Eur Biophys J (2006) 35: 517–522
DOI 10.1007/s00249-006-0060-z

ARTICLE

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Proteins as micro viscosimeters: Brownian motion revisited

Received: 21 December 2005 / Revised: 23 February 2006 / Accepted: 14 March 2006 / Published online: 13 April 2006

Abstract Translational and rotational diffusion coefficients of proteins in solution strongly deviate from the Stokes–Einstein laws when the ambient viscosity is induced by macromolecular co-solutes rather than by a solvent of negligible size as was assumed by A. Einstein one century ago for deriving the laws of Brownian motion and diffusion. Rotational and translational motions experience different micro viscosities and both become a function of the size ratio of protein and macromolecular co-solute. Possible consequences upon fluorescence spectroscopy observations of diffusing proteins within living cells are discussed.

Keywords Brownian motion · Microviscosity · Microscopic hydrodynamics · Diffusion in the cell

Introduction

To accomplish most of their functions biomolecules must meet and recognize each other. Brownian motion provides the random translations and rotations necessary for the reacting partners to encounter and to adopt the adequate mutual orientation required for subsequent docking and/or reaction. Translation and rotation of a Brownian particle occur simultaneously as a consequence of the spatial imbalance of random collisions with solvent molecules within a short time interval. The statistical laws of Brownian motion were derived exactly one century ago by Einstein (1905). They connect the macroscopic translational or rotational diffusion coefficient $D_{t,r}$ to the thermal bath kT and to the friction $f_{t,r}$ experienced by the particle, by $D_{t,r} = k\mathrm{T}/f_{t,r}$, respectively. The practical interest of studying diffusion processes follows from Stokes' hydrodynamic expressions $f_t = 6\pi\eta\mathrm{R}$ and $f_r = 8\pi\eta\mathrm{R}^3$ for the translational and rotational friction of a spherical particle of radius R in a solvent of viscosity η. Knowledge of the diffusion coefficients thus permits to calculate one of these quantities when the other is known.

The Stokes–Einstein relations have been of considerable historical interest. In addition to providing a final explanation of the mysterious random motion of pollens reported by the British botanist R. Brown in 1827, they produced the first direct evidence in favor of the molecular theory of matter as well as the theoretical basis for the determination of Avogadro's number by J. Perrin in 1907. For decades, they have been used in physical biochemistry for estimating the size of macromolecules by a variety of techniques such as centrifugation (translation) or fluorescence depolarization (rotation).

Using modern confocal microscopic techniques and fluorescence correlation spectroscopy (FCS), diffusion coefficients are currently measured even in living cells. Here, the ''Brownian particle'' is generally a fluorescent protein that can be expressed in engineered cells. Such measurements are necessary for quantifying intracellular displacements as well as the ''rigidity'' of the local environment by means of the viscosity dependence of the diffusion coefficients.

Unfortunately, the characteristics of the intracellular medium are incompatible with those that must be assumed to establish the laws of Brownian motion, namely those of a Brownian particle immersed in an ideal, homogeneous and isotropic, solvent whose molecular size is so small that it can be regarded as practically continuous. On the contrary the cytosol is extremely crowded (Ellis 2001) and its local viscosity does not result from that of a small solvent but from the presence of macromolecular cosolutes such as proteins, ribosomes, RNA's etc. with a large molecular weight dispersion.

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This work focuses on the consequences on diffusional properties of protein solutions brought about by dilute but macromolecular cosolvents. To this purpose we measured D_t and D_r over a wide range of proteins (MW from 68 to 4,000 kDa) and cosolvents (Dextrans, MW from 0.2 to 2,000 kDa) at viscosities varying between 1 and ≈100 cP. We present evidence that the Stokes–Einstein laws become invalid when the viscosity of the medium in which a Brownian particle is immersed is caused by the presence of macromolecular cosolvents such as dextrans rather than by a "small" and quasi continuous solvent.

Methods

Rotational diffusion

Rotational diffusion is usually measured by recording the decay of the fluorescence anisotropy induced by a polarized light pulse (photoselection). However, the method works only when rotational diffusion is faster or at most of the same order of magnitude as the probe's fluorescence lifetime. It breaks down with the large proteins and the high viscosities considered here, because rotational motion is so slow (μs–ms) that any fluorescence would have decayed long before the protein even starts rotating. We therefore used the "triplet absorption probe" technique and its variants that we introduced long ago to measure the slow rotational correlation time of particles as large as ribosomes (Lavalette et al. 1977) or Earthworm Haemoglobin (Gros et al. 1984).

In heme proteins, the chromophore is the heme in its liganded and deliganded state. It is firmly anchored in the protein. With the extrinsic labels used with the other proteins the optical signal is provided either by the triplet–triplet absorption or by the corresponding ground state depletion, both having the lifetime of the triplet state in deaerated solution, i.e. 100 μs to ms. As a result, a possible wobbling motion (if any) of the probe is averaged out and results only in a slight decrease of the anisotropy value at $t = 0$. This is of no consequence upon the correlation time because the latter depends only on the relative changes of the anisotropy with time, not on its absolute initial value. In this work, we consider essentially globular proteins. As long as the anisotropy decay is a single exponential (which was indeed always the case), the usual assumption is that it represents the correlation time of the sphere having the same volume as the hydrated protein.

Rotational diffusion coefficients were obtained by using the triplet–triplet (or alternatively ground-state depletion) absorption depolarisation technique (Lavalette et al. 1977) of BSA and $\alpha 2-$macroglobulin, labelled with tetramethyl-rhodamine-isothiocyanate (TRITC) (Pochon et al. 1978). For EW-Hb and F(EW-Hb) the absorption signal simply resulted from the photodissociation of the CO complexes of these hemoproteins (Lavalette et al. 1999; Gros et al. 1984). The absorption anisotropy was induced by pulsed photoexcitation of BSA-bound TRITC into its excited triplet state or by photodissociation of carbon monoxide from the CO complexes of EW-Hb and F(EW-Hb). Photoselection was achieved by the polarized output of a pulsed YAG laser (Quantel, Orsay, France) (532 nm, 10-ns pulse width), and the anisotropy was measured by a dual beam device as described earlier (Gros et al. 1984). Correlation time measurements were performed at 5°C with a protein working concentration in the range 10–100 μM. Cosolutes and viscosity measurements were as previously described (Lavalette et al. 1999).

Translational diffusion coefficients

The most appropriate and convenient method for measuring translational diffusion is FCS in which one counts the number of photons emitted by a protein-bound fluorescent probe crossing the confocal volume per unit time.

Translational diffusion coefficients were determined using FCS (Magde et al. 1972; Skakun et al. 2005). FCS measurements were performed on ConfoCor (Carl Zeiss & Evotec Biosystems, Germany), a single channel system based on a Zeiss Axiovert 135 inverted microscope with standard confocal epi-fluorescence microscope optics. The output of an air-cooled Argon ion laser (488 and 514 nm) is fibre-coupled to the back of the microscope. Inserting neutral density filters in front of the laser output attenuated the intensity of the excitation light. Excitation dichroic and emission filters mounted in filter sliders were employed to select the proper excitation wavelength and separate the fluorescence from excitation light. A water immersion C-Apochromat 40x objective lens (N.A. 1.2) (Carl Zeiss, Germany) focused the excitation light to a diffraction-limited spot and collected the fluorescence. Samples were stored in eight well Labtek chambered cover glasses (Naglenunc, USA) with borosilicate bottom that was positioned in a home-made holder to control the temperature of the sample. To limit the size of the observation volume a size adjustable and motor-controlled pinhole was placed in the image plane of the detection path. The SPCM-AQ avalanche photodiode (APD) (Perkin Elmer, USA) detector was placed directly behind the pinhole and coupled to a fast digital ALV5000E correlator card (ALV, Germany) that calculates the real-time autocorrelation function. Access 1.0.12 software (Carl Zeiss & Evotec Biosystems, Germany) controlled the system. The autocorrelation curves were fitted to a multi-component 3D Brownian motion model including triplet kinetics using FCS Dataprocessor softwares (Skakun et al. 2005).

Materials

Correlation time measurements were performed at 5°C with a protein working concentration in the range

10–100 μM. All proteins were extensively purified by gel chromatography and were found to display one unique band in SDS electrophoresis. The cosolutes were Dextrans (Pharmacia & Fluka) with a nominal dispersion range of MW ± 10%. With the series of dextrans used, there was no MW overlap between successive dextrans.

Viscosity measurements were as previously described (Lavalette et al. 1999).

Results

According to the Stokes–Einstein formulae, the relative decrease of the diffusion coefficients should be proportional to the relative increase of the viscosity. In contrast, experiments (Fig. 1) show a power law dependence:

$$\frac{D^{s}_{t,r}}{D_{t,r}} = \left(\frac{\eta}{\eta_s}\right)^{q_{t,r}} \quad (1)$$

with fractional exponents $q_{t,r} \leq 1$ that differ with the type of motion (the subscript s denotes the quantities measured in the pure water solvent). The deviations become more pronounced as the molecular weight of the viscous cosolvent increases. For a given cosolvent, the rotational motion is always more affected than the translational one, i.e. $q_r < q_t \leq 1$. When the macromolecular cosolvent becomes much larger than the protein the diffusion coefficients tend to become independent of viscosity, indicating that the protein molecules do not experience the bulk viscosity anymore but behave as if they were immersed in the pure solvent.

Measurements performed with proteins of various size indicate that the fractional exponents $q_{t,r}$ are not only a regular function of the cosolvent's dimension, but that they also vary with the protein used as a probe (Fig. 2a, b). A dependence of diffusion on probe protein size has been claimed in several F.R.A.P. (Fluorescence recovery after photobleaching) studies, though with conflicting conclusions (Luby-Phelps et al. 1987; Hou et al. 1990; Furukawa et al. 1991; Sesek et al. 1997). This dependence was unambiguously established in our preliminary study of the rotational diffusion (Lavalette et al. 1999) in which the data were parameterized as a function of the cosolvent molecular weight and of the protein hydrodynamic radius R_h (the latter being easily obtained by measuring diffusion coefficients in the pure solvent). This was not entirely satisfactory because hydrodynamic quantities are based on spatial dimensions and not on mass. The recent report of the hydrodynamic radius of dextrans (Weiss et al. 2004) now permits to account simultaneously for rotational diffusion and for the present new translational diffusion data.

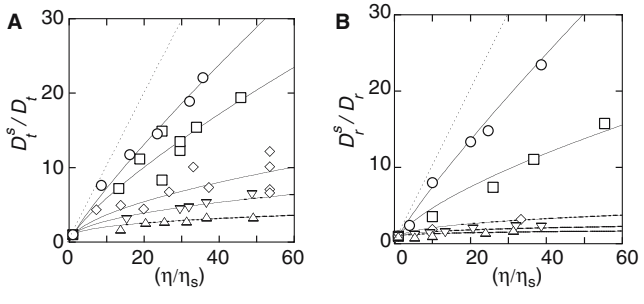

Fig. 1 Deviation of diffusion coefficients from the Stokes–Einstein law in presence of macromolecular cosolvents. Relative decrease of the translational (**a**) and the rotational (**b**) diffusion coefficient of bovine serum albumin (BSA) versus the relative viscosity increase in solutions of dextrans with various molecular weights. The *dotted line* shows the Stokes–Einstein prediction as it is usually observed with small cosolvents such as glycerol or sucrose. The fits through the dextrans data points are power laws. The molecular weights of the dextran polymer: 6 kDa (*circles*), 17.5 kDa (*squares*), 119 kDa (*diamonds*), 500 kDa (*down triangles*) and 2,000 kDa (*up triangles*). Temperature 4°C

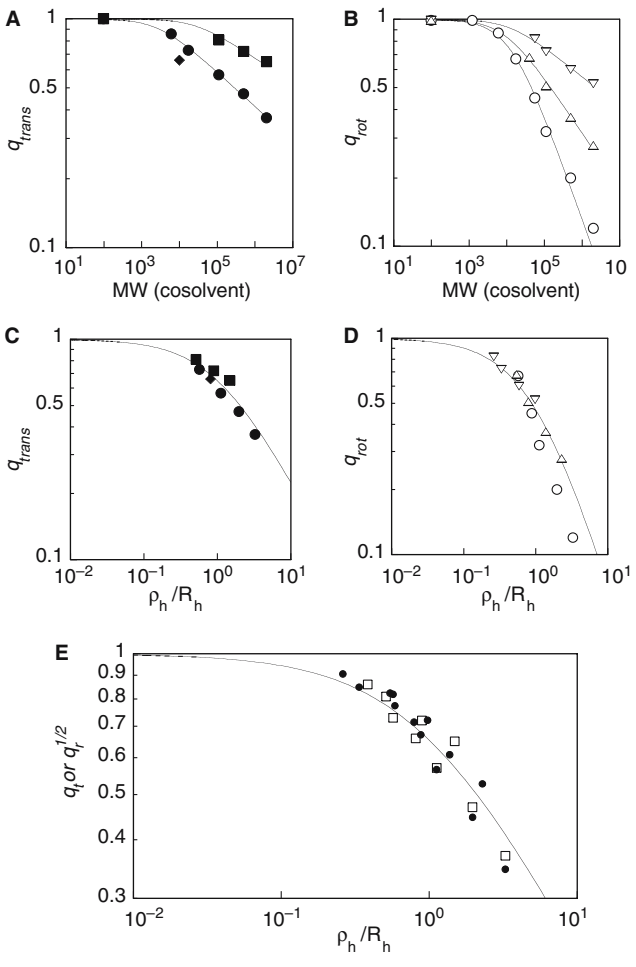

Fig. 2 Fractional translational (**a**) and rotational (**b**) viscosity exponents (Eq. 1) of proteins in dextran cosolvents. The proteins were: GFP, R_h = 23 Å (*diamond*) (Hink et al. 2000); BSA, R_h = 40 Å (*circles*) (Lavalette et al. 1999); α2 – macroglobulin, R_h = 88 Å (*squares*) (Pochon et al. 1978); Earthworm haemoglobin subunit, R_h = 57 Å (*up triangles*) (Lavalette et al. 1999); integral Earthworm haemoglobin, R_h = 134 Å (*down triangles*) (Lavalette et al. 1999; Gros et al. 1984). **c**, **d** The same data as a function of the ratio of the hydrodynamic radius of the cosolvent, ρ_h and of the protein R_h. **e** Unique correlation of q_t (*squares*) and $q_r^{1/2}$ (*circles*) with the size ratio of cosolvent and protein

A priori the data are a function of two independent variables, protein and cosolvent (Fig. 2a, b). Introducing the ratio of the cosolvent's hydrodynamic radius, ρ_h, and that of the protein, R_h, permits to use a trial function containing one adjustable parameter only i.e.: $q_{t,r} = [1 + (\rho_h/R_h)]^{-m_{t,r}}$. Figure 2e shows the simplification achieved: all data points now collapse into one unique curve, irrespective of the protein/cosolvent pair considered. The correlations, however, are still different for translation and rotation. The final step towards unifying both motions is suggested by the data obtained with BSA, the only protein whose intermediate size allowed both q_t and q_r to be measured in the same solvent/cosolvents mixtures (Fig. 1). For this protein a plot (not shown) of q_r against q_t reveals practically a quadratic relation, i.e. $q_r = q_t^2$. Figure 2e shows that this relation is quite general: q_t and the square root of q_r merge into one single correlation with the unique parameter: $m = 0.6 \approx 2/3$. In other words,

$$q_r^{1/2} = q_t = \left[1 + \left(\frac{\rho_h}{R_h}\right)\right]^{-2/3}. \tag{2}$$

These results imply that the Stokes–Einstein relations must be modified. They can be generalized, while keeping their form invariant, by rewriting the friction coefficients as

$$f_t = 6\pi\mu_t R_h \quad \text{and} \, f_r = 8\pi\mu_r R_h^3, \tag{3}$$

in which a new microscopic viscosity parameter

$$\mu_{t,r} = \eta_s \left(\frac{\eta}{\eta_s}\right)^{q_{t,r}} \tag{4}$$

is substituted for the solvent macroscopic viscosity η. The Stokes–Einstein laws are recovered when $q_t = q_r = 1$, a circumstance that, according to Eq. 2, holds when the protein is significantly larger than the cosolvent, in agreement with the classical assumptions. The present definition of microviscosity is therefore consistent with the whole body of data presently available.

Discussion

In contrast to the bulk macroscopic viscosity η, microviscosity is *not* an intrinsic property of the solvent–cosolvent system. It depends on scaling factors between the Brownian particle and its environment. In addition, a new feature appears: the two motions experience a different microviscosity. The rotational microviscosity relative to solvent is always less than the translational one.

We shall now consider the consequences of the modified laws for two widely different particles: the green fluorescent protein (GFP) (R_h = 23 Å), a small protein commonly used in microscopic investigations within cells and ribosomes (R_h = 133 Å) that are very abundant in the cell medium. As shown by the simulation of Fig. 3, data are expected to show severe discrepancies among laboratories working with different techniques and/or protein probes because the diffusion coefficient is dependent on the size of the protein of interest, on the (usually unknown) molecular size of its local environment as well as on the type of observation (rotation vs. translation). This is a real problem for in vivo investigations in which a change of « cosolvent » is obviously impossible. We suggest that combined FCS measurements involving the simultaneous observation of the rotational and translational diffusion of the same protein (Egrenberg and Rigler 1974) should be able to remove the ambiguity. Provided the diffusion coefficients in the pure solvent and the hydrodynamic radius are known, this should permit to calculate the exponent q_t

$$q_t = \frac{\log(D_r^s/D_r)}{\log(D_t^s/D_t)} \tag{5}$$

as well as the local macroscopic viscosity

$$\eta = \eta_s \left(\frac{D_t^s}{D_t}\right)^{1/q} = \eta_s \left(\frac{D_r^s}{D_r}\right)^{1/q^2}. \tag{6}$$

Finally an effective size of the macromolecular environment can be estimated:

$$\rho_h = \frac{R_h(1 - q^{3/2})}{q^{3/2}}. \tag{7}$$

Further consequences follow from the fact that macromolecules impede rotational motion less than translation. Facilitated transport occurs when a carrier protein is able to pick up a small ligand (particularly

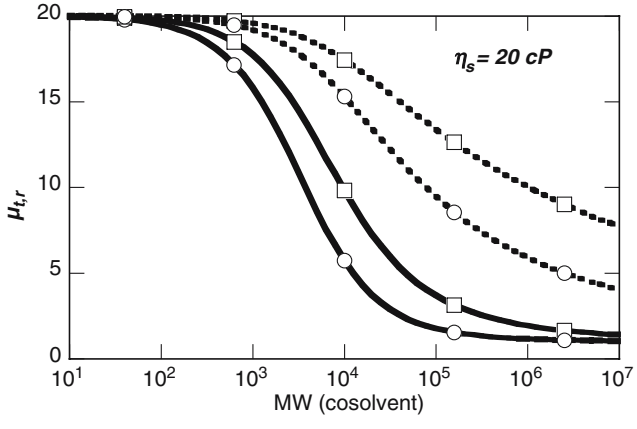

Fig. 3 Microviscosity predictions. Translational (*squares*) and rotational (*circles*) microviscosity predicted for GFP (R_h = 23 Å, *full lines*), a small protein commonly used in microscopic investigations within cells and for 70S ribosomes [R_h = 133 Å, *dotted lines* (Lavalette et al. 1977)]. A moderate external bulk viscosity. η_s = 20 cP was assumed in the simulation. The data are plotted in terms of cosolvent molecular weight, i.e. more familiar than the hydrodynamic radius

protons) that can be released after translational diffusion at a distance. Less familiar is the fact that Brownian rotation is able to provide an additional contribution to facilitated transport since a 180° rotation of the carrier protein is equivalent to a spatial displacement of the ligand by one protein diameter. The apparent diffusion coefficient, D^*, of this additional "rotation facilitated" transport is (Gros et al. 1984)

$$D^* = \frac{2}{3} R^2 D_r. \tag{8}$$

Whereas for ideal solutions the ratio $D^*/D_t = 1/2$ is independent of viscosity, it may increase up to threefold to fourfold in a macromolecular cosolvent even at moderate bulk viscosity, with the consequence that when the average MW of the cosolvent reaches a sufficient value, more ligands could be transported by rotation than by translation.

The same decoupling of rotational and translational motions with respect to viscosity may also be of some consequences for molecular recognition processes. The average angular rotation taking place during the time required for a protein molecule to move by one radius is normally about 0.7 radian for ideal solutions. But because rotation is less affected than translation, this value may become larger in macromolecular solutions thus increasing the probability of a correct orientation to be found during that time.

What is the physical meaning of the fractional exponents affecting the bulk viscosity? Einstein proposed that the viscosity induced by particles much larger than the solvent is due to the perturbation of flow lines and that energy is dissipated to maintain a steady flow. He was able to derive a simple formula for the viscosity induced by a dilute suspension of spherical particles as a function of a hydrodynamic factor, ν, and of the total volume fraction, φ, occupied by the particles.

For more realistic situations, semi-empirical corrections (Mooney 1951; Ross and Minton 1977) taking into account a self-crowding factor, κ, lead to

$$\eta = \eta_s \exp\left[\frac{\nu\phi}{1-\kappa\phi}\right] \tag{9}$$

an expression that has been shown to represent experimental data [especially dextrans (Lavalette et al. 1999)] at finite dilution quite satisfactorily. With our present definition, microviscosity becomes

$$\mu_{t,r} = \eta_s \exp\left[\frac{q_{t,r}\nu\phi}{1-\kappa\phi}\right] \tag{10}$$

showing that the role of the fractional exponents is to achieve a reduction of the hydrodynamic factor.

Conclusion

In our experiments neither the time course of the anisotropy decay (rotation) nor that of the diffusional autocorrelation function (translation) did show any sign of anomalous diffusion. Thus the deviations of the Stokes–Einstein laws are due to the failure of hydrodynamics at the microscopic scale and not to the statistical laws derived by Einstein. Equation 2 has been derived empirically. The fact that it describes all data over a wide range of proteins (from 68 to 4,000 kDa) and cosolvents (from 0.2 to 2,000 kDa) so well suggests that it may be of a sufficient generality to serve as a touchstone for future theoretical investigations of microscopic hydrodynamics.

In conclusion, we have explored one of the parameters characterizing the cell internal environment: the microscopic hydrodynamics resulting from the presence of macromolecules interfering with diffusion. Whereas the exclusion volume associated with crowding lowers the translational diffusion of tracers in *concentrated* protein solutions (Muramatsu and Minton 1988), the present work reveals an antagonist effect whereby translation and rotation of proteins are accelerated in *dilute macromolecular cosolvents* compared to their value in small solvents of the same viscosity.

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