Microscopic Viscosity and Rotational Diffusion of Proteins in a Macromolecular Environment

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ABSTRACT The Stokes–Einstein–Debye equation is currently used to obtain information on protein size or on local viscosity from the measurement of the rotational correlation time. However, the implicit assumptions of a continuous and homogeneous solvent do not hold either in vivo, because of the high density of macromolecules, or in vitro, where viscosity is adjusted by adding viscous cosolvents of various size. To quantify the consequence of nonhomogeneity, we have measured the rotational Brownian motion of three globular proteins with molecular mass from 66 to 4000 kD in presence of 1.5 to 2000 kD dextrans as viscous cosolvents. Our results indicate that the linear viscosity dependence of the Stokes–Einstein relation must be replaced by a power law to describe the rotational Brownian motion of proteins in a macromolecular environment. The exponent of the power law expresses the fact that the protein experiences only a fraction of the hydrodynamic interactions of macromolecular cosolvents. An explicit expression of the exponent in terms of protein size and cosolvent's mass is obtained, permitting definition of a microscopic viscosity. Experimental data suggest that a similar effective microviscosity should be introduced in Kramers' equation describing protein reaction rates.

INTRODUCTION

One reason for the continuing interest in rotational Brownian motion studies is to obtain information about either protein dimensions or the ambient viscosity, e.g., in vivo. This possibility is based on the fact that the random torque exerted upon a particle (or protein molecule) by the colliding solvent molecules causes a rotatory motion which is submitted to frictional damping. Consequently, solvent viscosity as well as the protein's size and shape affect the rate at which Brownian motion restores isotropy in an initially oriented ensemble of protein molecules. According to the differential law for Brownian motion derived by Einstein, the mean square angular random deviation of the infinitesimal angle $\Delta\theta$ by which a vector bound to a particle rotates during the time interval Δt is $\langle\Delta\theta^2\rangle = 4D_{\text{rot}}\Delta t$. The rotational diffusion coefficient is $D_{\text{rot}} = kT/f_{\text{rot}}$, assuming a velocity-dependent damping with a friction coefficient f_{rot}. The integrated form of the motion (Carrington and McLachlan, 1967) expresses the fact that the anisotropy parameter (Eq. 1) of an initially oriented population of particles decays exponentially with a rotational correlation time ϕ:

$$\left\langle \frac{3\cos^2\theta - 1}{2} \right\rangle = \exp(-6D_{\text{rot}}t) = \exp(-t/\phi). \qquad (1)$$

The left side of Eq. 1 equals the difference between the vertical and any horizontal projection of a radial vector R inclined by an angle θ with the vertical Oz axis of an orthonormal coordinate system. Nuclear magnetic resonance as well as electron spin resonance techniques provide indirect means for estimating ϕ from proton relaxation times or from the linewidth of spin labels, respectively. Optical methods monitor, in real time, the induced fluorescence or absorption anisotropy proportional to the left side of Eq. 1. The dipole transition moment (fluorescence or excited states absorption) of an attached or intrinsic probe serves as a reference vector.

The practical applications of Eq. 1 are based on the Stokes approximation for the hydrodynamic friction coefficient f_{rot} of a spherical particle. The correlation time is then expressed by the well known Stokes–Einstein–Debye (SED) equation,

$$\varphi = \frac{\eta_s V}{kT}, \qquad (2)$$

in which η_s is the solvent's viscosity and V the solvated volume of the rotating molecule.

Whereas the derivation of Eq. 1 only implies rather broad assumptions about the collision frequency between solvent and protein because of the discontinuous nature of the solvent, Eq. 2 is based on classical hydrodynamics and requires the hypothesis of a continuous and homogeneous solvent. Its validity has been verified experimentally with small, nonprotein molecules in pure solvents like alcohols and alkanes of different viscosity (Ben-Amotz and Drake, 1988). However, some moderate but significant deviations from the SED equation have been reported for small molecules (i.e., of the size of anthracene) in polymeric organic solvents (Hyde and Ediger, 1990; Gisser and Ediger, 1993), and the nature of the hydrodynamic boundary conditions leading to Eq. 2. has been questioned (Moog et al., 1982; Mikosch et al., 1994).

Because a change of solvent is impossible with proteins, the viscosity is usually adjusted by adding some viscous

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cosolvent, such as glycerol or sucrose, to explore a reasonable viscosity range. Although the validity of Eq. 2 has never been seriously questioned, the use of mixed cosolvent/water solutions departs from the hypotheses underlying the Stokes approximation.

Similarly, the local viscosity in vivo is likely to be governed by the high density of macromolecules rather than by small cosolvents. Often, the most relevant information is to know how fast a particular molecule diffuses in cells compared to its diffusion rate in water. The ratio of the correlation times is then characteristic of the apparent or microscopic viscosity experienced by the molecule of interest under the particular in vivo environment. Three recent works may be mentioned here as representative examples, each of them illustrating a different aspect of the problem and a different methodological approach. Genaro et al. (1996) used electron spin resonance to measure the correlation time of spin-labeled glutathion to test the hypothesis that the internal viscosity of erythrocytes is governed by intracellular Hemoglobin (Hb). Wang et al. (1997) used paramagnetic nuclear magnetic resonance relaxation rates of the proximal histidine of deoxy Myoglobin (Mb) and Hb to obtain the correlation time in the perfused myocardium and in erythrocytes, respectively. Swaminathan et al. (1997) monitored the fluorescence dichroism of the green fluorescent protein (GFP) expressed in CHO cells to estimate the cytoplasmic viscosity. Though the idea of a microscopic viscosity is not new, it is not easily quantified and, consequently, a reexamination of the rotational Brownian motion of proteins in mixed viscous solutions of macromolecular cosolvents has become appropriate.

Because an appreciation of nonhomogeneity depends on the relative scale of particle and cosolvent dimensions, significant information can only be obtained by exploring a wide range of particle and cosolvent size or molecular weight (MW). In this work, we used the transient optical absorption anisotropy method to measure the isothermal rotational relaxation of bovine serum albumin (BSA, MW = 66,000), earthworm (*Lumbricus terrestris*) hemoglobin (EW-Hb, MW = 4,000,000), and of a fragment thereof, [F(EW-Hb), MW = 330,000] in the viscosity range 1.5 to 200 cP, using a series of cosolvents presenting chemically similar groups: glycerol and dextrans with molecular weight between 1.5 and 2,000.

METHODS AND MATERIALS

Glycerol and dextrans were from Fluka (St.-Quentin, France). BSA and tetramethyl-rhodamine-isothiocyanate (TRITC) were from Sigma (St.-Quentin, France). Labeling was performed at pH 9.5 in borate buffer. The TRITC-BSA conjugate was passed on an ACA202 (Biosepra, Paris, France) column to eliminate unreacted dye and further purified on a trisacryl DEAE column eluted using a 0–300 mM NaCl gradient. The conjugate contained an average of 1 dye per BSA molecule. EW-Hb was prepared from local sources of live worms according to known procedures (Shlom and Vinogradov, 1973), with final purification by repeated centrifugation at 362,000 × g. All steps were performed using the CO complex to avoid oxydation. Subunits F(EW-Hb) were obtained as described by Kapp et al. (1984), separated and stabilized by gel permeation chromatography (TSK HW55S) (Touzart, Orsay, France), and finally concentrated by centrifugation for 6 h at 362,000 × g. According to the correlation time measurement, for the fragment F(EW-Hb), we obtain a hydrated volume 12.8 times smaller than for EW-Hb. This is in agreement with EW-Hb consisting of 12 major subunits (Kapp et al., 1984). Viscosities were measured at 5°C using a series of Ubbelohde viscosimeters. Densities were obtained by weighing a certain volume of solution taken at 5°C.

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^{-5} through 10^{-4} M.

RESULTS AND DISCUSSION

Viscosity dependence of correlation time

In agreement with Eq. 1, the anisotropy of the three globular proteins investigated was found to relax exponentially (Fig. 1). In glycerol, the correlation time was directly proportional to the viscosity. The reduced correlation times ϕ/η and hydrodynamic radii R_{prot} listed in Table 1 were computed using Eq. 2.

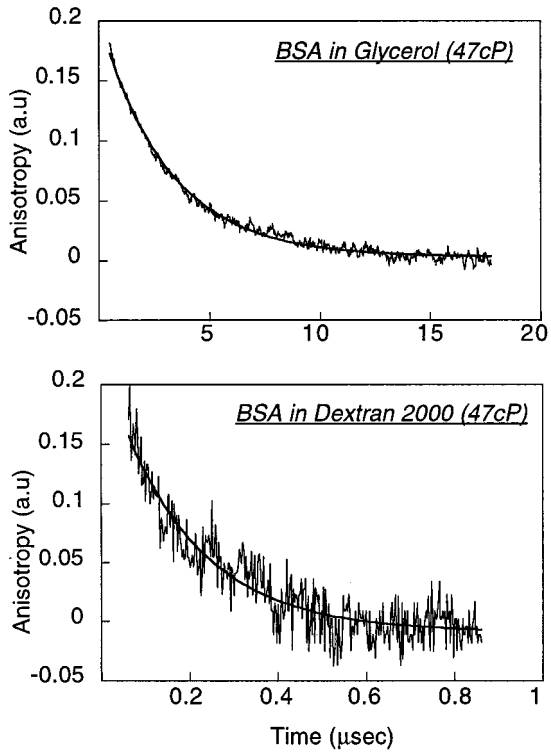

FIGURE 1 Anisotropy decay of TRITC-labeled BSA in isoviscous (47 cP) buffer solutions of glycerol (*top*), and dextran 2000 (*bottom*). Note the change in the time scale. The dual beam absorption setup gives a signal proportional to the anisotropy. The lines are single exponential fits to the data points.

TABLE 1 Correlation times, hydrodynamic radii, and fitting parameters (Eq. 5)

	Molecular Weight	ϕ/η (278 K) (ns/cP)	R_{prot} (Å)	M_0	n	$R_{prot} \times$ n
BSA	66,000	68	40	13200	0.46	18.4
F(EW-Hb)	330,000	198	57	9200	0.24	17.2
EW-Hb	4,000,000	2530	134	12800	0.13	13.7

(1 cP = 10^{-3} Pa/s).

Strong deviations from the Stokes–Einstein law were observed with macromolecular cosolvents (Fig. 2). For the three proteins, a power law best fits the data,

$$\frac{\phi}{\phi_s} = \left(\frac{\eta}{\eta_s}\right)^q, \tag{3}$$

in which η and η_s are the viscosity of the mixed and pure solvent, respectively (η_s = 1.5 cP for water at 278 K). Except for glycerol, q is smaller than unity and is distinctly cosolvent and protein dependent (Fig. 2 and Fig. 3 *A*). These experiments only permit determination of the functional dependence of ϕ/ϕ_s on η/η_s. Because the Stokes–Einstein approximation is satisfied in pure solvents, one may insert the value of ϕ_s given by Eq. 2 to obtain the desired generalization to mixed solvents:

$$\phi = \left(\frac{\eta}{\eta_s}\right)^q \frac{\eta_s V}{kT}. \tag{4}$$

In Eq. 4, $(\eta/\eta_s)^q$ is a pure number that may be regarded as correcting for the noncontinuous and nonhomogeneous nature of the mixed solvent. The dependence of q on cosolvent and protein size should therefore not be surprising.

For a given protein, the variation of q with the cosolvent's MW tends toward a power law with large cosolvents (Fig. 3 *A*). The data are correctly described by

$$q = (1 + M/M_0)^{-n}, \tag{5}$$

in which M is the cosolvent's MW. The fitting parameters M_0 and n listed in Table 1 present remarkable regularities. The product $R_{prot} \times n$ of the exponent n and the protein hydrodynamic radius and M_0 have very close values for the three proteins. This suggests that all data can be globally parametrized by

$$q = \left(1 + \frac{M}{M_0}\right)^{-R_0/R_{prot}}, \tag{6}$$

where R_{prot} is the protein hydrodynamic radius and M_0 and R_0 are constants common to all proteins–cosolvent systems. The global fit of Fig. 3 *B* supports the hypothesis.

At 278 K, the best estimates for the common parameters are M_0 = 8,800 and R_0 = 15.3 Å. Interestingly, these values indicate a possible mutual dependence of R_0 and M_0. The molecular weight of a liquid substance of specific mass δ is $M = v\delta N$, where N is Avogadro's number, and v is the spherical volume occupied by one single molecule. With R_0 = 15.3 Å and δ = 1, one obtains M = 9000, which is very close to M_0 and suggests that R_0 is the radius of the spherical space occupied by the mass 8800 if the density remains close to unity. Such a space can be occupied by approximately 488 molecules of water, 96 molecules of

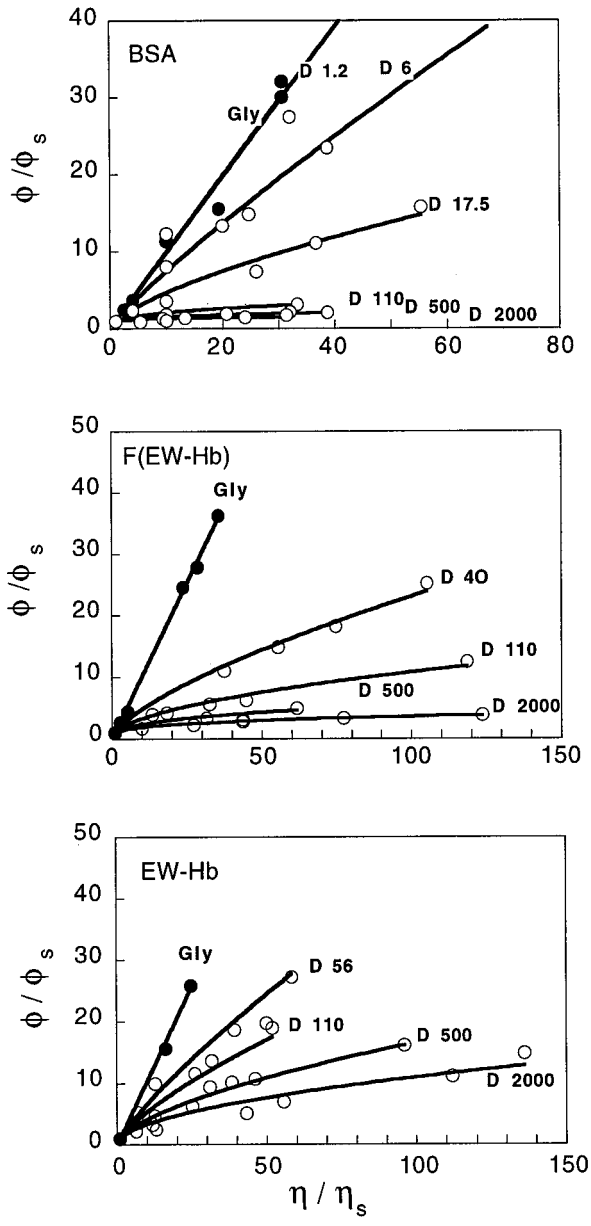

FIGURE 2 Relative increase of the correlation time of BSA, F(EW-Hb), and EW-Hb as a function of the relative viscosity in various water/dextrans, and water/glycerol mixed solvents. Temperature: 278 K. *Closed circles*, glycerol; *open circles*, dextrans. The dextran molecular mass is expressed in kD.

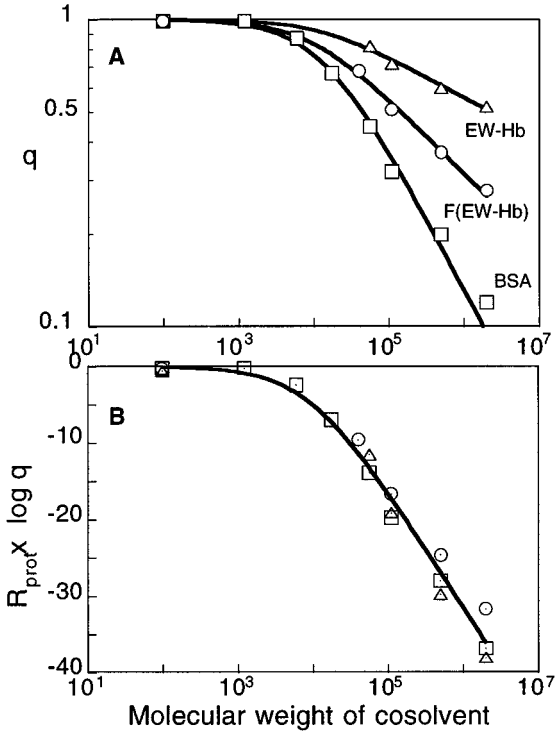

FIGURE 3 (*A*) Dependence of q (Eq. 3) on protein, and cosolvent molecular weights. Lines are least square fits using $q = (1 + M/M_0)^{-n}$. (*B*) Plot of $R_{prot}\log q = -R_0\log(1 + M/M_0)$ (see Eq. 6) against cosolvent molecular weight showing that one unique correlation is obtained with only two parameters R_0, and M_0. The *solid line* is the global, two-parameters fit of all data. *Squares*, BSA; *circles*, F(EW-Hb); *triangles*, EW-Hb.

glycerol, or by 48 glucose monomers of the dextrans. Although the argument indicates that R_0 and M_0 are numerically related, it does not permit one to decide whether the parameters are connected to the solvent or to the cosolvents.

The average distance between two protein molecules is of the order of 200 Å in our experimental conditions. At the maximum cosolvent fractional concentration of 0.2 used, a protein molecule is surrounded on the average by 10^6 molecules of water, 2×10^4 of glycerol, 2×10^3 of D 1.5 kD and only 2 molecules of D 2000 kD. Intuition suggests that, if the individual protein molecule were exposed mainly to solvent, the apparent viscosity should be less than the macroscopic viscosity. To reconciliate this view with the fact that q is concentration independent and to explain why a power-law of the viscosity is observed, a connection between macroscopic viscosity and solute concentration must be established.

Connection between viscosity and cosolvent concentration

The relative viscosity of concentrated protein solutions has been shown to follow an empirical generalization of a formula first proposed by Mooney (Mooney, 1951; Ross and Minton, 1977). In terms of solute mass fraction c ($0 < c < 1$), Mooney's relation is

$$\frac{\eta}{\eta_s} = \exp\left(\frac{Ac}{1 - Bc}\right). \tag{7}$$

A is characteristic of the cosolvent hydrodynamic interactions, and B is proportional to a self-crowding factor. In Mooney's original work, the relative viscosity was expressed as a function of the volume fraction of the cosolvent. Here, we use the mass fraction c, because the hydrodynamic volume of the cosolvents is not known. Both variables are simply proportional if, as was the case here, the density of the mixed solutions remains close to unity. In this work, we are not concerned with the particular values of the parameters A and B, but only with the discriminative power of the functional dependence of the viscosity on c.

As shown in Fig. 4, the generalized Mooney equation describes the viscosity of glycerol/water and dextran/water mixed solvents very well. Combining Eq. 3 and Eq. 7, one obtains, for the relative increase of the correlation time,

$$\frac{\phi}{\phi_s} = \exp\left(\frac{Aqc}{1 - Bc}\right). \tag{8}$$

This result, obtained by bringing together two experimental correlations, unveils the origin of the viscosity power law. The exponent q appears as a reduction factor applied to A, the hydrodynamic interactions of the cosolvent.

One may wonder whether our data are able to discriminate this result from the alternative possibility, according to which the protein would sense only a fraction of the number of cosolvent molecules. One would then expect

$$\frac{\phi}{\phi_s} = \exp\left(\frac{Aqc}{1 - Bqc}\right). \tag{9}$$

This question is not a trivial one, because the right hand sides of Eqs. 8 and 9 are close when the molecular weight of the cosolvent decreases. Even then, because the product Bqc is small, both tend toward an exponential law. Indeed,

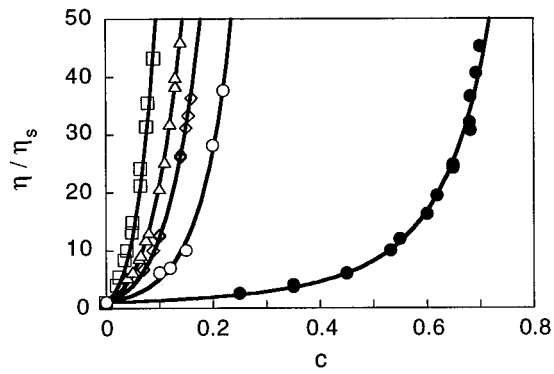

FIGURE 4 Least square fits of the relative macroscopic viscosity of cosolvents/water mixtures as a function of the cosolvent mass fraction according to Eq. 7. *Squares*, D 2000 kD; *triangles*, D 500 kD; *diamonds*, D 110 kD; *open circles*, D 40 kD; *closed circles*, glycerol. Temperature: 278 K.

plots of ϕ/ϕ_s against c (not shown) are roughly compatible with exponentials, although with an exponent different from A.

The comparison of experimental data points with the simulated curves shown in Fig. 5 is, beyond doubt, in favor of Eq. 8 and definitely confirms that the reduction factor q applies to the parameter A and not to the mass fraction c. So, it may be concluded that an individual protein molecule remains exposed to the full cosolvent concentration but feels only a fraction of its hydrodynamic interactions. For small dye molecules, there are clear indications that the SED Eq. 2 does not accurately describe rotational diffusion in extreme situations. So, for rhodamine B, the different linear viscosity dependence found in monoalcohols and in polyalcohols has been attributed to a change from "stick" to "slip" hydrodynamic boundary conditions, characterized by a reduction of the correlation time (Moog et al., 1982). For pyrene derivatives in water–glycerol mixtures, the SED Eq. 2 showed a saturation effect as a function of η/T at high viscosity, which was similarly attributed to a breakdown of the hydrodynamic model (Mikosch et al., 1994). However, the correction for the hydrodynamic boundary conditions appears in the SED equation as a multiplicative factor and not as a power law of the viscosity.

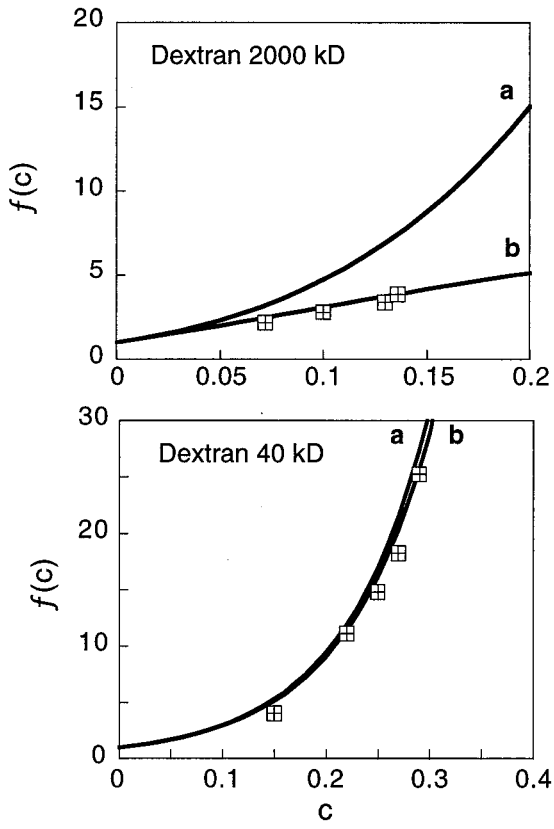

FIGURE 5 Experimental values of ϕ/ϕ_s for F(EW-Hb) (*squares*) as a function of cosolvent mass fraction c compared to (*a*) Eq. 9, and (*b*) Eq. 8 for dextran 2000 kD (*top*), and dextran 40 kD (*bottom*). The functions were calculated using the parameters A and B of the respective Mooney's fit of the cosolvent viscosity curves. Whereas both expressions are almost indistinguishable with low molecular weight cosolvents, they are clearly discriminated in dextran 2000 kD. Experimental data suppport the power law.

We are not aware of a microscopic hydrodynamic theory that could explain the particular dependence of the reduction factor q on cosolvent mass and protein hydrodynamic radius. The empirical parameter R_0 appearing in Eq. 6 might possibly represent some critical distance for the cosolvent hydrodynamic forces. Because viscosity is characteristic of the transfer of momentum, such a critical distance might be also related to a critical mass M_0.

Effective or microscopic viscosity of a macromolecular cosolvent

The fundamental connection between correlation time and friction coefficient $\phi = f_{rot}/6k_BT$ is independent of Stokes law. In terms of friction coefficients, Eq. 3 is equivalent to

$$\frac{f_{rot}}{f_{s,rot}} = \left(\frac{\eta}{\eta_s}\right)^q \tag{10}$$

independently of the explicit value used for $f_{s,rot}$. The equivalent of Eq. 4 is

$$f_{rot} = 6V\eta_s\left(\frac{\eta}{\eta_s}\right)^q \tag{11}$$

if Stokes friction is substituted for $f_{s,rot}$.

The generalized Stokes–Einstein equation (Eq. 4) and Eq. 11 may be recasted as

$$\phi = \mu V/kT \tag{12}$$

and

$$f_{rot} = 6\mu V, \tag{13}$$

in which we have defined an effective microscopic viscosity, μ, experienced by a particle in a mixed solvent as

$$\mu = \eta_s(\eta/\eta_s)^q. \tag{14}$$

This definition respects the form of the basic equations without changing the variable dimensions (which would not be the case if one were writing, for instance, $\phi = \eta^p V/kT$).

For practical purposes, it may be useful to estimate q directly from the protein and cosolvent molecular weights without prior knowledge of the protein correlation time or hydrodynamic radius. This can be done using an empirical correlation based on our past experience with rotational measurements of proteins over a wide range of MW: $\phi = 2 \times 10^{-4}\ M^{1.11}$ in which ϕ is the correlation time (in ns) measured at 1 cP and 20°C (Lavalette, unpublished results). The uncertainty about ϕ is generally better than 20%. Using Eqs. 2 and 6, Fig. 6 can be constructed, permitting a rapid estimation of q for a protein of interest in a given cosolvent.

Equations 12 and 6 are well behaved in two limiting situations of major practical interest. For a small cosolvent ($M \ll M_0$) and for a large protein or a macroscopic particle ($R_0 \ll R$), $q = 1$ and ϕ/ϕ_s is proportional to the macro-

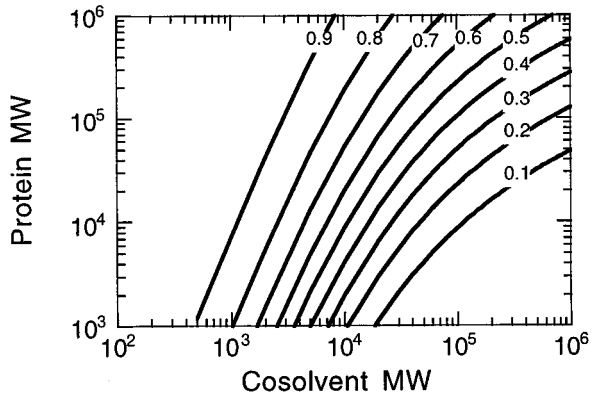

FIGURE 6 Plots of equal q values for protein–cosolvent systems in terms of protein, and cosolvent molecular weights.

scopic viscosity of the mixed solvent ($\mu = \eta$). The Stokes–Einstein equation for a continuous solvent is valid in this limit, which corresponds to most in vitro investigations using principally small cosolvents. In contrast, for a small particle or a large cosolvent, $q = 0$. The relative correlation time ϕ/ϕ_s is only proportional to the solvent viscosity ($\mu = \eta_s$) independent of the cosolvent macroscopic viscosity even though the latter may be considerable. This is illustrated in Fig. 7, which give the calculated effective microviscosity experienced by two proteins of differing size in a series of isoviscous solutions as a function of the cosolvent molecular weight.

The situation depicted in Fig. 7 is directly relevant to the estimation of local viscosity in vivo. For instance, at the estimated physiological Hb concentration of 33 g/dL in erythrocytes (Gennaro et al., 1996), the relative macroscopic viscosity of a hemoglobin solution is about 7 cP (at 37°C) (Ross and Minton, 1977). Considering Hb as its own cosolvent and using the correlation time of Hb to calculate R_{prot}, Eq. 6 yields $q = 0.37$. The apparent intracellular

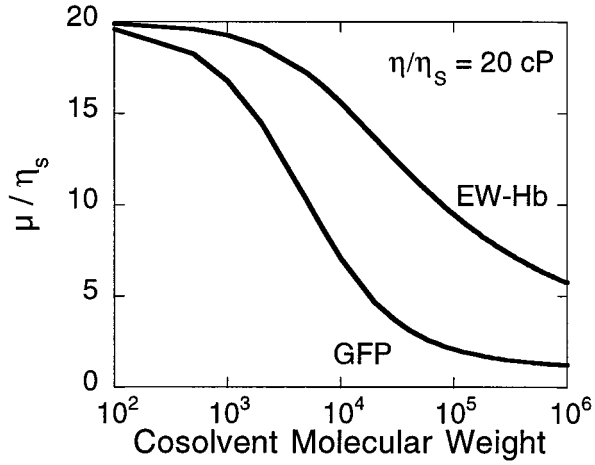

FIGURE 7 Calculated relative microviscosity μ/η_s experienced by GFP, and EW-Hb in isoviscous solutions (20 cP) of dextrans. For GFP, the parameter q was calculated using the rotational correlation time reported by Swaminathan et al. (1997).

microviscosity is thus expected to be approximately $7^{0.37} = 2$ cP, in good agreement with the reported ratio of 2.2 for the correlation times of Hb in the erythrocyte compared to water (Wang et al., 1997). Swaminathan et al. (1997) reported that the correlation time of GFP in CHO cells is only 1.5 that found in water and that the same ϕ value was obtained in 12% (w/w) 70 kD dextran. Using, again, Eq. 6 and the correlation time of GFP, one gets $q = 0.28$ for GFP in 70 kD dextran. From our own viscosity measurements on dextran solutions, we may estimate the macroscopic viscosity of 12%–70 kD dextran to be about 20 cP, giving an apparent viscosity of $20^{0.28} = 2.3$ (see also Fig. 7), which is comparable to the experimental value of 1.5, given the uncertainties of the viscosity extrapolations.

In all similar works, it should be kept in mind that microscopic viscosity is not a property of the environment per se, and that the answer depends on the size of molecular probe used.

Translational diffusion

Nonlinearity on solvent viscosity of translational diffusion coefficients of small solutes has been reported several times in the past. Whereas the apparent diffusion coefficient of microspheres in dextrans was reported to deviate only slightly from a linear T/η dependence (Phillies and Quinlan, 1992), the microscopic viscosity experienced in the presence of rod-shaped polymers has been found to be up to 50% smaller than expected (Tracy et al., 1993). Examples and the theories aiming at describing friction effects in term of free volume or of time and frequency-dependent friction have been recently reviewed (Gavish and Yedgar, 1995). Nonhomogeneity of viscous cosolvent mixtures has been proposed as a cause, and the translational diffusion of water tracers has been shown to be affected by cosolvents according to a power law of the cosolvent molecular weight (Barshtein et al., 1995). But, because the tracers consisted of isotopically labeled water molecules, a relation similar to Eq. 6 could not be elaborated. The quantitative relations established here for rotational diffusion might well provide a touchstone for an adequate theory of friction on a microscopic scale, which remains to be developed.

Prospects in protein dynamics

The definition of the effective microscopic viscosity (Eq. 14) may be also relevant to frictional effects observed in protein dynamics. When crossing over an energy barrier is driven by Brownian motion, Kramers' theory predicts that friction plays a pivotal role in the evaluation of the rate of barrier crossing (Kramers, 1940).

In the high friction limit, the rate parameter is given by $k = (A/\beta)\exp(-H/RT)$, in which β measures the friction along the reaction coordinate and the other symbols have their usual meaning. (The intermediate friction case involves a more complicated function of β.) The difficulty is

to find an adequate and explicit form for β in terms of measurable quantities.

Because protein structural fluctuations are clearly driven by Brownian motion, Kramers' theory has been invoked to describe the rate of entry and escape of small ligands (Beece et al., 1980; Lavalette and Tetreau, 1988) and enzymatic reactions (Gavish and Werber, 1979; Ng and Rosenberg, 1991) or hydrogen exchange (Rosenberg et al., 1989; Somogyi et al., 1988). In the high friction limit, and assuming classical hydrodynamic friction $\beta \sim \eta$, the rate parameter is usually parametrized by

$$k = \frac{A}{\eta_s} \exp\left(-\frac{H}{RT}\right). \tag{15}$$

With proteins, experiments generally report a power law on viscosity $k \sim \eta^{-p}$ with $p < 1$. In early works, the fractional exponent was hypothesized to result from some kind of shielding of the frictional effects by the protein matrix (Beece et al., 1980). In a recent investigation of the escape rate of oxygen from the respiratory protein, hemerythrin, in the presence of dextrans, we found that the exponent varied with the mass M of a macromolecular cosolvent according to $p = 1.52\ M^{-0.23}$, suggesting that hydrodynamic effects might be more important than initially thought for explaining the fractional viscosity exponent (Yedgar et al., 1995). A straightforward manipulation shows that the hemerythrin result may be rewritten as $p = (1 + M/M_0)^{-n}$ with $M_0 = 6.37$ and $n = 0.23$, in line with Eq. 5. A further analysis of the exponent n, similar to that performed for rotational diffusion, is not possible here, because the size of that part of the protein responsible for the fluctuating motion is not known. The analogy with the rotational diffusion problem suggests that a suitable generalization of Eq. 15 could be

$$k = \frac{A}{\eta_s(\eta/\eta_s)^p} \exp\left(-\frac{H}{RT}\right), \tag{16}$$

in which the effective microscopic viscosity μ can be recognized in the denominator of the prefactor. It should not be surprising that p for oxygen escape from hemerythrin is different from q for rotational Brownian diffusion because the protein fluctuations involved in ligand escape correspond to localized motions, whereas rotational motion is representative of the whole molecule. Nevertheless, both problems formally permit a similar definition of microviscosity. If the argument is valid, one might anticipate that q and p will become approximately equal for large conformational changes involving a protein as a whole.

Kramers' theory has been repeatedly tested using unsaturated organic polymers isomerization reactions monitored by nuclear magnetic resonance or optical probes. (Glowinkowski et al., 1990; Adolf et al., 1992; Zhu and Ediger, 1995, 1997). Such reactions are localized in the polymer and, in a sense, they may be compared to ligand escape from proteins. They systematically exhibited a power law on viscosity, indicating a breakdown of Kramers' approximation when solute reaction and solvent reorganization do not take place on well separated time scales. However, the exponent (0.40–0.76) was found to be a characteristic of the polymer being investigated rather than of the solvent. Bowman et al. (1988) tested the picosecond excited state isomerization of 1–1′-binaphthyl as a model reaction. When friction was evaluated by using a hydrodynamic model in which $\beta \sim \eta$, agreement was satisfactory for a series of n-alcohols solvents, but significant deviations were observed with n-alkanes. Complete agreement with Kramers' theory was restored, however, when the authors estimated the friction coefficient directly from the measured rotational correlation time of binaphthyl itself rather than from the macroscopic viscosity of the solvents. Though no attempt was made in the binaphthyl isomerization case to derive a formula similar to our Eq. 3, the excellent agreement obtained by using the rotational correlation time ϕ to parametrize the friction coefficient β suggests that μ is likely to be the same for the rotational motion and for the particular reaction, provided the latter involves a conformational change affecting the molecule as a whole. We take this as the first piece of evidence that hydrodynamic deviations may indeed, in some cases, affect rotational diffusion and reaction rates in a similar way.

The difficulty with the study of rate coefficients of proteins is twofold. First, those parts of a protein undergoing conformational fluctuations are not precisely known. Second the use of cosolvents may possibly induce correlative changes in other physical parameters such as dielectric constant or osmotic pressure, which may also affect the dynamics of protein fluctuations. The present work was actually initiated in an attempt to evaluate the importance of purely hydrodynamic effects using a well defined microscopic process, Brownian motion, which should be insensitive to such changes, while remaining on comparable spatial and time scales.

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