Thrombosis Research 132 (2013) e48–e53

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Hydrodynamic characterization of recombinant human fibrinogen species

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ARTICLE INFO

Article history:
Received 17 January 2013
Received in revised form 21 March 2013
Accepted 1 April 2013
Available online 30 April 2013

Keywords:
Recombinant Fibrinogen
Blood Coagulation
Analytical Ultracentrifugation
Hydrodynamics

ABSTRACT

Introduction: Fibrinogen is a key component of the blood coagulation system and plays important, diverse roles in several relevant pathologies such as thrombosis, hemorrhage, and cancer. It is a large glycoprotein whose three-dimensional molecular structure is not fully known. Furthermore, circulating fibrinogen is highly heterogeneous, mainly due to proteolytic degradation and alternative mRNA processing. Recombinant production of human fibrinogen allows investigating the impact on the three-dimensional structure of specific changes in the primary structure.
Methods: We performed analytical ultracentrifugation analyses of a full-length recombinant human fibrinogen, its counterpart purified from human plasma, and a recombinant human fibrinogen with both Aα chains truncated at amino acid 251, thus missing their last 359 amino acid residues.
Results: We have accurately determined the translational diffusion and sedimentation coefficients ($D^0_{t(20,w)}$, $s^0_{(20,w)}$) of all three species. This was confirmed by derived molecular weights within 1% for the full length species, and 5% for the truncated species, as assessed by comparison with SDS-PAGE/Western blot analyses and primary structure data. No significant differences in the values of $D^0_{t(20,w)}$ and $s^0_{(20,w)}$ were found between the recombinant and purified full length human fibrinogens, while slightly lower and higher values, respectively, resulted for the recombinant truncated human fibrinogen compared to a previously characterized purified human fibrinogen fragment X obtained by plasmin digestion.
Conclusions: Full-length recombinant fibrinogen is less polydisperse but hydrodynamically indistinguishable from its counterpart purified from human plasma. Recombinant Aα251-truncated human fibrinogen instead behaves differently from fragment X, suggesting a role for the Bβ residues 1–52 in inter-molecular interactions. Overall, these new hydrodynamic data will constitute a reliable benchmark against which models of fibrinogen species could be compared.

Introduction

Fibrinogen (FG) is a high molecular weight (~340,000), very elongated (length ~45 nm) plasma glycoprotein playing a central role in the blood coagulation system of vertebrates. FG is also implicated in various pathologies like thrombosis, hemorrhage, and cancer [1–3]. In plasma, FG is a heterogeneous mixture of several variants that differ mainly in primary amino acid structure. At the basic level, all variants are composed of two pairs each of three different chains (Aα, Bβ and γ) whose N-termini reside in a central globular domain ("E" region), from which they symmetrically depart forming two triple coiled-coil connectors held in register by two "disulfide rings" [4–7]. At the end of each connector, after the second disulfide ring, the Bβ and γ chains C-terminals fold separately forming two outer globular domains ("D" regions), while the Aα chains revert their direction forming a fourth coiled helix at least up to residue Aα200 and Aα219 in the human and chicken species, respectively, as determined by X-ray crystallography [7–9]. The structure and location of the remaining 411 C-terminal residues (human numbering) of the Aα chains (AαC regions) is still undetermined, and has been alternatively proposed to behave as a free-swimming appendage [4,8,9], or to form a fourth globular domain on top of the E region [10–14]. Only a small portion of the bovine species C-terminus (corresponding to residues Aα425-502 in the human species) has been proved to be

Abbreviations: FG, fibrinogen; FpA, fibrinopeptide A; FpB, fibrinopeptide B; AUC-SV, analytical ultracentrifugation sedimentation velocity; hpHMW-FG, human plasma high molecular weight FG; hpFrX-FG, human plasma FG fragment X; cpFG, chicken plasma FG; hrHMW-FG, human recombinant high molecular weight FG; hrα251-FG, human recombinant FG with Aα chains truncated after residue 251.

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http://dx.doi.org/10.1016/j.thromres.2013.04.005

at least partially structured by NMR and other biophysical methods [15–18]. An additional unique feature of the AαC regions is the presence among species of a variable number (*e.g.*, eleven in the human, none in the chicken) of 13-amino acids repeats [19–21], located between the end of the fourth coiled helix and the partially structured region. Furthermore, the AαC region is also highly susceptible to proteolytic degradation (in 25 % of the plasma FG the C-terminus of the Aα chain is degraded on one side of the molecule, and in 5% both Aα chains are degraded), which, coupled with other post-transcriptional and post-translational modifications like partial AαSer3 and other Ser/Thr residues phosphorylation [22], renders circulating FG highly polydisperse [23]. The N-terminal 16 and 14 amino acid residues of the Aα and Bβ chains, respectively, are cleaved off by thrombin during FG enzymatic activation yielding fibrin monomer ($\alpha_2\beta_2\gamma_2$) [1,2]. The released peptides are known as fibrinopeptides A and B, respectively (FpA and FpB).

Hydrodynamic parameters, such as the infinite-dilution translational diffusion and sedimentation coefficients ($D^0_{t(20,w)}$ and $s^0_{(20,w)}$), can be effectively used in multi-resolution modeling of biomacromolecules, helping to discriminate between alternative conformations (*e.g.*, [24]; see also [25]). In a previous publication, we have reported the hydrodynamic and mass spectrometric characterization of a highly purified, nearly intact human plasma FG fraction (hpHMW-FG), of a controlled plasmin proteolysis human FG product lacking the AαC regions, termed fragment X (hpFrX-FG), and of chicken plasma FG (cpFG) [26]. We present here the results of new analytical ultracentrifugation sedimentation velocity (AUC-SV) experiments on a similar hpHMW-FG preparation, and on two human FG recombinant species, full-length (hrHMW-FG, from the commercial source ProFibrix), and with the Aα chain truncated after residue 251 (hrα251-FG, prepared in our laboratories). We were able to determine $s^0_{(20,w)}$ and $D^0_{t(20,w)}$ accurately, and complemented them with Aα chain polydispersity and Fp content analyses. Together these results establish benchmark data that are a reliable basis for further structural analyses of human natural and recombinant fibrinogen species.

Materials and Methods

General

All chemicals were reagent grade from Merck (VWR International, Milano, Italy; http://www.merck-chemicals.com/) or from Sigma-Aldrich (St. Louis, MO; http://www.sigmaaldrich.com/united-states.html), unless otherwise stated, and double-distilled water was used in the preparation of all the solutions. DU640 and DU800 spectrophotometers (Beckman Coulter, Fullerton, CA; http://www.beckmancoulter.com) were used for all spectrophotometric operations. Quality control and characterization of the samples by polyacrylamide (PAA) gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) and Western blotting, followed by densitometric analyses, was done as previously extensively reported [26].

Human Plasma High Molecular Weight Fibrinogen Purification

hpHMW-FG was purified by $(NH_4)_2SO_4$ fractional precipitation from lyophilized human fibrinogen (hFG; plasminogen depleted, type FIB1 from Enzyme Research Laboratories, South Bend, IN; http://www.enzymeresearch.com/), after reconstitution and dialysis into an appropriate buffer, as previously described in detail [26].

Human Recombinant High Molecular Weight Fibrinogen Preparation and purification

hrHMW-PG was obtained from ProFibrix, Leiden, The Netherlands (http://www.profibrix.com/), and was prepared and purified as previously described [27].

Human Recombinant Aα251 Truncated Fibrinogen Preparation and Purification

Media with recombinant hrα251-FG, having intact Bβ and γ chains and the Aα chain truncated after residue 251 [28], were purchased from the National Cell Culture Center (Biovest International, Minneapolis, MN, USA; http://www.biovest.com/) and purified by salt precipitation and immunoaffinity chromatography using the calcium-dependent FG-specific monoclonal antibody IF-1 (Kamiya Biomedical, Seattle, WA, USA; http://www.kamiyabiomedical.com/) as described [29,30].

Briefly, hrα251-FG was precipitated from the medium overnight at 4 °C with 33% saturated $(NH_4)_2SO_4$. After centrifugation, the pellet was dissolved in loading buffer (Tris (tris(hydroxymethyl)aminomethane) 20 mM, NaCl 0.3 M, pH 7.4) with 10 mM $CaCl_2$ and loaded onto the pre-equilibrated IF-1 column. After washing steps with Tris 20 mM, NaCl 1 M, $CaCl_2$ 10 mM, pH 7.4, and CH_3COONa 50 mM, NaCl 0.3 M, $CaCl_2$ 10 mM, pH 6.0, hrα251-FG was eluted with Tris 20 mM, NaCl 0.3 M, EDTA-Na_2 5 mM, pH 7.4, dialyzed once against loading buffer with 1 mM $CaCl_2$ and then twice against Tris Buffered Saline (TBS; Tris 50 mM, NaCl 104 mM, aprotinin 10 KIU/ml, pH 7.4). Several batches were purified. Each was tested by SDS-PAGE under reducing and non reducing conditions. Subsequently, Western blotting was performed using rabbit polyclonal antibodies to human FG (Dako Corp, code N A0080, Carpinteria, CA; http://www.dako.com/) and the monoclonal antibody Y18 (a gift from Dr. Nieuwenhuizen, Leiden, The Netherlands) specific for the N-terminus of the Aα-chain [31]. All batches were found to be > 97% clottable.

Different hrα251-FG batches were pooled together, concentrated by ultrafiltration (Amicon Ultra-15, MWCO 100, Millipore, Billerica, MA; http://www.millipore.com) to 10 mg/ml (concentration above which hrα251-FG tends to precipitate), added with 1 mM AESBF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, Sigma–Aldrich) and stored at -80 °C until use.

Fibrinopeptides Analysis

Fibrinopeptides release and analysis by RP-HPLC was done essentially as previously reported [32], with some modifications. In short, 140 μL of reaction mixture contained FG species at 0.5 mg/ml and thrombin (Sigma-Aldrich) at 0.25 nominal NIH units/ml in TBS. After 2 h of incubation at 25 °C, the reaction was stopped by immersion in boiling water for 1 min, followed by centrifugation at 16,000 *g* for 15 min. The supernatant was filtered on polyethylene sulfone 0.45 μm pore-size filters (Millipore) and 40 μL were injected on a 1.0 × 150 mm, 300 Å pore size, 3.5 μm particle size Symmetry 300 C18 column, protected by a Symmetry 300 C18 2.1 × 10 mm pre-column (Waters Corp., Milford, MA, USA; http://www.waters.com). The HPLC set-up consisted of an Agilent 1200 series system, equipped with G1379B degasser, G1376A capillary pump, G1377A micro autosampler, G1316A thermostatted column holder, and G1315B diode array detector. It was equilibrated in 97% 25 mM CH_3COONH_4, brought to pH 6 with diluted H_3PO_4 (buffer A), and 3% 50 mM CH_3COONH_4, brought to pH 6 with diluted H_3PO_4 and then diluted 1:1 with CH_3CN (buffer B). CH_3COONH_4 was from Fluka (Sigma-Aldrich) and CH_3CN was LiChroSolv from Merck. The flow rate was 50 μl/min, and the elution was performed in this sequence: isocratic 97% A - 3% B for 8 min, then three linear gradients, the first in 5 min to 12% B, the second in 45 min to 27% B, and the third in 6 min to 100% B, and finally maintained at 100% B for 5 min. The elution was monitored at 211 nm, and the columns were thermostatted at 30 °C.

Analytical Ultracentrifugation

AUC-SV experiments were performed using a Beckman Coulter (Palo Alto, CA, USA) Optima XLI Analytical Ultracentrifuge. In each