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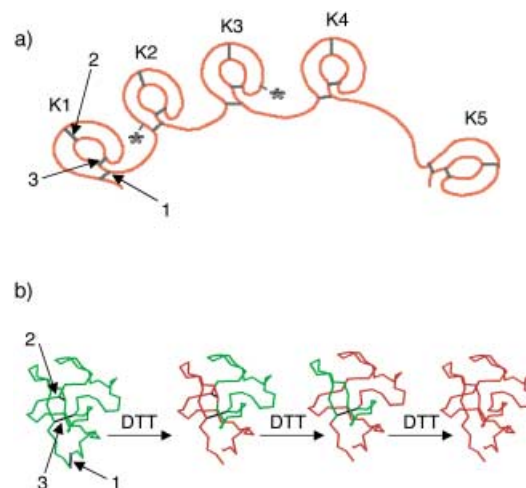


Figure 1. a) Schematic representation of the Ang(1–5) structure with the triple-loop topology of its five Kringle domains (K1–K5), determined in each case by a sequence of three disulfide bonds: cys 1–cys 80 (1), cys 22–cys 63 (2), and cys 51–cys 75 (3). The two asterisks (*) indicate the position of an additional, inter-Kringle disulfide bond. b) Pathway of the sequential reduction of the most exposed (1) and then of the more interior (2) and (3) bonds. This reduction pathway leads to gradually lengthening topological loops (red) that are exposed to the force exerted by the SFM tip at the two ends of the Kringle domain.

The Mechanical Properties of Human Angiostatin Can Be Modulated by Means of Its Disulfide Bonds: A Single-Molecule Force-Spectroscopy Study**

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Human angiostatin 1–5 [Ang(1–5)], a 57 kDa proteolytic fragment of human plasminogen, consists of five compact globular modules called Kringle domains with very similar gross architecture and remarkable sequence homology. They are built around a hydrophobic core and exhibit a triple-loop topology defined by three internal disulfide bonds: cys 1–cys 80 (1), cys 22–cys 63 (2), and cys 51–cys 75 (3) (Figure 1 a).^[1] Here we report on a single-molecule force-spectroscopy study that shows how the redox environment can control the topology and mechanical properties of angiostatin by modifying the extent of pairing of the internal disulfide bonds.

Ang(1–5) molecules were deposited on a polystyrene surface; the tip of a scanning force microscope (SFM) was brought into contact with this surface and retracted after reaching a force of 1 nN. This cycle was repeated many hundreds of times, first under phosphate buffer saline (PBS),

and then under different reducing conditions by using dithiothreitol (DTT) in concentrations ranging from 5 to 100 mM. Whenever the tip physically adsorbed and picked up a chain segment of an Ang(1–5) molecule, a molecular bridge was established between the two moving surfaces, and the force acting on this bridge was plotted versus the tip displacement in force curves such as those in Figure 2.

Two representative curves, recorded under PBS buffer prior to any addition of reducing agent, are shown in Figure 2 a. Their profiles are very irregular and typical of deadsorption processes of globular or short inextensible proteins.^[2] These curves do not exhibit the characteristic sawtooth pattern that was previously recognized to be typical of multimodular proteins, and first demonstrated by Gaub and co-workers to result from the sequential stepwise unfolding of their individual domains.^[3] This was due to the fact that Ang(1–5) disulfide bonds acted as mechanical barriers to mechanical unfolding. In fact, being covalent bonds, they can withstand forces up to a few nanonewtons,^[4] that is, much higher than those involved in the experiments reported here, which were in the range of 30–150 pN. Therefore, the mechanical stress exerted on the disulfide bonds, both under nonreducing and reducing conditions (see below), could never succeed in breaking any of them.

The PBS buffer in the fluid cell was then replaced with a 5 mM DTT solution, and force curves were recorded 30 min after DTT injection. This experiment was also performed with 50 and 100 mM DTT, and in the latter case the incubation time was doubled. The force curves recorded under these three sets of reducing conditions exhibited the characteristic sequences of sawtooth peaks (Figure 2 b–d). Each peak resulted from the unfolding of a single module. Each Ang(1–5) module could unfold until an internal unreduced disulfide bond was met along its unfolding pathway. In fact only the disulfide

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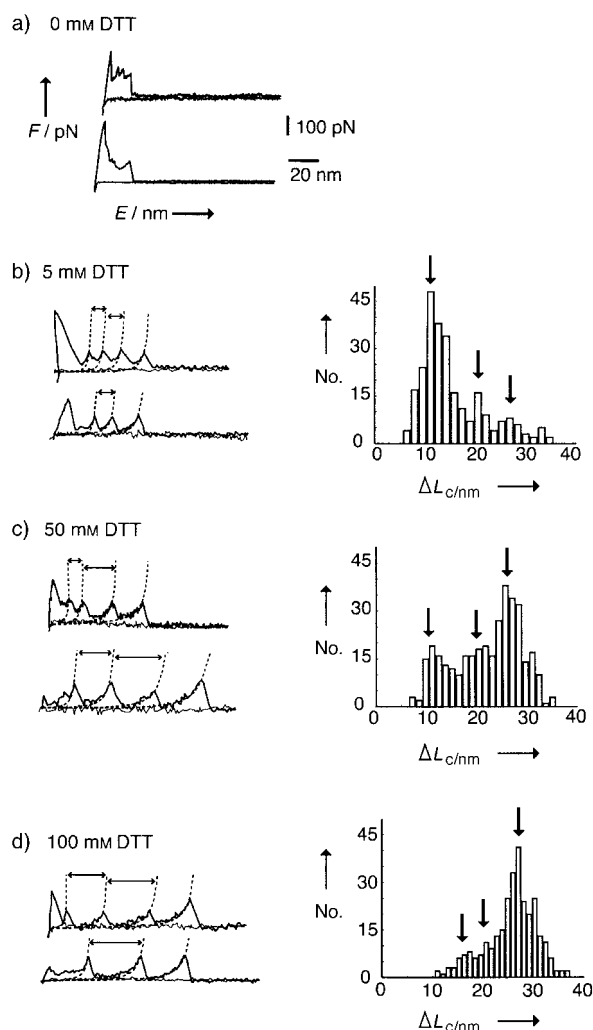


Figure 2. Left: Representative force F vs extension E curves recorded in PBS buffer prior to addition of the reducing agent (DTT) into the fluid cell (a) and in PBS buffer with 5 (b), 50 (c), and 100 mM DTT (d). The length increments of the protein after each unfolding event were estimated by fitting the force curves with the WLC model and assuming a persistence length of 0.36 ± 0.02 nm. This value is in the range of values determined for a modular protein composed of immunoglobulin domains containing an internal disulfide bond.^[5] Right: the relative histograms of the length increments ΔL_c after each unfolding event; they are characterized by three main peaks (↓) that correspond to the different thiol/disulfide intermediates.

bonds that were already chemically opened by DTT no longer acted as points of resistance.^[5] The number of peaks in each force curve ranged from two to four; it was determined by the number of Kringle domains contained in the molecular bridge under tension in each approach–retract cycle, and therefore by the position along the chain at which the protein was picked up by the tip.

As long as the reduction proceeded inside the Kringle domains (Figure 1b), additional topological loops were exposed to the force exerted by the SFM tip, and the length of the resulting unfolded domains increased (Figure 2). The spacing between two subsequent peaks reflects the number of amino acids that each unfolding event added to the total length of the molecular bridge between the tip and the polystyrene substrate.^[6] The behavior of polymers under

stress can be predicted by using the wormlike-chain (WLC) model of entropic polymer elasticity.^[7] This model made it possible to estimate the length increase after each unfolding event, as shown in Figure 2b–d.

The distributions of these length increments (Figure 2, right) were characterized by three peaks centered at about 12, 20, and 27 nm. The heights of these peaks depended on the reducing conditions: after reduction with 5 mM DTT the highest peak was that at about 12 nm, and the other two were much smaller; after reduction with 100 mM DTT the highest was that at 28 nm. The heights of the three peaks were on the same order of magnitude after reduction with 50 mM DTT.

On the basis of the Kringle domain structure (Figure 1b) we could assign these peaks to different thiol/disulfide intermediates. The reduction of the most exposed cys1–cys80 (first) disulfide bond of each Kringle domain opens a topological loop of 40 amino acids that corresponds to an increase in length of 14 nm (Figure 2b). On increasing the concentration of the DTT, reduction can proceed further to include the second and third bonds, and longer sections of the domain can unfold, with length increments of 21 and 29 nm, respectively (Figure 2c,d).

Note that uncertainty still remains in these assignments. Since the unfolding of each domain is blocked by the first unreduced disulfide bond, the peak at about 12 nm could also correspond to domains in which the first and third disulfide bonds, but not the second, were reduced. The disulfide bond between Kringle domains 2 and 3 (Figure 1a) was found to play no role in this dependence of domain topology on the reducing conditions. As an interchain bridge, it is very likely that it was already completely reduced with 5 mM DTT.^[8]

The distributions of intermediates obtained under the different reducing conditions could be estimated from the heights of the three main peaks (see arrows) in the length-increment histograms of Figure 2 only after we had ruled out a potential artifact. These distributions could have been modified during the mechanical unfolding and refolding cycles because of possible thiol/disulfide interchange reactions and/or because of increased accessibility conferred on interior disulfide bonds. We could rule out major contributions from these two possible side reactions because the histograms in Figure 2 did not change significantly after we had chemically trapped the free thiol groups with iodoacetamide. Treatment with this alkylating agent was carried out after reduction with DTT and before recording the force curves in PBS buffer, in this case in the absence of any reducing agent. The length increment distributions of the histograms in Figure 2 can thus be considered to reflect those of intermediates obtained under the different reducing conditions.

The presence of partially reduced intermediates in large amounts after reduction with 50 mM DTT, and still in significant quantities after treatment with 100 mM DTT, demonstrates that the Kringle domain disulfide bonds are not reduced by DTT with an “all-or-none” mechanism, as in other proteins,^[9] but in a sequential manner starting from the most exposed bond (cys1–cys80). This result is in perfect accord with gel-filtration studies on reductive cleavage and denaturation of Kringle domains.^[10]

To our knowledge this is the first report on the use of single-molecule atomic-force spectroscopy to study the reduction pathway of multiple disulfide bonds in proteins and to evaluate the distributions of intermediates obtained under different reducing conditions without separating them and without any blocking and fractionation steps. The characterization of these intermediates has so far been accomplished by first blocking them with reagents such as alkylalkanethiosulfonates and then by fractionation by ion-exchange chromatography, 2D or capillary gel electrophoresis, or gel filtration.^[11] The determination of thiol groups and disulfide bonds in a polythiol systems has always been a very challenging problem.^[12]

The single-molecule force-spectroscopy data presented here show: 1) how a redox environment can modulate the mechanical properties of angiotensin; 2) how this modulation relies, at the single-molecule level, on the extent of reduction of the disulfide bonds; and 3) how, at the level of a large sample of molecules, the distribution of the different thiol/disulfide intermediates after reduction can be estimated by statistical analysis of the force curves.

Experimental Section

Ang(1–5), purchased from Calbiochem with purity greater than 95 %, was adsorbed onto a Petri dish from a 20–100 µg/mL PBS (pH 7.4) buffer solution for 3–5 min and then rinsed with the same buffer. The Petri dish was previously cleaned and exposed to high-energy nitrogen plasma for 15 min. The force-spectroscopy experiments were performed with a Nanoscope IIIa, Digital Instrument, Santa Barbara, CA, with silicon nitride (Si_3N_4) probes (Microlevers, Thermomicroscopes) with a spring constant of 40–55 pN nm⁻¹, determined by a thermal oscillation method.^[13] A velocity of 1 µm s⁻¹ during tip approach and retraction, a loading force of about 1 nN, and a contact time between the tip and the surface of 200 ms were used. We started recording the force curves 30 min after injection of 5 or 50 mM DTT (Aldrich), and 60 min after injection of 100 mM DTT into the fluid cell.

The same experiment was repeated with the following two modifications: First, after reduction with 5, 50, or 100 mM DTT, we injected a 100 mM solution of iodoacetamide (Fluka) into the fluid cell, and allowed it to stand for 20 min. Second, the iodoacetamide solution was subsequently replaced with PBS, under which the force-spectroscopy measurements were then carried out.

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Colorimetric and Fluorometric Detection of Nucleic Acids Using Cationic Polythiophene Derivatives**

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Simple and reliable sequence-specific methods are needed for the rapid detection of oligonucleotides, to diagnose infections and various genetic diseases. In this regard, interesting optical and electrochemical DNA-hybridization sensors have been proposed.^[1–5] The recognition capabilities of DNA are well established but, to transduce the recognition event into a physically measurable value, a fluorescent or electroactive tag is often bound to the analyte. Electrochemical and optical sensors based on conjugated polymers have also been reported^[6–9] and some oligonucleotide-functionalized conjugated polymers can also transduce hybridization events into an electrical signal without labeling of the oligonucleotide target.^[10–12] The detection relies on a mod-

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