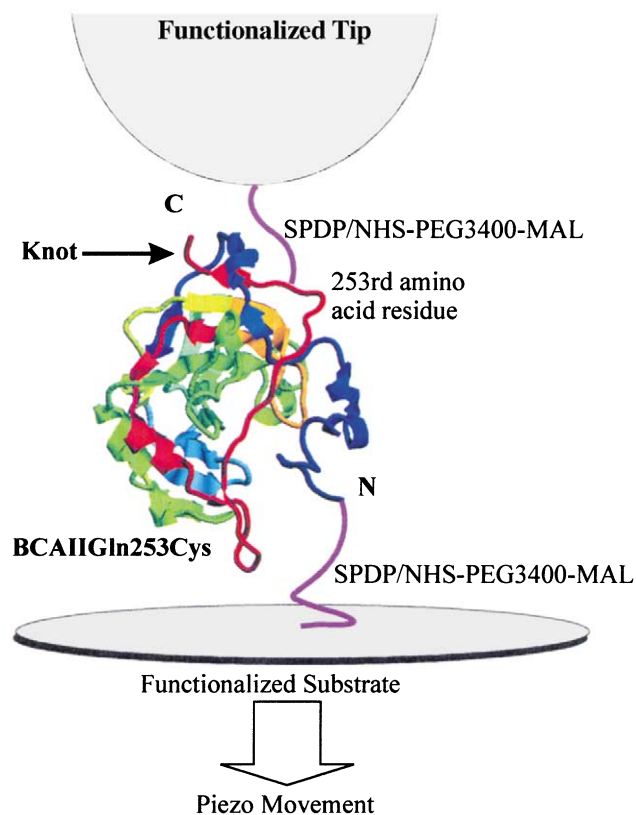


Fig. 1. The strategy for stretching the engineered protein by avoiding knot tightening illustrated using the crystallographic structure of BCAII [16].

and the other not, we suspected the second possibility to be true. In order to clarify the situation, an affinity matrix was constructed by immobilizing the well-known inhibitor of the enzyme, *p*-aminomethylbenzenesulfonamide, on an activated agarose gel. The resultant matrix fractionated a solution of BCAII Gln253Cys into a flow-through (type II conformer) and a column-bound (type I conformer) fraction with an approximate ratio of 1:1. The matrix-bound protein was subsequently eluted by the addition of sodium azide (0.4 M) as a

Table 1
Esterase activity of bovine carbonic anhydrase I and II and its variant and conformers

Protein	$V_o/[E]_o$ (min^{-1})	Relative activity (%)
Commercial BCAII	54.0 ± 1	100
BCAII Gln253Cys	26.5 ± 1	49
Type I	52.4 ± 1	97
Type II	0.0 ± 1	≈ 0

Note: V_o is the rate of *p*-nitrophenol production in $\mu\text{M}/\text{min}$ and $[E]_o$ is the initial concentration of enzyme in μM unit. Due to the low solubility of *p*-nitrophenyl acetate, V_o was measured at different protein concentrations and averaged. The error indicates the standard deviation.

releasing agent [23]. Thus, our hypothesis that the particular mutation employed in this experiment caused an accumulation of two conformers, one having an active site for inhibitor binding and the other not, was supported. It was further confirmed that the type I conformer was enzymatically fully active while the type II conformer was not (Table 1). The finding that the two conformers co-existed in a ratio of 1:1 was consistent with our earlier observation that the specific activity of the engineered protein was about 49% of that of the wild-type enzyme.

3.1. Spectroscopic properties of the separated conformers

BCAII contains seven Trp residues in mainly apolar environments and upon excitation at 280 nm, it gives a fluorescence emission spectrum with $\lambda_{\text{max}} = 342$ nm. Any alteration of the above environment results in a red shift in emission maxima. Fig. 2a shows the fluorescence spectrum of BCAII along with those of the two newly found conformers (type I and II). Close similarities in λ_{max} among the three indicate that Trp residues of both conformers are situated in similar apolar environments to those of the wild-type BCAII. Furthermore, as the seven Trps are positioned in different parts of the conformers, the observed fluorescence spectra indicated similarities in their global conformations. The far-UV CD spectra of the two conformers were almost identical to each other and to that of the wild-type enzyme with very similar α -helicities; 7.8–8.2% for all three. Fig. 2b shows the near-UV CD spectra of the native BCAII along with those of the two

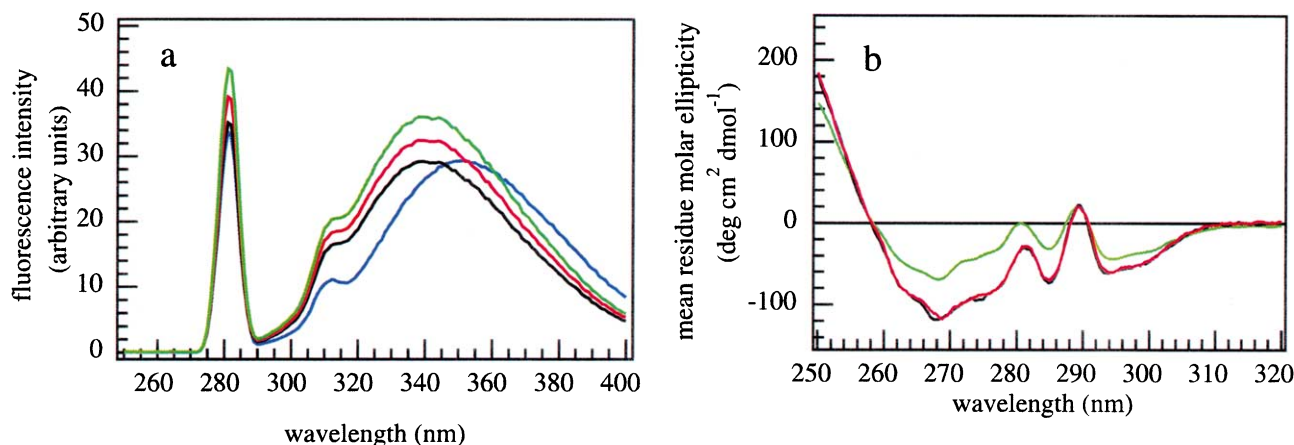


Fig. 2. Spectroscopic similarities between separated type I and II conformers of engineered BCAII. a: Intrinsic fluorescence spectra of native (black), random coil (blue), type I (red) and type II (green) conformers in Tris- SO_4 buffer at pH 7.5. The excitation wavelength was 280 nm and the protein concentration was slightly different for each spectrum. When normalized with respect to concentration, the fluorescence intensities of the three spectra (black, red and green) were virtually identical. b: Near-UV CD spectra of native (black), type I (red) and type II (green) conformers in the same buffer.

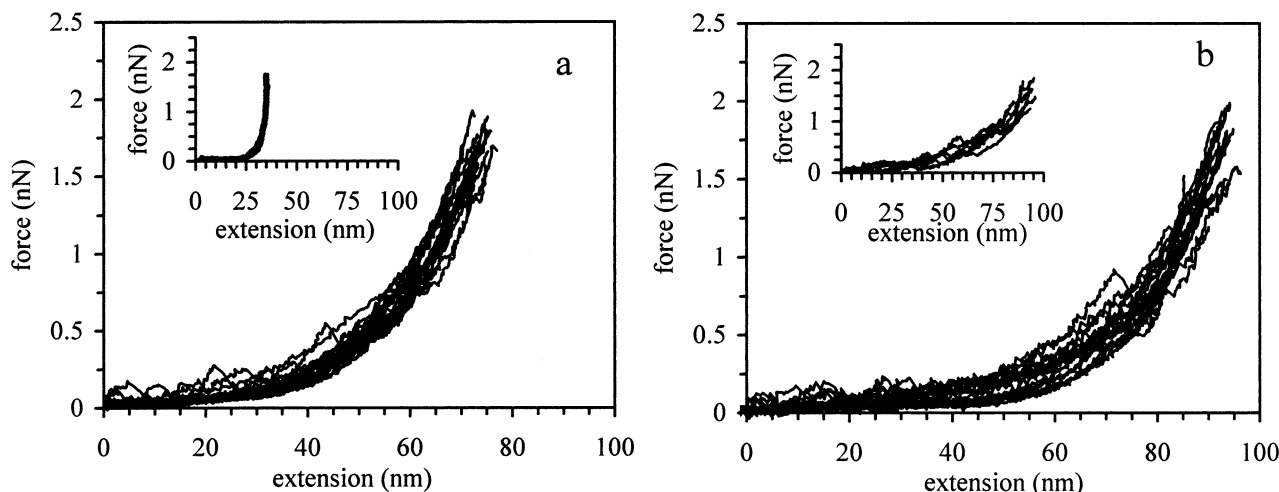


Fig. 3. Force-induced unfolding of two independently separated conformers in a neutral buffer in terms of F–E curves. The engineered protein was stretched from precisely determined sites (residue numbers –1 and 253) after being sandwiched between the tip and the substrate of AFM. Sample proteins were extended with a mean and S.D. of 73 ± 2.7 nm ($n=82$) for type I (a) and 91 ± 3 nm ($n=75$) for type II (b), both having similar final rupture forces of 1.7 ± 0.2 nN. The insets represent the effect of inhibitor that was added to the protein solutions for force measurement. Type II F–E curves remained unchanged, whereas type I curves exhibited a dramatic change.

conformers. The CD data in this region are known to provide information on the tertiary structure of the protein. Comparison of the near-UV CD spectra indicated that the characteristic CD bands for the wild-type enzyme and type I were almost identical to each other but recognizably different from those of type II for which all the peaks and troughs were kept almost unchanged with respect to their positions while some of the troughs are shallower for type II. The result indicates that all three forms had similar tertiary structures with minor conformational deviations in type II. When the asymmetry around the aromatic residues is partially lost as in the case of the ‘molten globule’ form, the spectrum in this region becomes flat and positive [14]. It is, therefore, likely that the type II conformer is more compactly folded than the ‘molten globule’ state, which is in accordance with the above fluorescence result.

3.2. Chaperonin-assisted refolding of type II

To investigate whether type II had a defective covalent structure and, therefore, was unable to form an active enzyme, we performed chaperonin-assisted refolding experiments using a denatured type II. It has been observed that chaperonin can facilitate rearrangements of misfolded structures during refolding and unfolding of HCAII [30,31]. Our reactivation experiment showed that the enzymatic activity was completely restored if a small amount of aggregated and/or dimerized material was discarded verifying that type II is a misfolded conformer with the same covalent structure as type I rather than a chemically defective species.

3.3. Mechanical unfolding of the conformers

After confirming the spectroscopic similarities, mechanical unfolding experiments on type I and II were performed in order to know the mechanics of knot-free stretching (for details, see Section 2.4 and Fig. 1). Since the stretchable part of the protein had 254 amino acid residues including an extra cysteine residue at its N-terminus plus two crosslinker molecules, we expected to obtain F–E curves having a full exten-

sion of 94 nm (where the length of one amino acid residue was taken as 0.37 nm). Nearly consistent with our expectation, the F–E curves of both conformers showed much longer extensions than the wild-type enzyme with some important differences for type I and II [17,18]. First, F–E curves of the type I conformer had a fairly flat extension in the initial 10–15 nm region (Fig. 3a) followed by a highly non-linear increase of force until it reached 1.7 ± 0.2 nN with an average extension of 73 ± 2.7 nm ($n=82$). We attributed this result to the successful avoidance of knot tightening during the stretching process. F–E curves for type II showed a relatively longer flat extension in the initial part (30–40 nm) followed by a non-linear increase in force until it again reached 1.7 ± 0.2 nN with an almost full extension (91 ± 3 nm, $n=75$) of the chain (Fig. 3b). The final rupture forces in both cases corresponded to the force to sever the covalent crosslinking system used in this experiment. The small irregular bumps in the initial part of

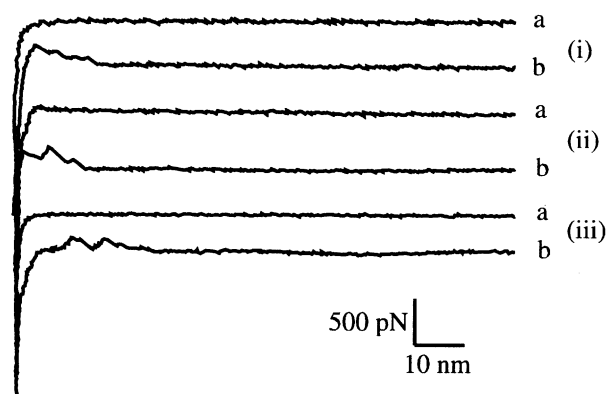


Fig. 4. Force curves obtained in an experimental buffer showing minor interactions between the tip and the substrate in the absence of protein under various tip and substrate conditions. (i) Bare tip and substrate, (ii) tip and substrate both functionalized with SPDP, and (iii) Polyethyleneglycol-functionalized substrate and SPDP-functionalized tip. Almost 70% of the force curve is similar to (a), while the rest is similar to (b).

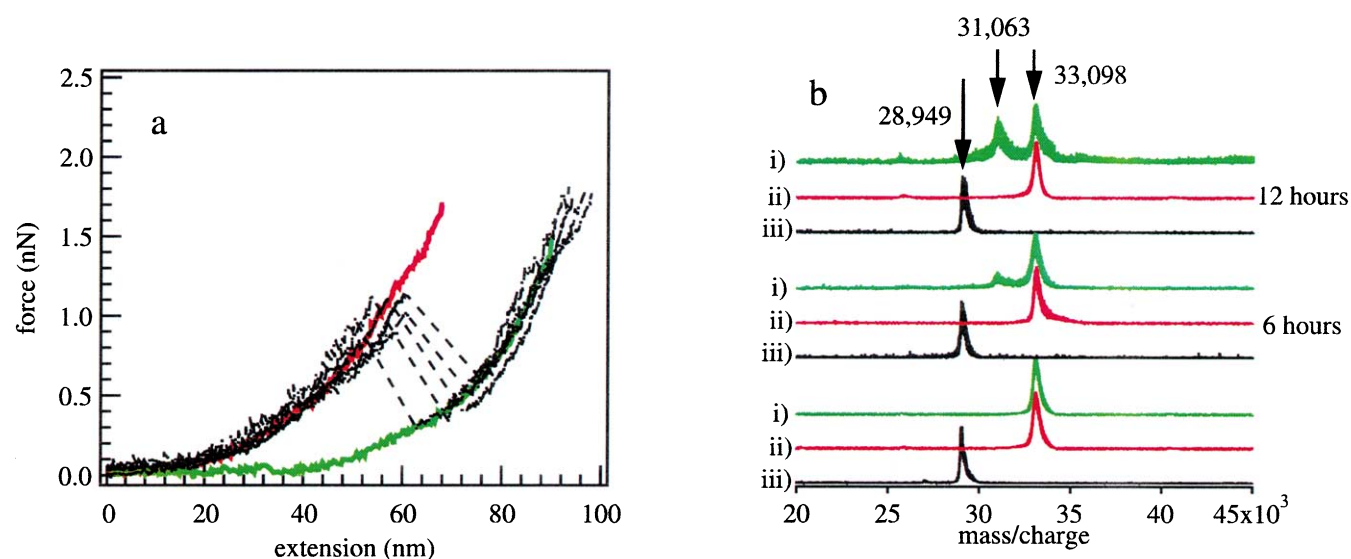


Fig. 5. a: Collection of F–E curves signifying a transition from type I to type II conformer occasionally observed during force measurement. The red and green lines represent F–E curves of type I and II, respectively. b: Partial digestion of three types of BCAII by CP-Y: (i) Type II conformer, (ii) type I conformer and (iii) wild-type BCAII. The samples were exposed to CP-Y and the digestion mixtures were subjected to MALDI TOF mass spectroscopic analysis at 6 and 12 h intervals (upper two panels). The lower panel shows the results of a control experiment without CP-Y.

some of the curves in Fig. 3 were not considered to be related to the unfolding mechanics of the protein because similar bumps were occasionally observed in reference experiments without protein molecules (Fig. 4).

The possibility that F–E curves representing type I stretching were due to simultaneous extension of multiple chains of type II molecules can be excluded because an integral multiplication of the latter F–E curves does not coincide with the former. However, occasional transitions from type I to type II curves were observed during stretching experiments (Fig. 5a), supporting that type I is mechanically convertible to type II.

3.4. Effect of inhibitor on mechanical unfolding pathway

To study the mechanical response of the type I and type II conformers to enzymatic inhibitors of BCAII, we added *p*-aminomethylbenzenesulfonamide to the protein solution during force measurements. In the presence of the inhibitor, a remarkable change was induced in the type I F–E curves indicating binding of the inhibitor to the protein, whereas type II curves remained almost unchanged (insets of Fig. 3). The new type I curves extended almost flatly in the initial 20–30 nm and then the tensile force rose abruptly from less than 200 pN to 1.7 ± 0.2 nN with 5–10 nm in further extension reaching a total extension of 30 ± 5 nm before the final rupture. The peak force at the end of the stretching corresponds to the rupture event in the covalent crosslinking system. The result is in good agreement with the mechanical response of the wild-type BCAII observed in the presence of the same inhibitor [19]. The change in the type I curves confirmed the presence of the active site in this conformer and creation of a ‘rigid core’ structure upon inhibitor binding. A partial explanation for the observed mechanical response may come from a comparison of the temperature factors of the crystal structure indicating that, after binding the inhibitor, the central part of the enzyme becomes more rigid and the outer shell more labile [5,32].

3.5. What is the structural difference between type I and II?

The mechanical response of the mutant protein was, generally speaking, consistent with what we expected when we decided to stretch BCAII from Cys-253 rather than from Cys-260, thus avoiding knot tightening under a tensile stress. Compared with a mere 13 ± 2 nm extension of the wild-type enzyme with a maximum force of 1.7 ± 0.2 nN [17,18], type I and II conformers were extended 73 and 91 nm, i.e. nearly 77 and 97% of the theoretical stretched length of the polypeptide (94 nm), respectively. This is clearly a consequence of the knot avoiding strategy. What was not expected was that the expressed protein was actually composed of two conformers with significant differences in their enzymatic activity and mechanical responses. Though unexpected, this is an interesting finding and we planned to examine the origin of these differences.

We hypothesized that the observed differences in mechanical and enzymatic properties between the two conformers resulted from a subtle structural difference due to the introduction of a Cys residue at the 253rd position leading to the complete (type I) and incomplete (type II) formation of the knot topology in the C-terminal region. This is in accordance with the fact that completion of the C-terminal folding including knot formation has been implicated as an important step in the reactivation of carbonic anhydrase [4]. Our hypothesis was supported when the reactivity of the two conformers towards CP-Y was studied. It has been reported that wild-type BCAII is poorly attacked by CP-Y [33], however, when the C-terminal tertiary structure is perturbed (as in the denatured state), it is readily hydrolyzed from the C-terminus [8]. We therefore analyzed the susceptibility of the two conformers to CP-Y by mass spectroscopy. Although the sensitivity of the mass spectrum in this region (≈ 30 kDa) is not high enough to identify the removal of individual amino acid residues, it clearly indicated that there was a gradual loss in mass until 31063 corresponding to the removal of 17 amino acids from

type II, whereas type I as well as the commercial sample remained unaffected by CP-Y after 12 h incubation (Fig. 5b). The digestion did not proceed beyond this stage, indicating that the remaining part of type II possessed a tight conformation inaccessible to CP-Y. In addition, our recent results of crystallization and X-ray analyses of type I showed that it has a very similar 3D structure to wild-type BCAII and HCAII including the C-terminal knot topology [16]. Although for the type II conformer we still lack concrete evidence, the result of the CP-Y treatment reported above showing that the C-terminal sequence of the type II conformer was freely accessible to the protease while that of type I was not, strongly supports an unknotted conformation for type II. Based on these findings, we conclude that the major differences in mechanical and enzymatic properties between the two conformers resulted from a subtle structural difference due to the different degrees of completion of C-terminal conformation, most notably, that of the knot topology.

In view of the immediate interest in the above result on the bovine enzyme of a closely related human homolog, we extended our experiments to examine a pseudo-wild-type variant of HCAII (HCAIIpwt) where a unique Cys at position 206 was replaced with Ser. It has been reported that the spectroscopic properties, stability and activity of this pseudo-wild-type are indistinguishable from those of the wild-type enzyme [34]. Two conformational isomers having similar enzymatic and mechanical properties to type I and II conformers as described for BCAII were found to accumulate in the population, when two Cys residues were introduced at the N-terminal and 253rd position as above. This indicates that a similar mechanism for knot formation might be involved in both cases, which is consistent with their similar refolding and kinetic properties as well as 3D structure. Details on the human homolog will be reported in the future.

We think our data present a more direct link between knot completion and the creation of the active site of the enzyme than the previous observations. It will be interesting to delineate the structural relationship between the knot completion and the active site formation processes through detailed crystallographic studies.

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References

- [1] Taylor, W.R. (2000) *Nature* 406, 916–919.
- [2] Takusagawa, F. and Kamitori, K. (1996) *J. Am. Chem. Soc.* 118, 8945–8946.
- [3] Boren, K., Freskgard, P.O. and Carlsson, U. (1996) *Protein Sci.* 5, 2479–2484.
- [4] Freskgard, P.O., Carlsson, U., Martensson, L.G. and Jonsson, B.H. (1991) *FEBS Lett.* 289, 117–122.
- [5] Eriksson, A.E., Jones, T.A. and Liljas, A. (1988) *Proteins* 4, 274–282.
- [6] Kannan, K.K., Notstrand, B., Fridborg, K., Lovgren, S., Ohlsson, A. and Petef, M. (1975) *Proc. Natl. Acad. Sci. USA* 72, 51–55.
- [7] Fransson, C., Freskgard, P.O., Herbertsson, H., Johansson, A., Jonasson, P., Martensson, L.G., Svensson, M., Jonsson, B.H. and Carlsson, U. (1992) *FEBS Lett.* 296, 90–94.
- [8] Carlsson, U., Henderson, L.E., Nyman, P.O. and Samuelsson, T. (1974) *FEBS Lett.* 48, 167–171.
- [9] Mitsui, K., Hara, M. and Ikai, A. (1996) *FEBS Lett.* 385, 29–33.
- [10] Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J.M. and Gaub, H.E. (1997) *Science* 276, 1109–1112.
- [11] Oberhauser, A.F., Marszalek, P.E., Erickson, H.P. and Fernandez, J.M. (1998) *Nature* 393, 181–185.
- [12] Oberhauser, A.F., Hansma, P.K., Carrion-Vazquez, M. and Fernandez, J.M. (2001) *Proc. Natl. Acad. Sci. USA* 98, 468–472.
- [13] Semisotnov, G.V., Uversky, V.N., Sokolovsky, I.V., Gutin, A.M., Razgulyaev, O.I. and Rodionova, N.A. (1990) *J. Mol. Biol.* 213, 561–568.
- [14] Henkens, R.W., Kitchell, B.B., Lottich, S.C., Stein, P.J. and Williams, T.J. (1982) *Biochemistry* 21, 5918–5923.
- [15] Ferrell, R.E., Stroup, S.K., Tanis, R.J. and Tashian, R.E. (1978) *Biochim. Biophys. Acta.* 533, 1–11.
- [16] Saito, R. (2002) Master's Thesis, Tokyo Institute of Technology.
- [17] Wang, T. and Ikai, A. (1999) *Jpn. J. Appl. Phys.* 38, 3912–3917.
- [18] Ikai, A. and Wang, T. (2000) *Jpn. J. Appl. Phys.* 39, 3784–3788.
- [19] Wang, T., Arakawa, H. and Ikai, A. (2001) *Biochem. Biophys. Res. Commun.* 285, 9–14.
- [20] Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- [21] Idiris, A., Alam, M.T. and Ikai, A. (2000) *Protein Eng.* 13, 763–770.
- [22] Pocker, Y. and Stone, J.T. (1967) *Biochemistry* 6, 668–678.
- [23] Khalifah, R.G., Strader, D.J., Bryant, S.H. and Gibson, S.M. (1977) *Biochemistry* 16, 2241–2247.
- [24] Provencher, S.W. and Glöckner, J. (1981) *Biochemistry* 20, 33–37.
- [25] Hutter, J.L. and Bechhoefer, J. (1993) *Rev. Sci. Instrum.* 64, 1868–1873.
- [26] Mitsui, K., Nakajima, K., Arakawa, H., Hara, M. and Ikai, A. (2000) *Biochem. Biophys. Res. Commun.* 272, 55–63.
- [27] Persson, M., Carlsson, U. and Bergenheim, N. (1997) *FEBS Lett.* 411, 43–47.
- [28] Galvani, M., Bordini, E., Piubelli, C. and Hamdan, M. (2000) *Rapid Commun. Mass Spectrom.* 14, 18–25.
- [29] Alam, M.T. and Ikai, A. (2001) *Appl. Phys. A* 72 (Suppl.), S121–S124.
- [30] Persson, M., Hammarstrom, P., Lindgren, M., Jonsson, B.H., Svensson, M. and Carlsson, U. (1999) *Biochemistry* 38, 432–441.
- [31] Hammarstrom, P., Persson, M. and Carlsson, U. (2001) *J. Biol. Chem.* 276, 21765–21775.
- [32] Boriack, P.A., Christianson, D.W., Kingery-Wood, J. and Whitesides, G.M. (1995) *J. Med. Chem.* 38, 2286–2291.
- [33] Nyman, P.O., Strid, L. and Westermark, G. (1968) *Eur. J. Biochem.* 6, 172–189.
- [34] Martensson, L.G., Jonasson, P., Freskgard, P.O., Svensson, M., Carlsson, U. and Jonsson, B.H. (1995) *Biochemistry* 34, 1011–1021.