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Biophysical Chemistry 116 (2005) 187 – 191

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Local rigidity of a protein molecule

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Received 25 December 2004; received in revised form 4 April 2005; accepted 5 April 2005
Available online 10 May 2005

Abstract

Distribution of soft and rigid substructures within a protein molecule has been implicated in several occasions and most recently from the imaging and indentation experiments using an atomic force microscope. In this paper, previously reported result of mechanical extension experiments on the recombinant bovine carbonic anhydrase II, Q253C, is re-analyzed to estimate the distribution of Young's modulus, *Y*, in this protein. The force vs. extension curve of the enzymatically active, type I conformer gave an estimate of *Y* increasing from 40 to 220 MPa as the polypeptide chain was extended from 10 to 75 nm indicating the presence of a rigid core structure. The enzymatically inactive type II, in contrast, gave an almost constant modulus of 55 ± 15 MPa in the same extension range in agreement with the previous proposal that it lacked a core structure.

Keywords: Protein rigidity; Protein stretching; Young's modulus; Rigid core; Flexible surface

1. Introduction

Molecular flexibility has been often emphasized as an important characteristics for proteins to function as enzymes, receptors, energy transducers, etc. Flexibility is, however, a difficult property to be numerically defined for a small and anisotropic material like a protein molecule. Various attempts to extract information on the flexibility of a protein molecule have been put forward including, (1) conformational changes observed in X-ray crystallography between the un-complexed and complexed forms of a protein with its specific ligands [1], (2) the thermal B-factors of individual atoms from X-ray crystallography [2], (3) pressure dependent chemical shift in NMR spectroscopy [3], and (4) changes in other spectroscopic measurements upon complex formation with specific ligands [4], among others. Flexibility or compliance is a complementary concept of rigidity which is also an important element of physical characteristics of a protein molecule. When a protein molecule functions as an enzyme to cleave a covalent bond in the substrate, it must keep the activated conformation of the substrate for a longer time than in free state [5]. In its activated state, the substrate is in a more or less distorted conformation with a higher energy than in the ground state. To keep such an activated conformation for a long enough time for the reaction probability to increase significantly, the enzyme must have a rigid substructure that can hold a distorted substrate. Since both flexibility and rigidity are required for a protein molecule to function as a life supporting polymer, it is most likely that it has a mechanically heterogeneous 3D structure composed of locally rigid and flexible sub-structures.

Such a heterogeneity in the mechanical design of a protein molecule is implied in the heterogeneous distribution of the thermal B-factor in its crystalline state but an actual measurement of localized rigidity or flexibility in a protein molecule as mechanical quantities has been almost impossible until the recent development of the force spectroscopic method based on the scanning probe microscopy, especially that of atomic force microscopy (AFM). AFM now allows us, in principle, to measure the mechanical rigidity of protein molecules and its local

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doi:10.1016/j.bpc.2005.04.003

variations in a direct way on the single molecule level. A protein molecule can be stretched from two defined sites on its surface, e.g., from its N- and C-termini, so that its tensile stiffness can be probed, or it can be compressed for the measurement of its apparent Young's modulus [6–13]. In this paper, we compare the results obtained from the stretching experiment with those obtained from the compressing experiments.

2. Results and discussion

2.1. Estimate of how rigid an enzyme molecule must be

To estimate the level of local rigidity required for a protein molecule to rapidly rupture or create covalent bonds as an enzyme, we must know the force required to mechanically sever a covalent bond as the upper limit of an estimate. The magnitude of the force to sever a covalent bond has been studied in two cases both giving similar values of nearly 2 nN per a single bond rupture at a given force loading rate of between 10 and 100 nN/s [14,15]. Grandbois et al. predicted that the bond ruptured most readily in their covalently bonded system was Si–C bond [14]. Other types of bonds such as C–C or C–O bonds were estimated to be ruptured with a force in the range of 2–3 nN. Alam et al. obtained a result showing that the covalently bonded system employed in their stretching experiment of bovine carbonic anhydrase (BCA II) was ruptured within 5–10 s, if the system was suspended at a tensile force of 100 pN (M.T. Alam and H. Arakawa, personal communication). Such observations, though highly preliminary yet, predict that a covalent bond may be ruptured within a short time if a tensile force of a few hundred pNs to 1 nN is continuously applied. Thus an enzyme participating in such a reaction is expected to have a sub-structure that can support the activated state of the substrate which tends to deform with a force in this range.

The importance of rigid local structure in enzymes in the above sense has been emphasized by Vanselow [5] but, of course there are other vital factors critically important for the functional expression of enzymes. Local geometry around the active site with precise arrangements of ionizable groups to relay the catalytic reaction is one and exclusion of bulk water from the active site by the concerted action of strategically positioned hydrophobic side chains is another, for example. The classical concept of the "lock-and-key" model in enzyme-substrate binding has been replaced by a more accommodating "induced-fit' model during a long history of enzymology and, nowadays, even the significance of "natively unfolded proteins" is discussed [16]. The concept of flexibility in a protein molecule has contributed enormously to our understanding of its function and structure, but for even natively unfolded proteins, some rigidity must be needed for their functional expression after binding with suitable ligands or substrates.

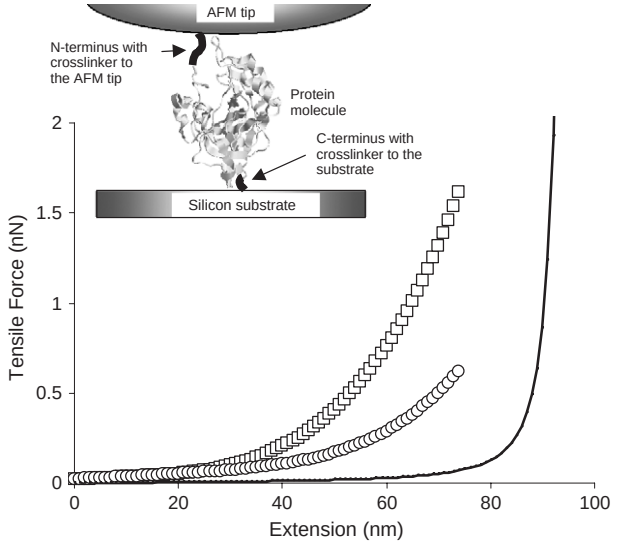

Fig. 1. F–E curves of type I (□) and II (○) forms of bovine carbonic anhydrase II using Eqs. (1) and (2), respectively, and a theoretical curve of WLC (solid curve) based on Eq.(3) with $p=0.3$ nm. The inset figure is a schematic representation of the experimental set-up for protein stretching.

2.2. F–E curve of Q253C mutant of BCA II

The result of pulling experiment on a Q253C mutant of BCA II with 260 stretchable amino acid residues performed by Alam et al. [9] is reproduced in Fig. 1 in the form of a polynomial fitting curve of the fourth order between the tensile force, F, and the extension length, E of the protein (F–E curve). The inset figure shows a conceptual view of the experimental setup for protein stretching as remodeled from Ref. [9]. The protein is covalently cross-linked to the tip and the substrate, and the cross-linking system together with the silanization reagent had an approximate total length of 2 nm and it was assumed to be flexible. This mutant protein had a cysteine residue prior to the N-terminus and a replacement of Gln253 to cysteine, which were used as cross-linking sites to the AFM tip and the substrate. Cys253 facilitated a "knot" free stretching and, when expressed in *Escherichia coli*, two conformers, enzymatically active type I and inactive type II were produced. Since the reversibility of the force curve was partially confirmed by Okajima et al. in their dynamic measurement of the same event [17], any irreversible energy dissipation is neglected in the present treatment.

The fitting curve for the enzymatically active type I used in this paper is,

$$F = 0.019332 + 0.001758E - 5.4843 \times 10^{-5}E^2 + 1.7224 \times 10^{-6}E^3 + 3.5525 \times 10^{-8}E^4 \quad (1)$$

and for the enzymatically inactive type II,

$$F = 0.023787 + 0.0007233E + 6.4902 \times 10^{-5}E^2 - 2.3049 \times 10^{-6}E^3 + 3.7383 \times 10^{-8}E^4 \quad (2)$$

As a reference, the following fitting curve of the worm like chain (WLC) [18] is also used

$$F = (k_B T/p)\left[0.25(1-(E/E_0))^{-2} - 0.25 + (E/E_0)\right] \quad (3)$$

where, k_B, T, E_0 and p are, respectively, the Boltzmann constant, temperature in Kelvin, total contour length, and the persistence length of the chain.

First, the derivatives of Eqs. (1) and (2) which give the chain stiffness were calculated. The stiffness of the type I protein increased from almost zero in the beginning of extension to about 80 pN/nm at an extension of 70 nm and that of type II from zero to about 30 pN/nm. These values compare favorably with the reported range of average stiffness of 15–50 pN/nm for α_2-macroglobulin by Mitsui et al. [6]. Although it is qualitatively quite clear that type I has a more rigid core structure compared with type II simply by observing the result in Fig. 1 and also from the comparison of stiffness, it is more desirable to extract some material constant independent of the size of the sample so that quantitative comparison will be possible with the rigidity of other proteins as well as with non-protein materials. For this purpose, Young's modulus is a most suitable mechanical parameter to be deduced.

To obtain an estimate of Young's modulus of the sample from the stretching curves in Fig. 1, we considered the following model which is also schematically explained in Fig. 2.

A protein molecule before stretching is approximated by a rod of length L_0 and cross-sectional area of A. Protein segments are folded in the rod so that the effective length of each segment to the direction of stretching is $1/b$ of the actual length. If we assume $L_0 = 6$ nm from the crystal structure of BCA II, $A = 6$ nm^2 since the molecular volume of the protein is about 36 nm^3 [19]. Upon application of a tensile force of F, the stress is F/A and the apparent extension of the chain after a local unfolding of the protein is ΔL. A most conspicuous contribution to ΔL comes from the realignment of main chain segments to the direction of tensile force and only a small part contributes to the real strain which is more likely to be confined to local deformations (such as strained internal rotations and/or small deformations in covalent bond angles and bond lengths, for example) with a cumulative length of $\Delta L/a$ with a (> 1) as a parameter. $\Delta L/a$ is likely to be large for the extension of less stiff local structures and small for that of stiffer ones, thus in the case of BCA II, a must be larger in the initial stage of extension than in the later stage according to the change in stiffness described above. As the chain is extended, the length of the unfolded part of the protein molecule is shortened from L_0 to $(L_0 - \Delta L/b)$. Thus the strain is equal to $(\Delta L/a)/(L_0 - \Delta L/b) = \Delta L/[(a/b)(bL_0 - \Delta L)]$. When the chain is fully elongated with $\Delta L = \Delta L_{max}$, the denominator should be reduced to zero, therefore, $b = \Delta L_{max}/L_0 \approx 96/6 = 16$. The value of a is more difficult to estimate because we do not know where and how the local strains accumulate and it is expected to be not constant as discussed above but here we assume that a has a constant

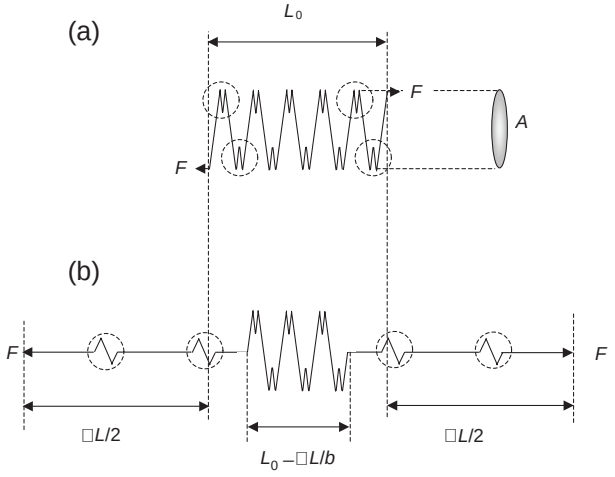

Fig. 2. Model used for extracting Young's modulus from the $F-E$ curves in Fig. 1. Before stretching, the protein is in the native state with a length L_0 and a cross sectional area of A (a). With an application of tensile force, F, the protein is stretched to a total length of $L_0+\Delta L$, without a change in the cross sectional area (b). In (b), the sample is extended symmetrically to both directions only for simplicity in presentation. The real contribution to stress and strain is confined to circled localized area which has a much shorter length of $\Delta L/a$ than apparent extension length of ΔL, i.e., $a \gg 1$.

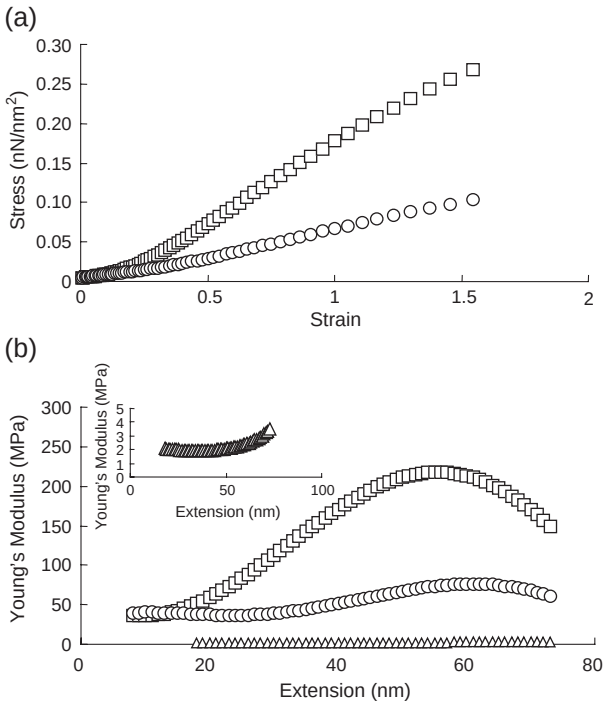

Fig. 3. Calculated relation between stress and strain as defined in the text (a) for type I (□) and type II (○) conformers; and apparent Young's modulus calculated from the result in (a) as a function of chain stretching for type I (□), type 2 (○) and for WLC (Δ) (b). The inset figure in (b) is an expanded version for WLC. For type I and II parameters are common: $L_0 = 6$ nm, $A = 6$ nm^2, and $a/b = 2.0$. For WLC: $p = 0.3$ nm, $L_0 = 17$ nm, $A = 30$ nm^2, and $a/b = 1.5$.

value and $a/b=2$, for simplicity. The value of Young's modulus thus obtained is directly proportional to the assumed value of a/b and, for a larger value of a/b, we should expect proportionally larger values of Young's modulus. In the following analysis, Young's modulus was calculated between 10 nm and 75 nm in extension for the part of the $F–E$ curve in Fig. 1. Experimentally most reliable range should be 30–70 nm.

The Young's moduli, Y, of two types of BCA II can then be obtained as derivatives of the stress against strain plot (i.e., $F/A=Y\Delta L/[a/\mathrm{b}(bL_0-\Delta L)]$) given in Fig. 3a according to the definition [20]. Numerically obtained Young's moduli are given in Fig. 3b vs. chain stretching rather than strain. In our model, only the length of the original sample was shortened as the chain is extended but the cross-sectional area was kept constant. This may be partially justified for a protein rich in β-sheet because the width of the sheet would not change during the stretching process. The result indicates that the Young's modulus of type I ranges between 40 and 220 MPa and that of type II is approximately constant at 55 ± 15 MPa with a small but notable increase in the large extension range. The gradual increase of the modulus of type I from ~40 MPa to ~220 MPa in accordance with a chain elongation from 10 nm to 70 nm can be taken as a sign of progressive exposure of a more rigid core structure. The presence of such a core structure has been implicated for BCA II [9]. The starting value of the Young's modulus of type I compares favorably with a recently reported value of 75 ± 10 MPa by Afrin et al. [13] for the wild type of BCA II based on a modified Hertzian compression analysis. The Young's modulus of type II is less than that of type I as expected from its incompletely folded conformation but larger that of the molten globule state of the same protein as reported by Afrin et al. [13].

The marked decrease of the calculated Young's modulus of type I beyond the extension of 65 nm is quite interesting as it implies softening of the rigid substructure after the maximum rigidness was reached. For an extensive discussion of this phenomenon based on the detailed molecular structure needs a further experimental confirmation.

For a denatured BCA II in 6 M guanidinium chloride, Afrin et al. [13] gave a diameter of 17 nm which leads to the volume expansion factor of ~30 from the native state. Consequently a denatured BCA II molecule is three times larger in linear scale than a native molecule, and the cross-sectional area of the former is ten times larger than that of the latter. It is noteworthy, therefore, that when the same mechanical model was applied to the $F–E$ curve of WLC, with $L_0=17$ nm, $A=60$ nm^2 and $a/b=1.5$, an almost constant value of $Y\sim1$ MPa was obtained. If the effective cross-sectional area of a randomly coiled chain is set to 30 nm^2, Y becomes approximately 2 MPa which compares favorably with the previously reported value of 2 ± 0.5 MPa for denatured BCA II in 6 M guanidinium chloride [13].

The reported values of the Young's modulus of proteins range between 200 MPa and several GPa [21,22]. Some of the high values obtained from the bulk compressibility (β) originate from the Poisson's ratio used in the conversion formula, $Y=3\beta^{-1}(1-2v)$, where v was often set to 0.3–0.33. Since v is normally between 0 and 0.5, Y becomes sensitively dependent on v when the latter is close to 0.5. For example, if $v=0.48$, which is not impossible for some proteins, Y becomes 1/10 of when v is assumed to be 0.3. Thus, depending on the value of v, Y can be easily calculated to be in the 100–200 MPa range.

The method used in this paper to deduce Young's modulus from the polymer extension curve needs further refinement especially in the estimate of a which is most likely a non-linear function of extension. In the present stage, the present method at least gave results in agreement with our previously reported values on the same protein.

Acknowledgements

I would like to thank Dr. Rehana Afrin for critically reading the manuscript. This work was partially supported by financial aids from JSPS (# 15101004) and from JST (SPM simulator project).

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