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Protein structure probed by polarization spectroscopy

I. Evidence for fibrinogen rigidity from stationary fluorescence *

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Two fluorescent derivatives of human fibrinogen have been synthesized, by the covalent bonding of 1-dimethylaminonaphthalene-5-sulphonyl and methylpyrene chromophores, to investigate the internal molecular dynamics of the protein in solution. The stationary fluorescence depolarization of these derivatives under isothermal conditions is described here while in an accompanying paper (part II) a time-resolved study is reported. From the static fluorescence data it is concluded that reorientational processes in the subnanosecond and microsecond time ranges account for all the observed depolarization. The faster motion was assigned to the restricted, localized oscillations of the label while the slow motion was ascribed to the overall rotation of the protein molecule. Consequently, the protein in solution appears considerably rigid in the 10–1000 ns range, in contrast with a previous conception of a flexible fibrinogen based on non-isothermal depolarization experiments. These previous experiments are, in fact, concordant with the rigid fibrinogen proposed here if they are reinterpreted using Weber's early ideas on thermally activated depolarization (G. Weber, J. Biochem. 51 (1952) 145).

1. Introduction

Rotational and segmental motions of biological macromolecules are often monitored by measuring the fluorescence anisotropy of chromophores attached to the biopolymers [1,2]. There are two variants of this technique: the static approach, in which the fluorescence anisotropy is recorded under photostationary conditions, and the kinetic approach, which entails time-resolved measurements of anisotropy. The value of the static anisotropy contains contributions from all the depolarizing motions operative under the prevailing experimental conditions. Accordingly, when different types of motion act together, it becomes almost impossible to treat them separately; nonetheless, since very accurate measurements of the static anisotropy are easily made, much ingenuity has been spent on extracting from static data interesting information concerning macromolecular structure [3]. The kinetic alternative yields results which are easier to interpret but harder to obtain [4], and the necessary equipment is rather costly. In this work, we address the question of the flexibility of the fibrinogen molecule by examining data on static anisotropy; the corresponding time-resolved study is reported in an accompanying article (part II).

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Abbreviations: 1,5-DNS, 1-dimethylaminonaphthalene-5-sulphonyl chloride; 1,5-DNS-FIB, human fibrinogen labelled with 1,5-DNS; HPSEC, high-performance size-exclusion chromatography; MePy-FIB, human fibrinogen labelled with the methyl pyrene chromophore; D/P, dye/protein molar ratio.

The plasma protein fibrinogen is the soluble precursor of the fibrin clot [5]; its size and shape under physiological conditions are still in dispute. A question of great importance in this context is whether fibrinogen, a multidomain protein [6,7], is rigid or supple; as has been pointed out before [8], flexible linkages between the domains can easily foil the analysis of the available hydrodynamic data. In fact, considerable pliability has been inferred from the appearance, in some electron micrographs [9], of bent and partially folded forms of the protein, and from the variation with temperature of the emission anisotropy of fluorescent conjugates of fibrinogen [10,11].

Johnson and Mihalyi [10] recorded the temperature dependence of the fluorescence polarization of a chemically labelled bovine fibrinogen containing approx. 12 molecules of 1,5-dansyl(1,5-DNS). More recently, Hantgan [11] incorporated dansylcadaverine into specific sites of human fibrinogen and measured the fluorescence anisotropy at different temperatures. They fitted their data (recorded under photostationary conditions) to the Perrin equation (eq. 1):

$$r_0/\langle r\rangle = \left(1 + \frac{kT}{V\eta}\tau\right) = \left(1 + \frac{\tau}{\phi}\right) \qquad (1)$$

which relates the steady anisotropy $\langle r\rangle$ of a spherical molecule of volume V and lifetime τ (suspended in a medium of viscosity η and temperature T) to the Boltzmann constant k and the limiting anisotropy r_0 recorded under conditions where Brownian rotation may be disregarded. From the slopes of the straight lines to which their data could be accommodated, they obtained values for the rotational correlation time $\phi = V\eta/kT$ in the range 50–60 ns. Now, a rigid sphere of volume equal to that of the unhydrated fibrinogen molecule would be characterized by a correlation time of 100 ns, and hydration or deviation from the spherical shape would only lengthen the correlation time; accordingly, Johnson and Mihalyi [10] stated that "the molecule is not rigid but possesses internal degrees of freedom", and Hantgan [11] concurred by concluding that "fibrinogen is not a rigid molecule but in fact can undergo bending or twisting motions in solution".

In this work we show that, if T is maintained at a constant value (293 K) and the viscosity of the medium is raised by the addition of glycerol, the corresponding isothermal Perrin plot does not follow a rectilinear trend. Our data speak well for the advice [12], to which little heed has been paid, that "it is a good idea to check each Perrin plot both ways, if possible, to avoid specific artifacts arising from temperature effects of the influence of the sucrose or other viscosity increasing solute". Previous non-isothermal Perrin plots need to be reinterpreted in the light of the new experimental evidence, which indicates that the protein has a rigid structure.

2. Experimental

2.1. Fibrinogen labelling

Human fibrinogen (Kabi, Stockholm) was dialysed several times before labelling. The fluorescent label 1,5-dansyl chloride (Sigma, USA) was conjugated to the protein by incubating a phosphate buffer solution (pH 7.9) containing 10 mg/ml of fibrinogen and the dye adsorbed on Celite (10%, w/w) in a 1:120 molar ratio. After removal of the dye carrier by centrifugation, the labelled protein (1,5-DNS-FIB) was purified by dialysis and column chromatography (Sephacryl S-300). The sedimentation coefficient and emission anisotropy of the fractions corresponding to the chromatographic maximum were routinely measured and found to be constant. Functional tests (clottability) and SDS-polyacrylamide gel electrophoresis were carried out before and after labelling the protein. The number of dye groups per protein molecule (D/P) was determined by absorption spectroscopy and ranged from 5 to 7. The distribution of the fluorescent label among the Aα, Bβ and γ chains and between the D and E fragments of fibrinogen was determined by HPSEC techniques and gel electrophoresis. Samples of 1,5-DNS-FIB were reduced under carefully controlled conditions [13] with 1% β-mercaptoethanol in SDS and analysed on two TSK-G 4000 SW and one TSK-G 3000 SW column in series. The elution profile of a 1,5-DNS-FIB sample with a D/P ratio of 7 (fig. 1A) shows the dye mole-

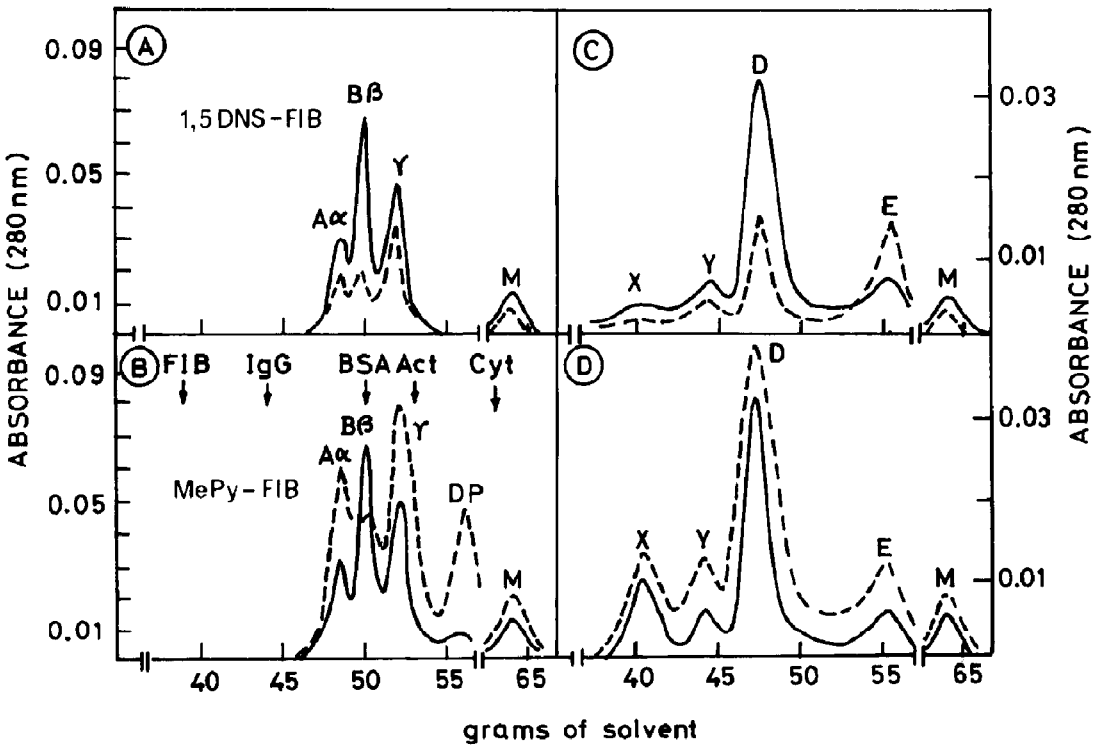

Fig. 1. HPSEC elution profiles showing the distribution of the fluorescent labels among the Aα, Bβ and γ chains and between the D and E fragments of fibrinogen, followed by the absorbance at 280 nm (———) and fluorescence (— — —). The emission of the dansyl derivatives was detected at 530 nm and that of the methylpyrene derivatives at 396 nm (A) 1,5-DNS-FIB reduced with β-mercaptoethanol. (B) MePy-FIB reduced with β-mercaptoethanol. (C) 1,5-DNS-FIB digested with plasmin. (D) MePy-FIB digested with plasmin. M. SDS micelles; DP, degradation products; FIB, human fibrinogen; IgG, immunoglobulin G; BSA, bovine serum albumin; Act, actin; Cyt, cytochrome *c*; D, E, X, Y, fibrinogen fragments after plasmin digestion.

cules spread over the whole protein. The dye distribution among protein domains was also determined by HPSEC analysis. Samples of the labelled fibrinogen were digested with plasmin [14] and analysed as above but under non-reducing conditions. The elution profile (fig. 1C) shows that the 1,5-DNS is bound to both the D and E fragments, with a higher specific content in the latter. The labelled fibrinogen, which shows an absorption band centered at 345 nm and a fluorescence maximum at 515 nm, was further characterized spectroscopically by determining the corrected excitation, emission and polarization spectra. The intrinsic fluorescence anisotropy, determined as described below, was $r_0 = 0.312 \pm 0.002$. The fluorescence decay was fitted to a double exponential with lifetimes of 9 and 20 ns and pre-exponential factors of 0.6 and 0.4, respectively.

MePy-FIB was prepared by forming first a Schiff base with 1-pyrenealdehyde (Aldrich recrystallized from ethanol) and the reacting amino groups of the protein. In a second step the Schiff base was reduced with sodium borohydride. Since the pyrenealdehyde is only marginally soluble in water, it was supported (~10%, w/w) on a silanized Celite (Chromosorb W, J. Malville, U.S.A.) which had been covered with a multilayer of 1,2-dimyristoyl-3-glycerylphosphatidylcholine. The phospholipid was added just to improve the subsequent dispersion of the Chromosorb W in water. To carry out the coupling reaction a

fibrinogen solution (10 mg/ml) in borate buffer (pH 8.5) was mixed with the dye supported on Chromosorb W in a 1:100 molar ratio. After incubation for 1–2 h in the dark at room temperature, the excess dye was centrifuged out and the Schiff base reduced (10 min) by adding sodium borohydride in a 2:1 molar ratio relative to the dye. The labelled protein was purified, analysed and controlled by the same procedures as those described above for the the 1,5-DNS-FIB samples. Fig. 1B shows the HPSEC elution profile of the reduced MePy-FIB samples, whereas fig. 1D illustrates the label distribution within the protein domains after plasmin digestion. Although the labelling ratio was always very low (1 dye molecule per 4–5 fibrinogen molecules) the label is quite homogeneously distributed.

The MePy-FIB derivatives were characterized spectroscopically as above [15]. The absorption spectrum shows maxima at 342 and 326 nm from the pyrene chromophore. The extinction coefficient at 342 nm (44000 $M^{-1}\,cm^{-1}$ [15]) was used for measuring D/P. The corrected fluorescence spectrum shows characteristic pyrene vibronic structure, with maxima at 376, 396 and 418 nm. The fluorescence decay is best described by a long-lifetime component ($\tau_{F1} = 105 \pm 5$ ns) and a shorter one ($\tau_{F2} = 15 \pm 5$ ns). The long-lifetime component accounts for 80% of the total fluorescence emission. The intrinsic anisotropy of MePy-FIB was $r_0 = 0.217 \pm 0.002$ ($\lambda_{ex} = 337$ nm; λ_{em}: cut-off filter at 389 nm).

All solvents were of fluorimetric grade. Glycerol (Carlo Erba) was distilled twice at reduced pressure (1 Torr, b.p. ~ 130 °C).

2.2. Fluorescence measurements

All stationary fluorescence measurements were carried out in an SLM 8000 D fluorimeter. The emission and excitation spectra were corrected using standard techniques. Polarization data were recorded by inserting two Glan prisms in a 90° configuration; the emission was isolated with 450 and 389 nm long-pass cut-off filters (Schott KV450 and KV389), for 1,5-DNS-FIB and MePy-FIB, respectively. The intrinsic anisotropies (r_0) were determined from a solution of the labelled protein in 96% glycerol at −20 °C. The viscosity of the aqueous glycerol solutions was obtained from refractive index measurements and tabulated data. 0.1% aprotinin was added to the samples used at $T > 20$ °C to inhibit protein degradation by residual plasmin. The light scattering correction of the experimental anisotropy was always less than 1% and the estimated total error in the quoted anisotropies is ±0.002.

Fluorescence lifetimes were determined in a time-correlated single-photon counting spectrometer with a nanosecond thyratron-gated flashlamp (EI 199). The decay analysis was based on a non-linear least-squares reconvolution numerical technique. Both 1,5-DNS-FIB and MePy-FIB showed a two-exponential fluorescence decay. Therefore, the fluorescence lifetime appearing in the simplified model analysis presented below was replaced by the second-order average lifetime $\langle \tau_F \rangle = \Sigma_i\, ai\, \tau_{Fi}^2 / \Sigma_i\, ai\, \tau_{Fi}$ as discussed elsewhere [15,16].

The integrity and homogeneity of the samples used in the spectroscopic measurements was checked before and after each experiment by HPSEC, electrophoresis, analytical ultracentrifugation and absorption spectroscopy.

3. Results

The depolarization of the fluorescence of 1,5-DNS-FIB as a function of T/η in phosphate buffer is shown in fig. 2. The anisotropy decreases by approx 0.09 when the viscosity of the solution is varied by changing the temperature from 5 to 35 °C. This is merely an iteration of the results obtained previously by Johnson and Mihaly [10] and by Hantgan [11] using the same technique. The three sets of data were included in fig. 2 and the straight lines drawn through the experimental points show virtually the same slope. In contrast, if the fluorescence depolarization of 1,5-DNS-FIB is recorded under isothermal conditions at 20 °C by modifying the buffer with purified glycerol, a completely different behaviour can be observed (fig. 3). In this case, large variations of the fluorescence anisotropy take place in the high-viscosity range, whereas in the low-viscosity range hardly

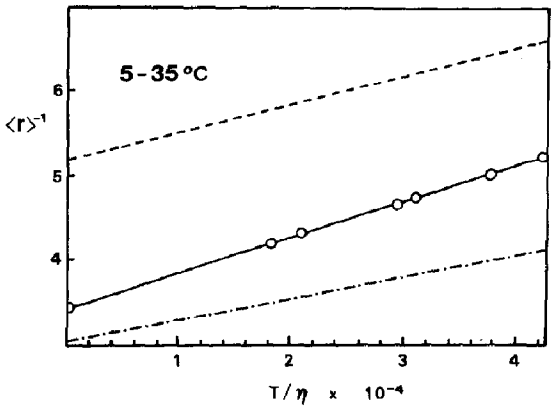

Fig. 2. Static fluorescence depolarization of 1,5-DNS-FIB in aqueous buffer as a function of temperature, represented as the reciprocal of the fluorescence anisotropy $\langle r \rangle^{-1}$ vs. T/η (in K P^{-1}). Data from this work (○) in 0.05 M sodium Phosphate, 0.1 M NaCl, 1 mM EDTA and 0.025% NaN_3 (pH 7) and those adapted from Johnson and Mihalyi [10] (— — —) and Hantgan [11] (·–·–·).

any alteration of the anisotropy can be recorded. The same results were reproduced with several independent preparations of the fluorescent protein derivatives. If the same kind of experiment is carried out with MePy-FIB (fig. 3), the dependence of the anisotropy on the viscosity at constant at constant temperature also differes drastically from that recorded under non-isothermal conditions (fig. 2) for 1,5-DNS-FIB. The experimental data for MePy-FIB under isothermal conditions resemble closely those of 1,5-DNS-FIB, although there is a significant difference: in the case of the methylpyrene derivatives the degree of polarization decreases appreciably even at the lower viscosities attained.

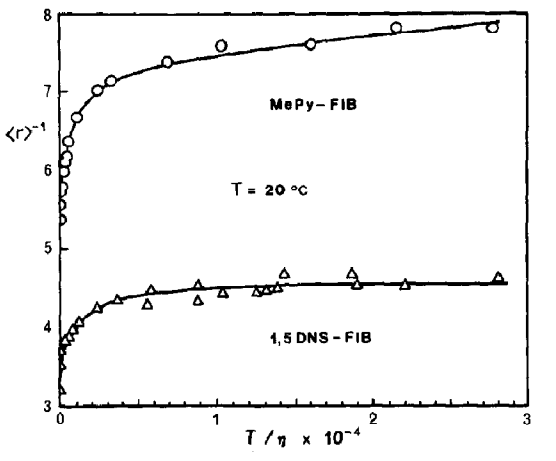

Fig. 3. Isothermal values of the stationary anisotropy $\langle r \rangle^{-1}$ of fibrinogen conjugates as a function of viscosity. Short emission lifetime ($\langle \tau \rangle = 15$ ns) 1,5-DNS-FIB (○) and long emission lifetime ($\langle \tau \rangle = 90$ ns) MePy-FIB (△) derivatives. The viscosity was varied by adding glycerol to a solution of the labelled protein in the same buffer as in fig. 2.

4. Discussion

4.1. Interpretation of the non-isothermal depolarization

The depolarization data of 1,5-DNS-labelled fibrinogen, recorded by changing the solution viscosity by heating the solution, are not reproduced when the viscosity is isothermally modified by glycerol addition. This is unlikely to be due to alteration of the protein structure by glycerol, as demonstrated by the time-resolved experiments (part II). Hence, the depolarization observed in the heating curves (fig. 2) should be mainly a temperature effect. Although the temperature can modify the rotational rate of a protein through the temperature dependence of the rotational diffusion constant [17], in the present case, however, this dependence would account only for a small fraction of the recorded anisotropy change. On the other hand, changes in the fluorescence lifetime in the temperature range studied here were also unimportant. Therefore, one is forced to conclude that a straightforward interpretation of the heating curves of fig. 2 based on the Perrin-Weber equation cannot be carried out. Accordingly, the fibrinogen rotational correlation time derived in refs. 10 and 11 should be viewed with caution. In fact, the hypothesis of temperature-dependent orientational motions was already considered by Weber [18] in his early work on rotational depolarization. In later work, a detailed account of thermally activated 'internal rotations' was presented by Wahl and Weber [19] and used by others. [20]. In terms of the models elaborated in

these papers, the depolarization observed in a heating experiment may result not only from the overall protein motions but also from internal motions which become more important as the temperature increases. These thermally activated motions are presumably the origin of the short apparent rotational times that are frequently obtained from that sort of experiment, as in the case of fibrinogen. Since nowadays the thermal effects can be observed in a direct way be determining the time-resolved anisotropy decay as a function of temperature (see part II), we shall not discuss them further.

4.2. Isothermal Perrin-Weber plots

In the isothermal depolarization of 1,5-DNS-FIB (fig. 3), the short-lifetime fluorescent derivative, when T/η is varied over a sufficiently wide range the Perrin-Weber plot takes a nonlinear shape and a residual anisotropy is still observed in the more fluid solution. This is indicative of the absence of nanosecond segmental motions in this fibrinogen derivative. In addition, the depolarization observed in the high viscosity range has to be produced by very fast restricted motions, probably in the picosecond time interval (in pure water), that can be assigned to the localized librational motions of the label. In fact, rotational correlation times of this magnitude have been measured recently [21] for the dansyl chromophore, both free and protein-bound, using kinetic spectroscopic techniques.

The experimental anisotropy measured with the long fluorescence lifetime derivative MePy-FIB (fig. 3) also shows similar subnanosecond orientational motions which, by analogy, are considered as arising from the fluorophore itself. On the other hand, these measurements differ from those obtained with the 1,5-DNS derivatives in that a weak depolarization (small slope) can still be recorded in the low-viscosity range. In this case the larger emission lifetime of the pyrenyl fluorophore makes it possible to detect the depolarization due to the overall Brownian rotation of the protein molecule. The linear segment of the MePy-FIB plot of fig. 3 can be analysed using the Perrin equation (eq. 1), where r_0 is replaced by the extrapolated limiting anisotropy $r(0) = 0.14$ and τ_F is the average lifetime of 90 ns. In this way, one can estimate an apparent correlation time of 1.1 ± 0.3 μs for the overall rotation of the protein.

5. Conclusions

(a) The rectilinear trend of the Perrin-Weber plots of chemically labelled fibrinogen, as described in previous work carried out under non-isothermal conditions, is not observed when the fluorescence depolarization is recorded isothermally. Hence, the notion of a nanosecond, segmentally flexible structure for this protein, apparently supported by the non-isothermal experiments, needs to be reconsidered.

(b) The viscosity dependence of the isothermal stationary fluorescence polarization of human fibrinogen, labelled with fluorophores of varying lifetimes, can be explained by the following depolarizing processes: (i) very fast orientational motions, within the subnanosecond time range, assigned to the restricted libration of the fluorophores; (ii) a microsecond rotational correlation time assigned to the overall rotation of the protein.

According to these experiments, the fibrinogen molecule is a rigid structure, in the 10–1000 ns time interval, in agreement with the time-resolved measurements reported in an accompanying paper (part II).

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