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Viscosity-Dependent Kinetics of Protein Conformational Exchange: Microviscosity Effects and the Need for a Small Viscogen

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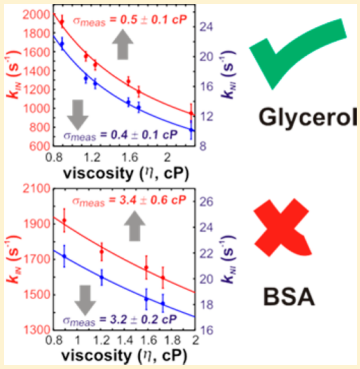
ABSTRACT: Conformational rearrangements are critical to a variety of biological processes including protein folding and misfolding, ligand binding, enzyme catalysis, and signal transduction. Viscosity-dependent kinetics measurements can provide crucial insights into the dynamics of protein conformational exchange by highlighting the relative importance of frictional forces derived from either solvent or from internal protein interactions in activating the exchange reaction. Here, we analyze the kinetics of interconversion between the native and intermediate states of the four helix bundle FF domain recorded in solutions containing the viscogens glycerol or bovine serum albumin (BSA), using the viscosity measured from the translational diffusion of probes of different sizes. In the large viscogen BSA, we demonstrate that vastly different internal friction values are obtained using the different viscosity measures, leading to conflicting interpretations of the role of solvent friction in the interconversion. We show that this can be a consequence of the small effective hydrodynamic radius of the protein conformational transition and differences between solution micro- and macroscopic viscosities that are germane in this case. In general, correct values of internal friction can only be obtained by carrying out measurements using small viscogens.



INTRODUCTION

Biological processes often involve conformational rearrangements occurring on a variety of different time and length scales. These changes are modeled as activated but diffusive barrier crossing events[1,2] for which the rate constants can be evaluated using Kramers' theory in the high friction limit.[3,4] Kramers' approach adopts a reduced dimensional picture of the conformational transition with a majority of both solvent and protein degrees of freedom not explicitly considered.[4] In the reduced dimensional formulation, a single viscosity term is used to account for the friction along the reaction coordinate.

In typical protein conformational exchange events, contributions to viscosity can arise from both solvent and protein degrees of freedom.[5,6] These two contributions are generally considered to be additive, although more complicated models are available.[7] The solvent contribution can be systematically increased by adding a viscogen such as glycerol, and this provides a route to separate the viscosity contributions from solvent and internal degrees of freedom. Viscous forces in solution originate from collisions between the solvent and the protein as the conformational transition progresses. These forces impede motion along the reaction coordinate but also provide the energy needed to cross the activation barrier and are related by the fluctuation dissipation theorem.[8,9] Consequently, experiments probing the dependence of conformational exchange kinetics on viscosity are valuable because they provide insight into the source of the energy needed for conformational exchange and the relative importance of solvent and protein degrees of freedom in activating the exchange process.[6,10−16] The measurement of the viscosity dependence of rates in two viscogens, one small and one large, can also provide information on the effective hydrodynamic radius (EHR) of the exchange reaction.[17] The EHR is a length scale characterizing the exchange process and can be interpreted as the average size of units in the protein that diffuse along the reaction coordinate at a given time.

Critically important to experiments probing the viscosity-dependence of protein conformational exchange kinetics is the choice of viscogen. Viscogens can be small molecules such as ethylene glycol, proteins like lysozyme and bovine serum albumin (BSA), or polymers such as Ficoll. In cases where the isostability adjustment[18] is not used, the choice is limited to viscogens that do not perturb the conformational free energy landscape. In this report, we demonstrate the need for a small molecule viscogen to obtain even qualitatively correct conclusions about internal friction and hence the relative importance of water and protein viscosity contributions to the exchange reaction. This is illustrated using viscosity-dependent kinetics measurements of the interconversion between intermediate (I) and native (N) states of the four helix bundle FF domain using either glycerol or BSA as viscogen. We highlight a pair of alternate models that follow from the interpretation of the data acquired with BSA that can only be resolved by employing the small molecule viscogen glycerol.

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MATERIALS AND METHODS

^{15}N-labeled WT FF domain from human HYPA/FBP11 was overexpressed in *E. coli* and purified as described in detail previously.[19] Samples for NMR were prepared in 50 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, 1 mM sodium azide, pH 5.7, 90% H_2O/10% ^2H_2O. Details of relaxation dispersion experiments from which FF domain interconversion kinetics were extracted (25 °C) have been reported.[15] Diffusion coefficients of acetate and the FF domain were obtained from pulsed field gradient diffusion NMR measurements using a water-suppressed longitudinal encode−decode (water-sLED) pulse sequence for acetate[20] and an ^{15}N-edited pulse scheme for FF.[21] Spectra were processed and diffusion coefficients extracted as detailed in Sekhar et al.[15] Diffusion coefficients were converted to viscosity values by applying the Stokes−Einstein equation (eq 5 below) and assuming the viscosity of buffer without viscogen to be 0.89 cP at 25 °C.[15,22]

RESULTS AND DISCUSSION

Kramers' equation[3,4] for the rate of an activated process in the high friction limit is given by

$$k_{A\to B} = \frac{\omega_A \omega^{\neq}}{2\pi\gamma_R}\exp\left(-\frac{\Delta G_{A\to B}^{\neq}}{RT}\right) = \frac{\delta\omega_A \omega^{\neq}}{2\pi\eta_R}\exp\left(-\frac{\Delta G_{A\to B}^{\neq}}{RT}\right) \quad (1)$$

where $k_{A\to B}$ is the rate of conversion from state A to state B, ω_A and $\omega^{\neq}$ are the curvatures of the free energy surface at the reactant well and the barrier, respectively, γ_R is the frictional force, η_R is the viscosity along the reaction coordinate ($\eta_R = \delta\gamma_R$) and $\Delta G_{A\to B}^{\neq}$ is the free energy of activation for the conversion $A \to B$. For reactions involving proteins, internal friction originating from solvent-independent protein degrees of freedom can contribute significantly to the overall viscosity.[5,6] Often, viscosity effects from solvent and internal friction are combined according to[6]

$$\eta_R = c\eta_s + (1-c)\eta_i = c\left[\eta_s + \left(\frac{1-c}{c}\right)\eta_i\right] = c(\eta_s + \sigma') \quad (2)$$

where c is the fractional contribution of the solvent viscosity (η_s) to the overall viscosity and η_i is the viscosity arising from solvent-independent internal degrees of freedom. The term proportional to η_i can be recast in terms of σ' as indicated in eq 2. Note that η_s, η_i, and σ' all refer to viscosities along the reaction coordinate. Inserting eq 2 into eq 1

$$k_{A\to B} = \frac{\delta\omega_A \omega^{\neq}}{2\pi c(\eta_s + \sigma')}\exp\left(-\frac{\Delta G_{A\to B}^{\neq}}{RT}\right) \quad (3)$$

that can be written as

$$k_{A\to B} = \frac{K}{\eta_s + \sigma'} \quad (4)$$

where it is most often assumed that K does not depend on viscosity.

The Kramers' model describes the reaction in terms of a diffusive passage over the activation barrier,[4] which in analogy with translational diffusion can be characterized by an EHR defining a length scale for this diffusive barrier crossing.[17] The exchange of energy between reacting moieties and the solvent molecules that are treated implicitly depends upon the EHR, with a larger EHR leading to more efficient exchange. In analogy to the fact that the viscosity of a solution can depend on the size of the probe that is used to measure it, the viscosity along the reaction coordinate can be sensitive to the EHR of the conformational exchange reaction (see below).

The relative contributions of η_s and σ' to the overall friction are usually determined by measuring rates of reactions in solutions where the viscosity is systematically varied through the addition of viscogens such as glycerol or polymers like Ficoll or poly(ethylene glycol). The viscosity, in turn, can be obtained from flow rates using viscometers (so-called macroscopic viscosity) or by measuring the translational diffusion coefficient (D) of a probe molecule from which η is calculated as

$$\eta = \eta_0\frac{D_0}{D} \quad (5)$$

where D_0 is the diffusion coefficient of the probe in a buffer solution of viscosity η_0. The value of η obtained using the diffusion of the probe is termed microscopic viscosity and is approximately equal to the macroscopic value for probes with hydrodynamic radii on the order of or larger than the viscogen radius.[23−25] In solutions containing "small molecule" viscogens such as ethylene glycol or glycerol, the method of translational diffusion gives the same viscosity value for any size probe (since any probe is of the approximate size or larger than the viscogen), and in this case macroscopic and microscopic viscosities are identical. By contrast, the viscosity of a solution containing a larger viscogen, such as sucrose or a protein, depends on the size of the probe that is employed to measure it because of the well-documented effects of micro- and macroviscosity on translational diffusion.[23−25]

Equations have been derived previously relating the viscosity of a viscogen-containing solution measured using a probe of hydrodynamic radius r_p, $\eta_{meas}(r_p)$, to the viscosity of a solution without viscogen, η_o, and the hydrodynamic radii of probe and viscogen (r_v) molecules.[23,25] Noting that in the general case $\eta_{meas}(r_p)$ lies between η_o and the macroscopic viscosity of the viscogen solution, η, we will express $\eta_{meas}(r_p)$ in what follows as

$$\eta_{meas}(r_p) = f_r\eta + (1-f_r)\eta_0 \quad (6)$$

where $f_r \in [0, 1]$ can be a complicated function of many parameters, including the concentration of viscogen, r_p and r_v with $f_r = 1$ in the limit $r_p \to \infty$. As we show below, from this particular form of $\eta_{meas}(r_p)$ it is particularly straightforward to see that extracted values of σ can depend very significantly on r_p that is a central result of the present work. It is noteworthy that η_s in eqs 2−4 is the viscosity along the reaction coordinate and not the macroscopic viscosity of the solution (η), and there appears to be no rigorous justification for replacing viscosity along the reaction coordinate by the measured solution viscosity.[26] However, since motion along the reaction coordinate exchanges energy with solvent through collisions, much as the diffusion of a particle in solution, the viscosity along the reaction coordinate may, to a first approximation, be considered linearly proportional to the viscosity measured using a probe of a hydrodynamic radius R that is equal to the EHR of the reaction

$$\eta_s = p\eta_{meas}(R) = p[f_R\eta + (1-f_R)\eta_0] \quad (7)$$

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where p is a constant of proportionality. Substituting η from eq 6 into eq 7 yields

$$\eta_s = p\left[f_R\left(\frac{\eta_{meas}(r_p) - (1-f_r)\eta_0}{f_r}\right) + (1-f_R)\eta_0\right] \quad (8)$$

that can then be inserted into eq 4

$$k_{A\to B} = \frac{K'(r_p)}{\eta_{meas}(r_p) + \left[\left(\frac{f_r - f_R}{f_R}\right)\eta_0 + \left(\frac{\sigma f_r}{f_R}\right)\right]} = \frac{K'(r_p)}{\eta_{meas}(r_p) + \sigma_{meas}} \quad (9)$$

where $K' = Kf_r/pf_R$, $\sigma = \sigma'/p$, and $\sigma_{meas} = [(f_r - f_R)/f_R]\eta_0 + (\sigma f_r/f_R)$ is typically reported for σ in the literature. In the case where rate measurements are carried out using a small molecule viscogen, $f_R = f_r = 1$, and eq 9 reduces to

$$k_{A\to B} = \frac{K'}{\eta_{meas} + \sigma} \quad (10)$$

Under these conditions $(\eta_{meas}/\sigma) = (\eta_s/\sigma')$ so that the relative contributions to the viscosity along the reaction coordinate from solvent and internal friction η_s/σ' can be obtained from the experimentally determined values of η_{meas} and σ.

The situation is more complex when larger molecule viscogens are used since the measured viscosity depends on the probe hydrodynamic radius. The choice of probe therefore becomes critical. If the EHR (R) of the reaction under study is known *a priori*, a probe with size $r = R$ can be chosen so that $f_R = f_r$, in which case eq 9 reduces to eq 10 and the correct value of η_s/σ' can once again be obtained. When the EHR is not known, as would normally be the case at least initially, the choice of a viscogen that is outside the small molecule limit (i.e., where $f_r, f_R \neq 1$) can be problematic. For example, in the case where the probe radius $r > R$ it follows that $f_r > f_R$ so that

$$\sigma_{meas} = \left(\frac{f_r - f_R}{f_R}\right)\eta_0 + \left(\frac{\sigma f_r}{f_R}\right) > \sigma \quad (11)$$

and the measured contribution from internal friction exceeds the correct value. In a similar manner when $r < R$ and hence $f_r < f_R$

$$\sigma_{meas} = \left(\frac{f_r - f_R}{f_R}\right)\eta_0 + \left(\frac{\sigma f_r}{f_R}\right) < \sigma \quad (12)$$

and the measured value of internal friction is an underestimate of the actual value. This ambiguity in σ_{meas} can only be resolved by using a small viscogen where the viscosity "felt" by any probe and also by the reaction as it proceeds is the same.

Differences between macroscopic and microscopic viscosity in the context of viscosity-dependent kinetics measurements have been discussed qualitatively earlier.[10,27] However, they come into prominence because of recent measurements showing that the EHR for a protein conformational transition can be much smaller than the hydrodynamic radius of the protein itself. The viscosity along the reaction coordinate, which depends on the EHR of the conformational transition, can thus be significantly smaller than the macroscopic solution viscosity when a large viscogen is used. Critically, we have observed an approximate 3-fold increase in the σ_{meas} value for a protein conformational transition when rates were measured in a solution of the viscogen sucrose, relative to glycerol, and where the macroscopic viscosities were used for η_{meas} in eq 9 (unpublished data).

The influence of probe size-dependent viscosity on σ_{meas} values obtained from viscosity-dependent kinetics studies is illustrated using experiments recorded on the four-helix bundle FF domain that interconverts between the native state (N)[28] and an on-pathway intermediate (I)[29] (Figure 1).[15]

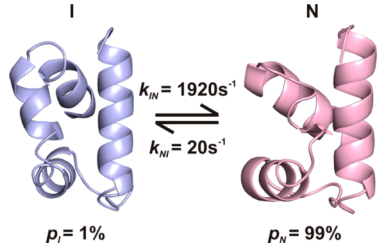

Figure 1. Cartoon showing the interconversion between an on-folding pathway intermediate state and the native state of the FF domain from HYPA/FBP11, as studied by relaxation dispersion NMR spectroscopy. Shown are the structures of the native (N, PDB ID: 1uzc[28]) and intermediate states (I, PDB ID: 2kzg[29]), their relative populations and the exchange rates in buffer solution, 25 °C.

An EHR for the process of less than 4 Å has been obtained previously. Kinetic measurements were made using either a small viscogen, glycerol, or a large protein viscogen, BSA. In the case where glycerol is used as a viscogen and the translational diffusion rates of either the FF domain (hydrodynamic radius of 13 Å) or acetate (2.24 Å)[30] are used to obtain the viscosity in eq 9, very similar σ_{meas} values are obtained (Figure 2A,B).

This is to be expected because both probes measure the macroscopic solution viscosity that is also relevant for the $I-N$ interconversion, since its EHR is on the order of the hydrodynamic radius of glycerol (2.6 Å).[31] In contrast, the situation is very different when BSA is used as a viscogen. For the case where viscosity is measured by the diffusion of the FF domain, $f_r > f_R$ and σ is overestimated by close to a factor of 10 (Figure 2C). However, when acetate diffusion is used to estimate viscosity the correct value of σ is obtained (Figure 2D), since the hydrodynamic radius of acetate (2.24 Å) is comparable to the EHR of the $I-N$ interconversion, $f_r \sim f_R$. These results are in keeping with expectations (eq 11). Assuming that the viscosity measured by acetate is a good proxy for viscosity along the reaction coordinate (η_s) and that the viscosity from diffusion measurements of the FF domain, η_{FF}, is a reasonable approximation of the macroscopic solution viscosity (η) of BSA solutions, then a linear fit of the relation between $\eta_{acetate}$ and η (Figure 3) gives

$$\eta_{meas}(R) \cong \eta_{acetate} = 0.28\eta + 0.64 \quad (13)$$

Comparing eq 13 with eq 7 yields $f_R = 0.28$, $\eta_o = 0.9$, and for $f_r \sim 1$ $(\eta_{FF} \sim \eta)$, $\sigma = 0.2$ cP, a value of 3.0 cP is calculated for σ_{meas} (eq 11) that is very similar to the experimentally observed value of 3.4 cP (Figure 2C). Note that very different conclusions are drawn from σ_{meas} values of 3.4 cP and 0.3 cP. In the former case it would be concluded that internal protein interactions contribute very significantly to the overall friction along the reaction coordinate, while a value of $\sigma_{meas} = 0.3$ cP points to solvent friction as the driving force for the $I-N$

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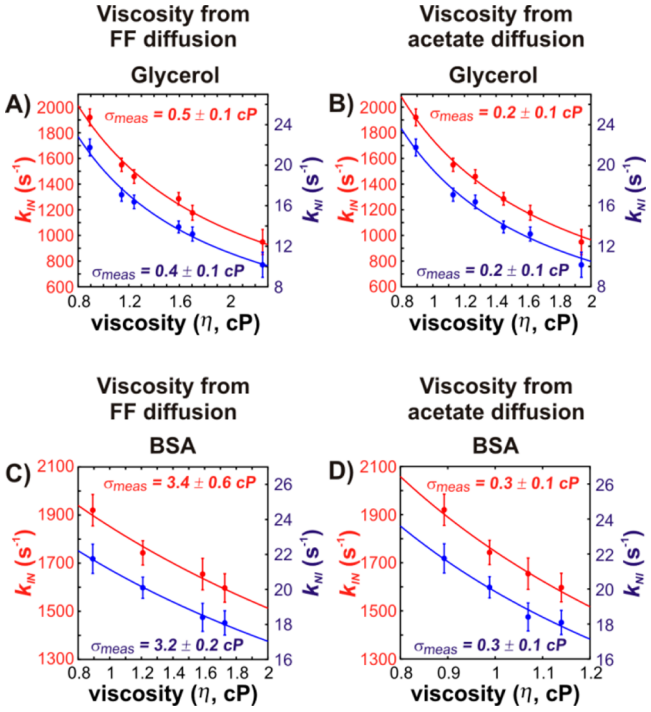

Figure 2. Rates of the FF domain I–N interconversion (25 °C) as a function of viscosity using the viscogens glycerol (A, B) or BSA (C, D), with viscosity measured using the translational diffusion rates of the FF domain (A, C) or acetate (B, D). Solid lines represent fits of $k_{I\to N}$ or $k_{N\to I}$ values to eq 9.

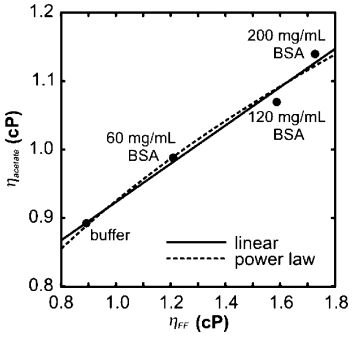

Figure 3. Plot of η_{acetate} as a function of η_{FF} measured in solutions with various concentrations of BSA, 25 °C. Values of η_{acetate} and η_{FF} were measured from the diffusion of acetate or FF domain "probes". The data can be equally well fit to a linear equation (eq 13, solid line) or a power law equation (eq 15, dashed line) because of the narrow range in viscosity over which measurements have been made, that is generally the case in most protein applications.

interconversion. Again, these ambiguous results are resolved by using a small viscogen such as glycerol where $f_r = f_R$.

Viscosity-dependent kinetics measurements in peptides and proteins have also been analyzed using a power law equation of the form[27]

$$k_{A\to B} = k^{o}_{A\to B}\left(\frac{\eta_0}{\eta_s}\right)^{\alpha} \tag{14}$$

where $k^{o}_{A\to B}$ is the rate constant in buffer of viscosity η_0. Significant deviations of the exponent α from 1 have been rationalized by invoking memory effects during barrier crossing and the breakdown of the Markovian assumption in Kramers' theory.[27]

As described above in the case where the rate–viscosity profile is given by eq 4, it is critical to use small viscogens in cases where the relative importance of solvent and internal frictional forces is to be evaluated. The microscopic viscosity measured by the diffusion of a probe of radius r_p can be related to the overall macroscopic viscosity (η) through

$$\eta_{\text{meas}}(r_p) = \eta_0\left(\frac{\eta}{\eta_0}\right)^{\beta_r} \tag{15}$$

In the case where the probe is of a similar size or larger than the viscogen, $\beta_r = 1$, and the measured viscosity is the macroscopic viscosity, η. Since the exchange reaction has an EHR value of R

$$\eta_s = p\eta_{\text{meas}}(R) = p\eta_0\left(\frac{\eta}{\eta_0}\right)^{\beta_R} \tag{16}$$

From eqs 14–16 it follows that

$$k_{A\to B} = \frac{k^{o}_{A\to B}}{p^{\alpha}}\left(\frac{\eta_0}{\eta_{\text{meas}}(r_p)}\right)^{\alpha\beta_R/\beta_r} = k'_{A\to B}\left(\frac{\eta_0}{\eta_{\text{meas}}(r_p)}\right)^{\alpha_{\text{meas}}} \tag{17}$$

Thus, if a small viscogen is used in the experiments $\beta_r = \beta_R = 1$ and the viscosity dependence of $k_{A\to B}$ gives the correct value of the exponent α. On the other hand, for a probe with $r > R$ and hence $\beta_r > \beta_R$, α_{meas} is smaller than α, with the opposite scenario for $r < R$.

Figure 4 shows rate viscosity profiles, focusing on the I–N interconversion of the FF domain as described above, with the data fit to eq 17.

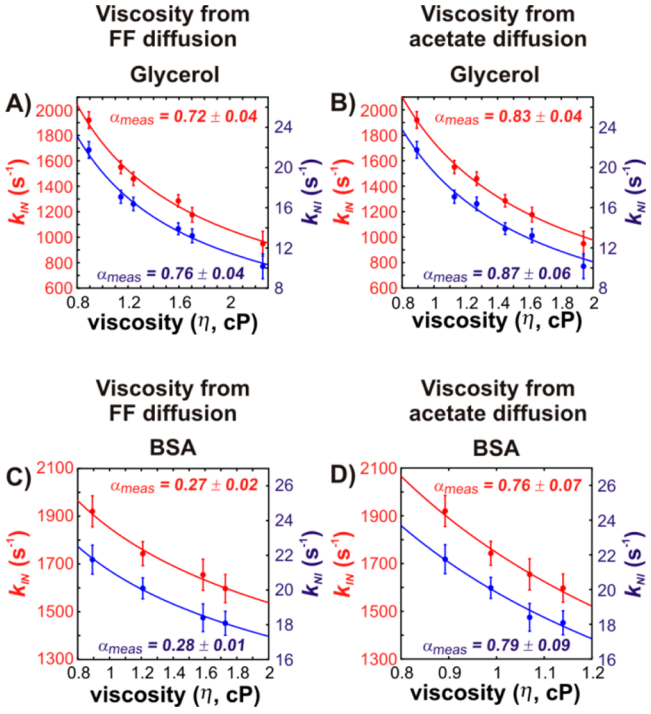

Figure 4. As Figure 2 but with fits of $k_{I\to N}$ or $k_{N\to I}$ values to a power law equation, eq 17.

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Notably, similar values of α_{meas} are obtained (0.7−0.8) using glycerol as a viscogen, irrespective of the probe size, as expected. By contrast, a value of 0.27 is measured when BSA is the viscogen, with the viscosity obtained from the diffusion of the FF domain. Assuming, as before, that $\eta_{acetate} \cong \eta_{meas}(R)$ and $\eta_{FF} \cong \eta$, the experimental viscosity data (η_{FF}, $\eta_{acetate}$) (Figure 3) can be fit to eq 15 with r_p set to R to give $\eta_0 = 0.8$ and $\beta_R = 0.35$. Since viscosity is measured from diffusion of the FF domain, $\beta_r = 1$ (since η_{FF} reports the macroscopic solution viscosity) and an α_{meas} value of $\alpha\beta_R/\beta_r = 0.30$ is predicted, essentially the same as that observed experimentally (0.27). The low value of α_{meas} does not imply a breakdown of Kramers' theory as might be naively interpreted. Rather, it can be readily explained on the basis of the EHR of the reaction studied and the choice of viscosity probes. Indeed, fractional power law fits have also been observed in translational diffusion measurements. In these cases, Zwanzig and Harrison have advanced arguments for retaining the $1/\eta$ dependence of the diffusion coefficient present in the Stokes−Einstein equation, instead interpreting the fractional power dependence as a microviscosity effect that depends on the radius of the diffusing particle.[32]

It is worth noting that the viscosity values used in the rate−viscosity profiles of Figures 2 and 4, described above, are those obtained from translational diffusion measurements. Despite the fact that protein conformational changes are essentially combinations of rotations about specific dihedral angles, it has been shown that the trajectory from one state to another can be described in terms of translational diffusion along a multidimensional conformational surface[33] and that viscosity values from translational diffusion coefficients are appropriate metrics when modeling viscosity-dependent reaction rates using Kramers' theory. For instance, the rate constants for isomerization of stilbene and 1,1′-binaphthyl in alkane solvents of varying chain lengths have been successfully modeled by Kramers' equations employing the translational diffusion of toluene[34] and naphthalene,[33] respectively, as probes of microviscosity, even though these reactions involve bond rotations in the rate-determining steps. The use of translational and rotational diffusion coefficients as measures of viscosity have been shown to be equally effective in modeling the above reaction rates,[35] since both coefficients scale in a similar fashion as a function of alkane chain length.[33,34] Assuming the rotation of stilbene as translation along a curved path, the frictional coefficients for rotation and translation have also been shown to be equivalent.[34] Here, we have employed translational diffusion coefficients because they can be measured accurately with PFG NMR methodology.

CONCLUSION

In summary, we have shown that the effects of microviscosity can significantly influence extracted values of σ or α obtained from an analysis of viscosity-dependent kinetics measurements. As described above, estimated parameter values depend on the relative sizes of the viscogen, the EHR of the conformational exchange reaction, and the probe used to measure the solution viscosity. Care must be taken even in studies involving "relatively small" viscogens in cases where the EHR of the reaction is not known. Errors in measurements can be minimized by using as small a viscogen as possible, for which the microscopic viscosity measured using the translational diffusion of all molecules is the same.

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Notes

The authors declare no competing financial interest.

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