Models of human platelet thrombospondin in solution

A dynamic light-scattering study

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The translational diffusion coefficient ($D_{20,w}$) of human platelet thrombospondin was measured by dynamic light-scattering. $D_{20,w}$, measured in 20 mM-Hepes buffer, pH 7.4, containing 350 mM-NaCl and 2 mM- CaCl_2 , was $1.73(\pm 0.02) \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$. After removal of bound Ca^{2+} by addition of EDTA, $D_{20,w}$ decreased to $1.56(\pm 0.04) \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$; this was not a consequence of aggregation. $D_{20,w}$ showed little sensitivity to NaCl concentration between 130 and 550 mM. Through hydrodynamic analysis combining $D_{20,w}$ and other parameters taken from the literature, two major types of models for thrombospondin can be proposed: either classic compact models (i.e. low degree of hydration) such as prolate or oblate ellipsoids with a high axial ratio (greater than 20) or models of low axial ratio made of multiple subunits with significant cavities (i.e. high degree of hydration).

INTRODUCTION

Thrombospondin is a high-molecular-mass glycoprotein (415–450 kDa) present in platelet granules, and is secreted when platelets are stimulated with thrombin [1]; for a review see ref. [2]. The secreted thrombospondin binds to the surface of activated platelets and is involved in platelet aggregation. Thrombospondin is also synthesized by a wide range of cells in culture. Thrombospondin is composed of three equivalent disulphide-linked chains of molecular mass 140–150 kDa. Each chain consists of several proteinase-resistant domains, which bind specifically to one or more of heparin, sulphated glycolipids, fibronectin, fibrinogen, type V collagen, laminin, histidine-rich glycoprotein, plasminogen, plasminogen activators, thrombin and osteonectin [2,3]. Thrombospondin from different sources showed different fragmentation patterns when exposed to proteolytic enzymes. This suggests thrombospondin polymorphism [4–7].

Thrombospondin has at least 12 binding sites for Ca^{2+} with an average dissociation constant 10^{-4} M [8]. The exact function of thrombospondin is as yet unknown, but this molecule is clearly involved in processes such as cell adhesion [9,10], migration [11,12] and proliferation [13,14].

To date there have been few data related to the three-dimensional structure of thrombospondin. The conformation appears to be sensitive to the presence of bound Ca^{2+} , as shown by electron microscopy [15], c.d. [16] and e.s.r. spectroscopy [17]. Most of the structural evidence was obtained from electron micrographs [15]. Observations of replicas obtained by low-angle rotary shadowing show structures presenting globular compact domains connected by fragments that appear to be more flexible. Electron microscopy could possibly lead to serious distortions in the structure by exposing the molecule to extreme conditions. Such distortions have been observed with another multi-domain adhesion glycoprotein of the extracellular matrix, namely fibronectin [18–20]. Despite the fact that thrombospondin can only be prepared in small quantities (a serious limitation to its study by physicochemical techniques), we have attempted to determine the low-resolution structure of thrombospondin from measurements obtained from molecules in solution. In this work, dynamic light-scattering after f.p.l.c. gel permeation was applied in order

to determine the translational diffusion coefficient of thrombospondin. This technique has several advantages for the study of the global conformation: thus it does not require any alteration of the molecule such as labelling or fragmentation. As opposed to many other physicochemical techniques, dynamic light-scattering can quantify the effects of polydispersity, thus enabling one to ensure that any observed change in diffusion properties is really due to a conformational transition and not only a consequence of aggregation. This technique also does not require large amounts of protein.

MATERIALS AND METHODS

Materials

Platelet concentrates were kindly provided by Dr. F. Robert (Centre de Transfusion Sanguine, Beynost, France). Thrombin was from Hoffmann-La Roche (Basel, Switzerland), and D-phenylalanyl-L-prolyl-L-arginyl-chloromethane was obtained from Calbiochem (Los Angeles, CA, U.S.A.). All other chemicals were of analytical grade and were obtained either from Prolabo (Paris, France) or from BDH Chemicals (Poole, Dorset, U.K.). Heparin-Sepharose and a pre-packed Superose 6 column were obtained from Pharmacia (Uppsala, Sweden). Microconcentrators (type YM 30) were from Amicon Corp. (Lexington, MA, U.S.A.). Two types of filters were used: Millipore filters (0.2 μm pore diameter) for buffers and Nucleopore filters (0.2 μm pore diameter) for protein samples.

Preparation of samples

Thrombospondin was prepared from thrombin-activated human platelets by affinity chromatography on heparin-Sepharose as described by Lawler *et al.* [15]. All buffers used in this work were degassed and filtered through 0.2 μm -pore-diameter filters. Fractions containing thrombospondin that were eluted from the heparin-Sepharose column were pooled and concentrated to a volume of 300–500 μl with the help of disposable microconcentrators. After filtration through 0.2 μm -pore-diameter filters, the concentrated fractions were loaded on a Superose 6 f.p.l.c. column equilibrated with 20 mM-Hepes

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buffer, pH 7.4, containing 350 mM-NaCl and 2 mM-CaCl₂ and eluted at a flow rate of 0.35 ml/min. The purpose of this gel-permeation step was to remove possible multimeric aggregates that could be present and to ensure buffer exchange with filtered and degassed buffer. Our experience in dynamic light-scattering studies with fibronectin had shown that gel permeation was essential to obtain samples showing very low polydispersity indexes with a 'log' correlator. Samples (50–200 μ l) were analysed in quartz fluorimeter microcells (internal dimensions 1 cm $\times$ 0.2 cm $\times$ 2 cm) (Hellma, Hülhein, Germany). Samples that were eluted from the f.p.l.c. column were loaded directly into the cells without use of a fraction collector. The lag between detection and collection was taken into account during collection. In order to obtain measurements in buffer containing 130 mM-NaCl, the peak fraction of the f.p.l.c. run (containing 350 mM-NaCl) was either concentrated on an Amicon microconcentrator (YM 30 type membrane) and diluted in 0.5 ml of 20 mM-Hepes buffer, pH 7.4, containing 130 mM-NaCl and 2 mM-CaCl₂, or diluted directly with 20 mM-Hepes buffer, pH 7.4, containing 2 mM-CaCl₂ to achieve an NaCl concentration of 130 mM. It was not possible to perform gel filtration at an NaCl concentration of 130 mM under the conditions described since thrombospondin is adsorbed on Superose 6 or Sephacryl S500. Because of the limited availability of material, measurements in 550 mM-NaCl were also obtained after microconcentration from a sample in 350 mM-NaCl and dilution in 20 mM-Hepes buffer, pH 7.4, containing 550 mM-NaCl and 2 mM-CaCl₂.

Photon correlation spectroscopy

The equipment for this work was a Malvern 4700c system (log correlator; Malvern Instruments, Malvern, Hereford and Worcester, U.K.) with an Innova 4 argon-ion laser (Innova, Palo Alto, CA, U.S.A.) operating at a wavelength of 514.5 nm and power from 80 to 250 mW. The apparatus was installed on an antivibration bench. The temperature was controlled to ± 0.1 °C. Since the observed values of Stokes radii were small compared with the wavelength, the contribution from the rotational diffusion coefficient was assumed to be negligible at the scattering angles chosen in this work (furthermore, polydispersity indexes were very low in buffers containing 350 mM- and 500 mM-NaCl). The fundamental time was set at 1.3–2.5 μ s for experiments performed at scattering angle 90° and temperature 25 °C with expansion factors of 2 or 4. Each dataset was made of the accumulation of at least 50 independent runs. Runs that showed scattering intensities above average were rejected by the software and not included in the analysis. The contribution of the scattering from particles too large to be included in the size distribution was negligible, as shown by the high value of the 'in-range index' (the ratio of the amplitude at the end of the correlation function to the theoretical baseline calculated from the average count rate). In the measurements presented here, this index was always over 98.5%. Analysis was performed by the cumulant method [21], and values of diffusion coefficients in this

work were Z-averaged diffusion coefficients [21]. The polydispersity index was a measure of the contribution from the second-order cumulant relative to the first-order contribution. For each different specimen, the polydispersity index showed a good consistency between different measurements.

The tabulated viscosities of water with respect to temperature [22] have been used as buffer viscosities. The observed diffusion coefficients were extrapolated to normalized diffusion coefficients, 20 °C in water ($D_{20,w}$).

RESULTS AND DISCUSSION

Fig. 1 shows correlation functions obtained at a scattering angle of 90° and a temperature of 25 °C with a thrombospondin specimen (concentration 0.1 mg/ml) in 20 mM-Hepes buffer, pH 7.4, containing 350 mM-NaCl and 2 mM-CaCl₂ and after addition of EDTA up to a final concentration of 9.5 mM. The average values obtained at 90° and 25 °C with a thrombospondin concentration of 0.1 mg/ml after normalization to 20 in 20 mM-Hepes buffer, pH 7.4, containing 350 mM-NaCl and 2 mM-CaCl₂ are shown in Table 1.

The polydispersity indexes were below 10%, hence showing that there was no noticeable contribution from flexibility, rotational motion or aggregates in the signal. The intensity of scattered light (corrected for the dilution) was approx. 2% lower after addition of EDTA. The determination of the diffusion coefficient was not affected by the protein concentration in the range 0.4 to 0.06 mg/ml (scattering angle 90° and temperature 25 °C in

Fig. 1. Correlation functions obtained at a scattering angle of 90° and a temperature of 25 °C with a thrombospondin specimen (concentration 0.1 mg/ml) in 20 mM-Hepes buffer, pH 7.4, containing 350 mM-NaCl and 2 mM-CaCl₂ (x) and after addition of EDTA up to a final concentration of 9.5 mM (+)

Table 1. Normalized diffusion coefficients of human platelet thrombospondin measured in 20 mM-Hepes buffer, pH 7.4, containing 2 mM-CaCl₂ under various conditions

Concn. of EDTA (mM)	Concn. of NaCl (mM)	Temperature (°C)	$10^7 \times D_{20,w}$ (cm ² ·s ⁻¹)	Polydispersity (%)
0	350	25	1.73(± 0.02)	2
9.5	350	25	1.56(± 0.04)	7
0	350	37	1.78(± 0.06)	4
0	550	25	1.74(± 0.05)	5
0	130	25	1.69(± 0.03)	25

Table 2. Molecular mass and hydrodynamic parameters of human platelet thrombospondin

Abbreviation: N.D., not determined.

	Ca ²⁺	EDTA	Calculated from composition
Molecular mass from <i>s</i> and <i>D</i> _{20,w} (kDa)	470(±5)	460(±9)	414
Molecular mass from sedimentation only (kDa) [15]	N.D.	420	
Stokes radius from <i>D</i> _{20,w} (nm)	12.4	13.7	5.6*
Stokes radius from η [15] (nm)	11.2	13.9	
Prolate-ellipsoid axial ratio from <i>D</i> _{20,w} , hydration < 50 %†	> 20	> 30	
Prolate-ellipsoid axial ratio from η [15], hydration < 50 %‡	> 10	> 15	
Oblate-ellipsoid axial ratio from <i>D</i> _{20,w} , hydration < 50 %†	> 35	> 45	
Oblate-ellipsoid axial ratio from η [15], hydration < 50 %‡	> 20	> 40	
Prolate-ellipsoid axial ratio from ref. [15]§	3.3	9.3	

* Assuming a partial specific volume of 0.71 cm³/g and a degree of hydration of 35%.

† From Perrin equations [25].

‡ From Simha factor [26].

§ From sedimentation and intrinsic viscosity.

20 mM-Hepes buffer, pH 7.4, containing 350 mM-NaCl and 2 mM-CaCl₂. In this buffer no angular dependence of either the diffusion coefficient or the polydispersity index was observed between 60° and 120°. The latter confirms the absence of aggregates in the sample or contributions from flexibility or rotation in the signal. The average value (normalized at 20 °C) obtained at a temperature of 37 °C with a protein concentration of 0.4 mg/ml in the same buffer was $1.78(\pm 0.06) \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$, showing the absence of any significant change in conformation with temperature.

Thrombospondin in 20 mM-Hepes buffer, pH 7.4, containing 350 mM-NaCl and 2 mM-CaCl₂ shows a noticeable change in conformation after removal of bound Ca²⁺. The decrease in translational diffusion coefficient corresponds to an increase by 11 % in the Stokes radius. This expansion of the molecule confirms the results obtained by intrinsic viscosity, centrifugation measurements and changes in limited-proteolysis patterns [15], modifications of the c.d. spectrum [16] and investigation by e.s.r. of the local motion of a probe [17]. A slight decrease in scattering intensity was observed, the normalized scattering after addition of EDTA being only 98 % of the initial value. This confirms that the observed decrease in diffusion coefficient was not due to aggregation. The decrease in intensity and increase in polydispersity suggests that not only does an increase of the molecule volume occur but also some degradation was probably induced by the removal of bound Ca²⁺. This degradation is confirmed by the decrease in the intensity of the thrombospondin peak after removal of bound Ca²⁺ followed by addition of Ca²⁺ as observed in centrifugation experiments [15]. It was not possible to determine whether this degradation was caused by a non-enzymic process due to the transition of thrombospondin into an unstable conformation or whether some sites sensitive to contaminating proteinases were unmasked by the transition.

When the NaCl concentration was raised to 550 mM a similar value of $1.74 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ (polydispersity 5 %) was obtained at a protein concentration of 0.2 mg/ml. Again, no angular dependence of the diffusion coefficient was observed between 60° and 120°.

Measurements were also obtained at a scattering angle of 90° with a thrombospondin specimen (concentration 0.1 mg/ml) obtained after exchange to 20 mM-Hepes buffer, pH 7.4, containing 130 mM-NaCl and 2 mM-CaCl₂. A contribution from slower-diffusing material representing approx. 25 % of the signal was detected. Analysis by the two-cumulant analysis method [21] yielded an average diffusion coefficient of $1.69(\pm 0.03) \text{ cm}^2 \cdot \text{s}^{-1}$ with a polydispersity index of 25 %. Theoretically, this high value of the polydispersity index could be a consequence of aggregation, rotational motion or flexibility. The Stokes radius corresponding to the observed diffusion coefficients are too small to allow the detection of rotational motion or flexibility in the signal. Furthermore the in-range [21] index was over 98 %, which would not have been possible if very large particles such as dust were present. Aggregation thus remains the most likely explanation for those high polydispersity values. The conformation of thrombospondin in the presence of Ca²⁺ does not seem to be affected by changes in NaCl concentration between 130 and 550 mM.

Table 2 presents various hydrodynamic parameters of thrombospondin models in the presence or in the absence of bound Ca²⁺. The models are derived from the diffusion coefficient determination of the present work, from the sedimentation coefficient (*s*) and intrinsic-viscosity (η) determination by Lawler *et al.* [15] and also from the combination of results from both pieces of work. The amino acid composition was deduced from the nucleotide sequence of the coding region for human endothelial-cell thrombospondin [23], the contribution from the carbohydrate groups being assumed to be 10 kDa [24].

The axial ratio of an ellipsoid model derived from a given hydrodynamic parameter (such as *D*_{20,w} or $[\eta]$) depends on the assumed degree of hydration. In Table 2 a value of 50 % hydration (which is higher than the usual value for proteins of about 35 %) was chosen. Table 2 shows that classic compact ellipsoid models derived from the determination of *D*_{20,w} or $[\eta]$ should have a high axial ratio. Thrombospondin would then present a classic disc or rod-like shape.

In order to be compatible with the measured translational

diffusion coefficients and intrinsic viscosities, low-axial-ratio ellipsoids (including models proposed by Lawler *et al.* [15]) should have a very high degree of hydration (up to several hundred percent). Thrombospondin would then be significantly different from a simple compact ellipsoid. The molecule could be made of multiple subunits and possesses significant cavities.

Thrombospondin could also be a hollow molecule (such a model has been described for fibronectin [20]) or even a 'sponge-type' protein. The latter would mean a rigid protein of low axial ratio with a very high degree of hydration. A large molecular volume (i.e. high degree of hydration) would mean that a relatively low-molecular-mass protein such as thrombospondin could be able to connect several ligands rather distant in space (about 20–30 nm apart). This model would allow connections in space where high-axial-ratio molecules (with low degree of hydration) cannot extend in three dimensions. Connections of several extracellular-matrix components by single molecules would be possible (as well as an easy proteolysis of those links). Connections could be realized quickly without the need of a complex (and slow) macromolecular assembly process. This last factor could be of some importance in a cell-adhesion protein that is secreted by activated platelets.

It must be kept in mind that, in considering the accuracy of the determination of the molecular mass and of the hydrodynamic parameters, one must always remain cautious about the validity of the models. To restrict the number of possible models, further studies are required to ensure that the molecule is globally rigid and to determine other parameters (such as the radius of gyration and the rotational diffusion coefficient) on the intact molecule as well as its subunits.

We thank Dr. P. Robert (Centre de Transfusion Sanguine, Beynost, France) for generously supplying platelet concentrates, Mr. I. Purdom at the University of Edinburgh (Edinburgh, U.K.) for providing expert technical assistance, and Dr. Wess at the University of Edinburgh and Dr. S. Harding from the National Centre for Macromolecular Hydrodynamics at the University of Nottingham (Nottingham, U.K.) for their advice and comments during the preparation of this manuscript. L.V. acknowledges the Imperial Cancer Research Fund (London, U.K.) for a postdoctoral fellowship.

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