Correlating Conformational Dynamics with the Von Willebrand Factor Reductase Activity of Factor H Using Single Molecule Force Measurements

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ABSTRACT: Activation of proteins often involves conformational transitions, and these switches are often difficult to characterize in multidomain proteins. Full-length factor H (FH), consisting of 20 small consensus repeat domains (150 kD), is a complement control protein that regulates the activity of the alternative complement pathway. Different preparations of FH can also reduce the disulfide bonds linking large Von Willebrand factor (VWF) multimers into smaller, less adhesive forms. In contrast, commercially available purified FH (pFH) has little or no VWF reductase activity unless the pFH is chemically modified by either ethylenediaminetetraacetic acid (EDTA) or urea. We used atomic force microscopy single molecule force measurements to investigate different forms of FH, including recombinant FH and pFH, in the presence or absence of EDTA and urea, and to correlate the conformational changes to its activities. We found that the FH conformation depends on the method used for sample preparation, which affects the VWF reductase activity of FH.
Dulbecco transfected into HEK 293 cells using Lipofectamine 2000 ligated to pSecTag2B his tag vector (Invitrogen, Grand Island, Manassas, VA) to generate FH. Full-length human FH has been reported. We used human embryonic kidney (HEK) 293 cells [American Type Culture Collection (ATCC), Houston, TX] to inactivate VH disease and reductase activity for VWF multimers. This hepatitis C virus (HCV) and human immunodeficiency virus (HIV) was released from the HEK (Enzo, Farmingdale, NY). rFH released from the HEK structure and reductase activity for VH multimers. This type of solvent–detergent (SD) treatment of plasma-derived components has been used in transfusion therapy to inactivate viruses with lipid envelopes [hepatitis B virus (HBV) and hepatitis C virus (HCV) and human immunodeficiency virus (HIV)].

■ EXPERIMENTAL METHODS

The production of rFH using baculovirus33 or yeast34 systems has been reported. We used human embryonic kidney (HEK) 293 cells [American Type Culture Collection (ATCC), Manassas, VA] to generate FH. Full-length human FH cDNA was generated by polymerase chain reaction with 5′ template. After confirming the sequence, the amplified DNA fragment was digested with Not I (5′) and Xho I (3′) and ligated to pSecTag2B his tag vector (Invitrogen, Grand Island, NY). The plasmid containing full-length FH cDNA was transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), which were maintained in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1 mM sodium pyruvate 1% minimal essential medium-nonessential amino acids, and the selection agent, Hygromycin B (250 μg/mL) (Enzo, Farmingdale, NY). FH released from the HEK 293 cells into the culture media was purified using his-select Ni2+-charged aminated agarose gel (Sigma Chemicals, St. Louis, MO) that binds to histidine-tagged rFH. Briefly, serum-free conditioned cell culture medium was purified using 6X his-select resin. His-tagged proteins were then eluted using 250 mM imidazole. pFH is commercially available (Comptech, Tyler, Texas and Quidel, San Diego, CA). The pFH has been chemically treated to maintain stability for commercial purposes.

FH molecules were equilibrated at 37 °C prior to being deposited onto a fresh gold surface at room temperature. All experiments were done in a fluid environment in phosphate-buffered saline (PBS) buffer. Segments of the FH molecules were elongated onto the substrate with pulling velocities ranging from 500 nm/s to 2 μm/s.

To test for FH-related VH-reducing activity, soluble VH multimers (containing ultralarge VH forms) released from human umbilical vein endothelial cells (HUVEC) were mixed with the various types of FH (pFH, rFH, pPH + urea, pFH + EDTA; 50/50 vol/vol) and incubated at room temperature for 15 min. The mixture was denatured using 8 M urea-Tris-1% sodium dodecyl sulfate (SDS), electrophoresed into SDS-1% agarose gel, and electrotransferred onto an immobilon poly(vinylidene difluoride) (PVDF) membrane (Millipore Corporation, Billerica, MA). The transferred protein was detected using rabbit anti-human VH (Ramco Laboratories, Houston, TX) and a secondary goat anti-rabbit antibody linked to horse radish peroxidase (HRP) (Thermo Scientific, Rockford, IL) and then chemiluminescent reagent (Thermo Scientific). Results were recorded using a BioRad Gel Doc XR imager (BioRad Laboratories, Hercules, CA).

The rFH and pFH samples were electrophoresed into a 7% nonreduced SDS-polyacrylamide gel, electrotransferred onto a PVDF membrane, and detected using goat anti-human FH (Complement Technologies, Tyler, TX) and a secondary rabbit anti-goat antibody linked to HRP (Thermo Scientific, Waltham, MA).

Ellman’s reagent (5,5-dithiobis-2 nitrobenzoic acid) (Sigma, Chemicals, St. Louis, MO) was used to quantify thiol groups in the samples of rFH (0.905–1.420 μg/mL) and pFH (100 μg/mL) by the absorption measurement. Free thiols were calculated from a standard curve constructed using known cysteine concentrations in a range of 0.125–1.5 mM. Iodoacetic acid (1 mM) was used to inhibit free thiols and blocked the disulfide bonds to prevent reformation (n = 4).

SD treatment of rFH consisted of treating his-tagged rFH with 1% solvent (tri-n-butyl phosphate) and 1% detergent (Triton-X) for 4 h at 30 °C to stabilize the VH-reducing activity and to eliminate HIV, HBV, and HCV. Added reagents were removed by extraction with 5% soybean oil, high-speed centrifugation, chromatography on Prep C18 resin, dialyzed, and concentrated.7 The amount of rFH before and after SD treatment was quantified using an enzyme-linked immunoassay.

■ RESULTS

We pulled single FH molecules using AFM and acquired their force-extension curves as shown in Figure 1. Typical force–extension curves of pFH and rFH, using a pulling speed of 0.5 μm/s, showed characteristic saw-tooth patterns resulting from protein domain unfolding.20,24 The unfolding peaks in the force curves were fitted with the worm-like chain (WLC) model17–19 as follows:

$$F(x) = \frac{k_B T}{L_p} \frac{1}{4(1-x/L_c)^2} + \frac{x}{L_c} - \frac{1}{4}$$

where x is the distance; F is the force; $k_B$ is the Boltzmann constant; T is the temperature; and $L_p$ and $L_c$ are the persistence length and contour length, respectively.

Histograms of the force peaks were compiled, and distributions were fitted to a Gaussian curve. The peaks of these Gaussian curves represent the most probable values. The force of the last peak in each force–extension curve is not included in the histogram because it corresponds to the detachment force from the substrate or AFM tip. The

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The distributions of unfolding forces and changes in contour length are shown in Figure 2. The change in contour length determined using $\Delta L_c$ corresponds to the added length from domain unfolding and is proportional to the number of amino acids per domain. $\Delta L_c$ ranges from 21 to 32 nm, which is consistent with the unfolding of a single 60-residue sushi domain of FH using 0.36 nm as the length of a residue. The unfolding force is also consistent with those of protein domain unfolding. The velocity-dependent unfolding forces of different forms of FH, shown in Figure 3, demonstrate that the peak unfolding forces of FH with VWF reductase activity (rFH, pFH + urea, pFH + EDTA) are consistently lower than that of untreated pFH, which has no VWF reductase activity. The difference in unfolding peak forces suggests different conformations, which results in different transition states.

Free thiols were quantified using Ellman’s reagent ($5,5’$-dithio-bis-2-nitrobenzoic acid). pFH contains fewer free thiols compared to rFH. rFH (0.905–1.420 $\mu$g/mL) at 1/100 of the concentration of pFH (100 $\mu$g/mL) has about 5-fold more exposed free thiols than pFH. pFH treated with either 1 M urea or 10 mM EDTA showed 25–40% increase in free thiols. However, the absolute number of free thiols remained small.

Figure 4 demonstrates that pFH migrates into SDS-polyacrylamide gels with a higher mobility (apparent $M_r = 130$ kD) than rFH (apparent $M_r = 150$ kD). pFH in PBS (310 ng/well final concentration) had an apparent $M_r$ of 130 kD in an unreduced SDS-7% polyacrylamide gel. After incubation overnight at 4 °C with either 10 mM EDTA or 1 M urea (that had been adjusted to pH 7.6), the apparent $M_r$ values of both chemically treated pFH samples were shifted to a slower migration with an apparent $M_r$ of 150 kD. When untreated pFH (31 $\mu$g/mL final concentration) was mixed 1:1 with soluble HUVEC-secreted VWF multimers (2–3% of normal human plasma VWF antigen), little or no VWF reductase activity was observed. In contrast, when VWF multimers were mixed 1:1 with either urea-treated pFH or EDTA-treated pFH, the chemically treated pFH showed VWF-reducing activity. Incubation with either urea or EDTA alters the conformation of pFH, slows the migration of pFH, and imbues the treated pFH with VWF reductase activity. This alteration in the
All of these rFH proteins, SD-treated or not, had apparent molecular mass (Mr) = 150 kD and detergent (Triton X-100) for 4 h at 30 °C, forced unfolding of di-molecule force studies. We compared data from the AFM soluble VWF multimers. Full-length FH determines the capacity of FH to reduce large VWF multimers. These are characteristics that are similar to those of rFH (treated or untreated with SD). Our data suggest that the conformation of pFH occurs in association with its shift to slower mobility (apparent Mr = 150 kD, which is similar to that of rFH).

After treatment of rFH with solvent (tri-n-butyl phosphate) and detergent (Triton X-100) for 4 h at 30 °C, the VWF reductase activity of the treated rFH was not changed (Figure 5). All of these rFH proteins, SD-treated or not, had apparent Mr values of 150 kD and VWF reductase activity when mixed with urea or EDTA converts the conformation of pFH to urea or EDTA. The other forms of FH evaluated in this study that either already have (rFH) or can be chemically altered (pFH + urea or pFH + EDTA) to obtain VWF reductase activity are in a less compact state and are less resistant to forced unfolding (Figure 6).20,45

Our single molecule AFM experiments indicate that FH prepared by different methods has different conformations that determine its capacity to reduce VWF multimers. Furthermore, the VWF reductase activity of rFH is not affected by SD treatment that is used to inactivate lipid envelope viruses. This latter finding may prove useful in the preparation of rFH or other full-length forms of FH with VWF reductase activity for therapeutic use.

**DISCUSSION**

Our results demonstrate that pFH has a smaller apparent molecular mass (130 kD), fewer exposed free thiols, and little or no reductase activity for soluble VWF multimers. Treatment with urea or EDTA converts the conformation of pFH molecules into a slower gel migration position at apparent Mr = 150 kD (with a slight increase in exposed free thiols) and the capacity to reduce soluble VWF multimers. These are characteristics that are similar to those of rFH (treated or untreated with SD). Our data suggest that the conformation of full-length FH determines the capacity of FH to reduce large soluble VWF multimers.

This interpretation was supported by the results from single molecule force studies. We compared data from the AFM forced unfolding of different forms of FH. The change in contour length of pFH without VWF reductase activity was greater than that of other forms of FH (rFH, pH + urea, and pFH + EDTA) that have VWF reductase activity. These results suggest that the domains of untreated pFH are in a more compact state than the domains of the other forms of FH with VWF reductase activity. A less compact conformation, essential for VWF reductase activity, can be induced in pFH by exposing the pFH to urea or EDTA. The other forms of FH evaluated in this study that either already have (rFH) or can be chemically altered (pFH + urea or pFH + EDTA) to obtain VWF reductase activity are in a less compact state and are less resistant to forced unfolding (Figure 6).

**CONCLUSIONS**

Our single molecule AFM experiments indicate that FH
that is similar to rFH in conformation and VWF reductase activity. Urea or EDTA switches pFH to a less compact form of FH (pathway 1), whereas the domain unfolding of rFH follows a different pathway (pathway 2). The barrier height of pathways 1 and 2 corresponds to the different unfolding peak forces. Treatment of urea or EDTA switches pFH to a less compact form of FH (pathway 3) that is similar to rFH in conformation and VWF reductase activity.

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