Viscosity Dependence of O_2 Escape from Respiratory Proteins as a Function of Cosolvent Molecular Weight

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ABSTRACT Laser photodissociation of respiratory proteins is followed by fast geminate recombination competing with escape of the oxygen molecule into the solvent. The escape rate from myoglobin or hemerythrin has been shown previously to exhibit a reciprocal power-law dependence on viscosity. We have reinvestigated oxygen escape from hemerythrin using a number of viscous cosolvents of varying molecular weight, from glycerol to dextrans up to 500 kDa. In isoviscous solutions, the strong viscosity dependence observed with small cosolvents is progressively reduced upon increasing the cosolvent's molecular weight and disappears at molecular weights greater than about 100 kDa. Thus, viscosity is not a suitable independent parameter to describe the data. The power of the viscosity dependence of the rate coefficient is shown here to be a function of the cosolvent's molecular weight, suggesting that local protein-solvent interactions rather than bulk viscosity are affecting protein dynamics.

INTRODUCTION

In the early 1980s, it was found that particular protein reactions that take place within a protein matrix happen to be sensitive to the viscosity of the solvent. The actual rate coefficient has been found to be adequately described by

$$k = \mathcal{A}(\eta/\eta_0)^{-p}\exp(-H/RT) \quad (1)$$

in which $\mathcal{A}$ is a frequency (or preexponential) factor, H is the activation enthalpy, η is the relative viscosity with respect to the solvent at $\eta_0 = 1$ cP, and p is an empirical exponent smaller than unity.

In the transition state theory of reaction rates, it is assumed that friction forces have no time to develop during barrier crossing; therefore, viscosity does not appear in the formula describing a first-order rate coefficient ($p = 0$ in Eq. 1). Kramers considered the escape of a Brownian "particle" over an energy barrier (the "reaction barrier" H) when the solvent collision rate is faster than barrier crossing (Kramers, 1940). In the high friction limit, which applies to all situations of practical interest (Frauenfelder and Wolynes, 1985), the rate parameter was found to scale linearly with the reciprocal friction coefficient. Under the usual assumption that friction is proportional to viscosity in liquids, Kramers result finally leads to a reciprocal viscosity dependence of the reaction rate (i.e., $p = 1$ in Eq. 1).

The application of Kramers theory to biochemical reactions as a probe of the reaction energetics (Gavish, 1978) was tested by measuring the rate of hydrolysis by carboxypeptidase (Gavish and Werber, 1979). This was followed by the observation that the rate of oxygen escape from myoglobin (Beece et al., 1980) and from hemerythrin (Lavalette and Tetreau, 1988) followed the fractional power dependence on viscosity shown in Eq. 1. Other cases of viscosity dependence were reported by Rosenberg and co-workers for ester hydrolysis by Subtilisin (Ng and Rosenberg, 1991) as well as for hydrogen isotope exchange in lysozyme and in other proteins (Rosenberg et al., 1989; Somogyi et al., 1988). It was found experimentally that p is generally of the order of 0.4–0.6.

The first experimental report of a deviation of p from unity (Beece et al., 1980) triggered a number of studies to understand the origin of the fractional power dependence in Eq. 1. It was suggested that p describes the way in which solvent viscosity is coupled with (Doster, 1983) or penetrates (Gavish, 1980) into the protein interior. However, the fact that Eq. 1 was found to hold in simple organic reactions (Flom et al., 1985; Rosenthal et al., 1991) suggests that the effect is rooted in a more fundamental phenomenon. Grote and Hynes (1980) showed that the rate dependence on solvent friction should be weaker than that predicted by Kramers. Zwanzig obtained a $\eta^{-1/2}$ dependence for a rate process controlled by passage through a bottleneck fluctuating in time according to a Langevin equation (Zwanzig, 1992). Recently, Ansari et al. proposed to modify Kramer's equation to include protein as well as solvent friction (Ansari et al., 1992). Despite all these efforts, there seems to be no general agreement yet about the origin of the fractional p value in Eq. 1.

Investigating the viscosity dependence of protein reactions requires the addition of viscous cosolvents to a buffered aqueous solution of the protein. Therefore, one is actually dealing with a mixed solvent. Viscosity is a function of the cosolvent's weight fraction w, and whereas w varies between 0 and 1, the viscosity increases by several orders of magnitude. In practice small molecules such as glycerol and sucrose were used almost exclusively as viscous cosolvents, a fact that, because of unavoidable experimental inaccuracies, is insufficient to warrant that "p" is unique for a given reaction. Because many other physical parameters of mixed

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solvents are also related to w, the precise knowledge of the physically relevant and independent variables is needed to guide the interpretation of these experiments.

A useful elementary process to address these issues is the escape of O_2 (and eventually of other ligands) after photodissociation of an oxygen carrier protein using laser photolysis. There is a general agreement that friction with the solvent governs the frequency at which local fluctuations of the protein matrix open transient gates permitting O_2 to leave the protein. This explains why the escape rate, although being kinetically an internal and first order rate, exhibits a marked viscosity dependence. In a given solvent of constant composition, this rate is strongly temperature-dependent, but it has been established that the apparent activation energy mainly reflects the temperature dependence of the solvent's viscosity in addition to the (generally much smaller) intrinsic reaction activation enthalpy H. At constant temperature and using glycerol as a cosolvent, the escape rate was found to depend on the mixture's viscosity according to Eq. 1 with $p \approx 0.5$–0.6 (Beece et al., 1980; Lavalette and Tetreau, 1988). This relation was established up to about 100 cP. Above this value, the yield of oxygen escape becomes too small for the reaction to be observed.

In the present study, we used the rate of oxygen escape from photodissociated oxyhemerythrin (HrO_2) as an internal, viscosity-dependent protein reaction to investigate a number of cosolvents of varying molecular weight and size from glycerol to Dextrans, keeping the macroscopic viscosity constant while changing the molecular weight (and hence w) by several orders of magnitude.

MATERIALS AND METHODS

Protein preparation

Hemerythrin is a non-heme protein that carries and stores oxygen in certain species of marine invertebrates. Its binding site consists of a binuclear Fe(II) center. The protein is octameric with a molecular weight M_r = 108,000, but oxygen binding does not exhibit cooperativity (Klotz and Kurtz, 1984). Marine worms *Sipunculus nudus* were obtained from the Centre National de la Recherche Scientifique Marine Biological Station at Roscoff (France). Oxyhemerythrin was extracted and purified as previously described in detail (Lavalette and Tetreau, 1988) and stored in liquid nitrogen until use.

Cosolvents and viscosity measurements

Glycerol (anhydrous) and polymeric dextrans were from Fluka BioChemika (Buchs, Switzerland), and glucose and sucrose were from Prolabo. Cosolvents were used as received. Because of the limited solubility of high molecular weight dextrans, we explored the viscosity range between 15 and 55 cP. Concentrated stock solutions were diluted until these nominal values were attained to within ±20%. The cosolvent weight fractions w required to reach 15 and 56 cP ranged from 0.545 (glycerol) to 0.075 (Dextran 500) and from 0.71 (glycerol) to 0.15 (Dextran 500), respectively. The viscosity was measured using a calibrated capillary viscosimeter immersed in a thermostated water bath (5°C).

Samples preparation

A few milliliters of a 0.5 mM hemerythrin stock solution were first dialyzed for 4 h against 1 l of deaerated 200 mM phosphate buffer containing 10 mM dithionite to eliminate traces of methemerythrin and to regenerate the iron-(II)-deoxy protein. Excess dithionite was then removed by dialysis against pure buffer. All solvents were deaerated, and the whole preparation was performed at 5°C under an argon atmosphere.

The required amount of protein was then diluted with the aerated cosolvent solution and water to reach a final protein concentration of 0.125 mM in 50 mM phosphate at a viscosity of about 55 or 15 cP. The protein was spontaneously converted to oxyhemerythrin during mixing.

Laser photolysis

The experimental setup was as previously described (Lavalette et al., 1979; Lavalette and Tetreau, 1988), except for some modifications performed to improve the accuracy. Photodissociation was performed using a Q-switched Nd/Yag laser ("Quantel") with a pulse width of 10 ns. The beam energy could be varied between 1 and 450 mJ at 532 nm. The detection of the transmittance changes was as before except for digital recording using a Lecroy 9450 digital oscilloscope. The data were transferred to an Apple MacIntosh II-CI computer via an IEEE-488 interface for conversion of the recorded signals into transient absorbance changes and further data processing.

Principles of measurement

A laser pulse dissociates the oxyprotein (see Fig. 1). Immediately after the laser pulse, O_2 may either internally recombine with rate k_{int} or escape into the solvent with rate k_{esc} (Fig. 1 *A*).

The time course (Fig. 1 *B*) of this geminate rebinding phase is exponential and independent on the concentration of oxygen. Its rate k_{gem}

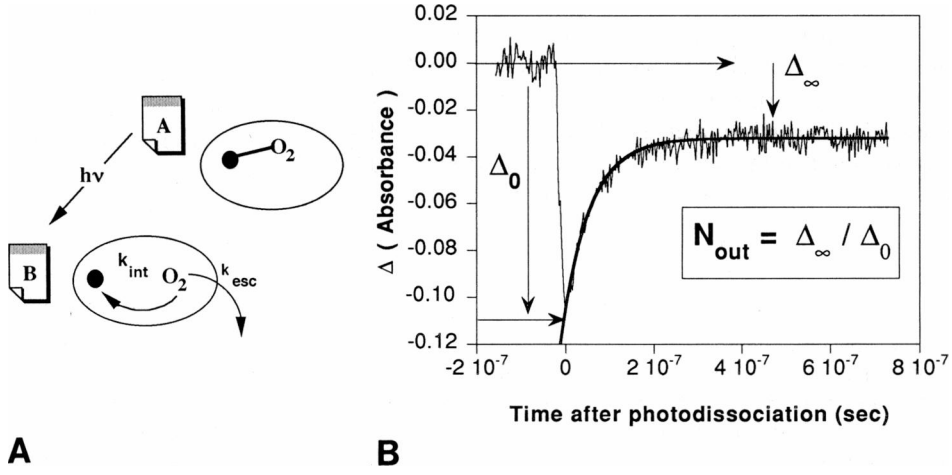

FIGURE 1 (*A*) Elementary processes following photodissociation of HrO_2. (*B*) Digital recording of the absorbance change during the geminate rebinding phase in sucrose (η = 18 cP, T = 278 K). Oxygen rebinding was monitored at 500 nm in the broad charge transfer band of HrO_2 (*pink*), which is absent in Hr (*colorless*). The time course of the absorbance change was fitted with an exponential function to obtain k_{gem} and N_{out}. The individual rates were then readily calculated using Eqs. 2–3.

is given by

$$k_{gem} = k_{int} + k_{esc}. \quad (2)$$

The ratio of the initial to final absorbance gives the fraction N_{out} of oxygen molecules that have left the protein:

$$N_{out} = (k_{esc}/k_{gem}). \quad (3)$$

These two relations are sufficient to extract the escape rate k_{esc} (Fig. 1 *B*).

RESULTS

Measurements in glycerol

Glycerol is the only cosolvent for which extensive data are available. To facilitate the understanding of the next section, we first summarize the temperature and viscosity dependence of k_{int} and k_{esc} of HrO_2 in glycerol/water as previously determined (Lavalette and Tetreau, 1988). Fig. 2 shows the viscosity dependence of both rates at the constant temperature of 278 K.

The internal rebinding rate follows a Transition State model:

$$k_{int}(s^{-1}) = 10^8 \exp(-H_{int}/RT) \quad (4)$$

with $H_{int} = 4$ kJ · mol^{-1}

The escape rate exhibits the behavior described by Eq. 1:

$$k_{esc}(s^{-1}) = 4 \times 10^9(\eta/\eta_o)^{-p} \exp(-H_{esc}/RT) \quad (5)$$

with $H_{esc} = 13$ kJ · mol^{-1} and $p = 0.54$.

Isoviscous solutions using various cosolvents

The escape rate from HrO_2 at 278 K, k_{esc}, is displayed in Fig. 3 at viscosities of about 15 and 56 cP in a series of isoviscous solutions containing different cosolvents. Because of the difficulty of precisely adjusting the solutions at a predetermined viscosity, some samples showed deviations as large as ± 20% from the nominal η value. This may account for some irregularities in Fig. 3, but the adequate correction was applied in the numerical data evaluation.

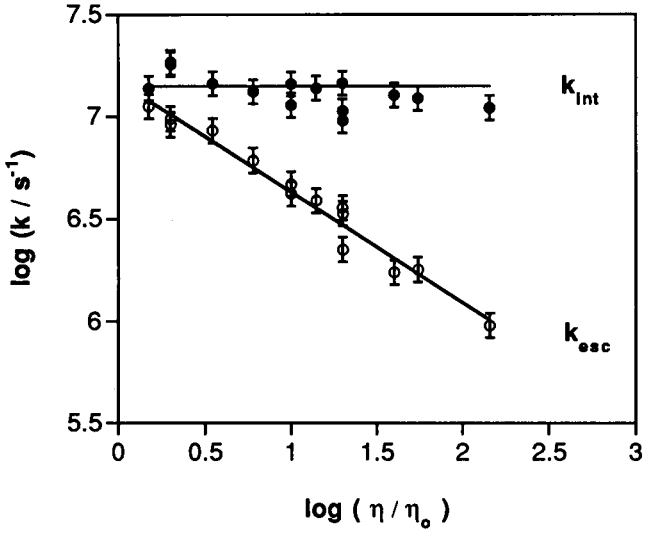

FIGURE 2 Viscosity dependence of k_{int} (●) and k_{esc} (○) for HrO_2 in glycerol-water mixtures at 278 K.

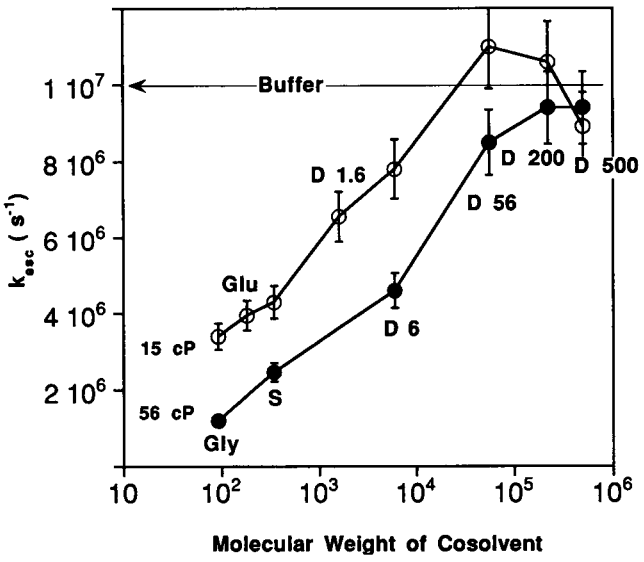

FIGURE 3 Escape rate of oxygen in two sets of nearly isoviscous solutions at 278 K as a function of the molecular weight of the cosolvent. (○) Measurements at 15 cP. (●) Measurements at 56 cP. Gly, glycerol; S, sucrose; Glu, glucose; D, dextrans. The numbers indicate the polymer M_r in kDa.

If viscosity were the independent variable, one would expect to observe only two distinct values of k_{esc}, one for $\eta = 15$ cP and another one for $\eta = 56$ cP, irrespective of the cosolvent used. The data show that this is clearly not the case. Taking the pure buffer as a reference, the drop of the escape rate observed with low molecular weight cosolvents such as glycerol gradually decreases as the cosolvent M_r increases and finally vanishes despite that the bulk viscosity is kept constant. This proves that the viscosity exponent "*p*" must be a function of the cosolvent M_r.

Dependence of "*p*" on cosolvent's molecular weight

The functional dependence of "*p*" on the M_r was obtained by calculating the exponent in each cosolvent (for which measurements could be performed at both viscosities). A rearrangement of Eq. 1 yields

$$p(M) = (\log k_o - \log k_{esc})/\log(\eta/\eta_o), \quad (6)$$

in which k_0 is the rate measured at 1 cP.

The wide range of molecular weight covered in the experiment (Fig. 4) suggests that the data can be fitted with a power law. A least-square fit gives

$$p(M) = 1.52\, M^{-0.23}, \quad (7)$$

where *M* is the molecular weight of cosolvent. The best estimate of "*p*" is given in Table 1 for commonly used cosolvents.

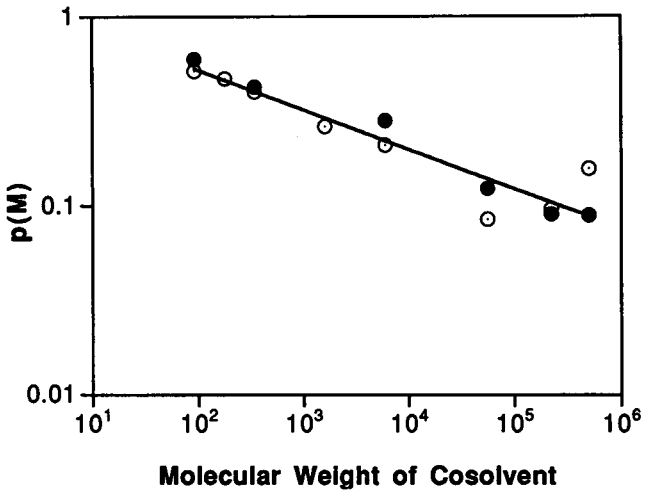

FIGURE 4 Variation of the viscosity exponent p, calculated according to Eq. 6, as a function of the cosolvent M_r. The line is a power-law fit to the data. (○) Measurements at 15 cP. (●) Measurements at 56 cP.

TABLE 1 Viscosity exponent p calculated according to Eq. 7 for various cosolvents

Cosolvent	Molecular weight	p (calculated)
Water	18	0.79
Ethyleneglycol	62	0.59
Glycerol	92	0.54
Glucose	180	0.46
Sucrose	342	0.40
Dextran 1.6	1,600	0.28
Dextran 6	6,000	0.20
Dextran 56	56,000	0.12
Dextran 200	200,000	0.09
Dextran 500	500,000	0.07

DISCUSSION

The exponent p is generally considered to represent the damping or "coupling" of frictional collisions by the protein matrix, leading to an "effective viscosity" experienced by the moving protein domains implied in the reaction. If this is so, p might be protein-dependent. But, surprisingly, the value $p \approx 0.5$–0.6 has been reported for different protein reactions investigated in glycerol (Beece et al., 1980; Lavalette and Tetreau, 1988; Ng and Rosenberg, 1991; Rosenberg et al., 1989). Thus, the protein (and reaction-) dependence of p, if any, does not seem to be appreciable. Further experiments are required to address this issue. Here, however, we show that the size of the solvent is an important factor.

The exponent p decreases by one order of magnitude from the pure solvent to the Dextran 500 (Table 1). The value for water ($p \approx 0.8$) is extrapolated. At this stage, we do not wish to introduce extra assumptions, and we do not find any compelling reason to assume that p should be unity in plain water. It is doubtful that the difference would be easily detected in an actual measurement anyway, because there is no direct means to estimate p without introducing an extra cosolvent! Note that the empirical relation Eq. 7 yields $P = 0.54$ for glycerol, which is precisely the value that was determined directly in this solvent (Fig. 2).

Equation 1 describes a variety of reactions quite well. In all previous investigations where a protein reaction was found to exhibit a viscosity dependence, it was tacitly assumed that the solvent viscosity was an independent parameter. Beece et al. showed that the escape of carbon monoxide from protoheme was a unique function of temperature and viscosity for a number of cosolvents (Beece et al., 1980). This is certainly true for isolated protoheme because the escape rate is purely diffusive (their Fig. 8) as subsequently confirmed (Marden et al., 1986). However, the argument may not be generally valid because photoejection from protoheme does not imply small scale restricted motions as in a protein. The effect of glycerol and ethylene glycol on hydrogen exchange has been compared (Rosenberg et al., 1989; Somogyi et al., 1988). It was concluded that the observed viscosity effect was independent on the solvent. A glance at Table 1, however, shows that the p values in these solvents are close to each other and that discrimination would have been certainly beyond the experimental accuracy.

Our results (Fig. 3) clearly indicate that the viscosity is not an independent parameter. In Fig. 5 we have replotted the escape rate as a function of $(\eta/\eta_0)^{-p}$. In contrast to Fig. 4, the data at 15 and 56 cP in all cosolvents now collapse into one unique linear correlation. This shows that the relative viscosity raised to the power p is an independent parameter, p being itself cosolvent-dependent according to Eq. 7.

Alternatively, as previously suggested (Gavish, 1980) one may consider that Kramers equation (with $P = 1$) is valid, provided that the bulk viscosity is replaced by an "effective" or "microscopic" viscosity:

$$(\eta/\eta_o)_{eff} = (\eta/\eta_o)^{p(\text{Mcosolv})}$$

Although the meaning of this relation is not clearly understood presently, it is supported by the present data. In the state of present knowledge, we may only speculate about the origin of the fractional power law and its solvent dependence.

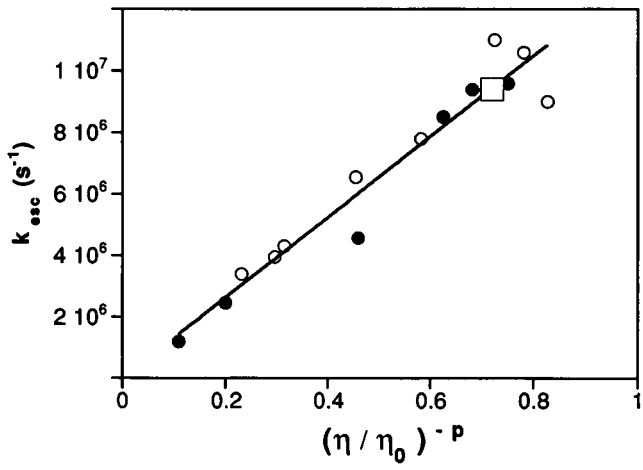

FIGURE 5 The original data of Fig. 3 follow a linear correlation when replotted against $(\eta/\eta_o)^{-p(\text{Mcosolv})}$. The regression line yields k_{esc} (s^{-1}) = 1.39 × $(\eta/\eta_o)^{-p}$. (○) Measurements at 15 cP. (●) Measurements at 56 cP. The open square corresponds to pure buffer.

Classical viscosity is defined under the assumptions that the solvent is homogeneous and isotropic. According to the Brownian theory, the collision frequency remains fast compared with the motion of a large particle. These conditions are fulfilled in ordinary rotational and translational Brownian motions of macroscopic particles. Because of the large size of the "particle," the solvent composition in the vicinity of its surface does not significantly differ from that of the bulk; also, the motion is slow compared with the collision frequency. Solvent-particle interactions are averaged out, and the medium appears as homogeneous both in space and time. Therefore, the classical friction laws are expected to be valid.

Consider now segmental fluctuations of a protein chain on a microscopic scale. Most of the time only a small number of cosolvent molecules are present in the immediate vicinity of the fluctuating segment. For a given viscosity, the higher the molecular weight of the cosolvent, the smaller the number of cosolvent molecules will be. This can be visualized by calculating the true average distance $\langle d \rangle$ (Chandrasekhar, 1943) between the cosolvent's molecules. Chandrasekhar's formula expressed in practical units gives

$$\langle d \rangle = 6.5 \times [C]^{-1/3},$$

in which $\langle d \rangle$ is in Å and the concentration [C] is in mol · dm^{-3}. Fig. 6 shows that k_{esc} reaches practically its value in the pure buffer when the cosolvent intermolecular distance exceeds 40 Å.

The dimensions of the octameric hemerythrin molecule are 75 × 75 × 50 Å (Ward et al., 1975). Its equivalent average radius is ≈40 Å. Fig. 7 suggests that the protein does not sense the presence of the cosolvent when the intermolecular distance exceeds the protein dimensions because the number of cosolvents molecules becomes too small in its immediate surrounding. Moreover, this number is expected to fluctuate in time. In addition, a large (polymeric) cosolvent may interact only locally with the protein. The collision frequency of the cosolvent may eventually become even smaller than the frequency of the protein's fluctuations. Collisions are therefore "heterogeneous" both in space and in time. In accord with this, Barshtein et al. have found evidence that viscous mixed solvents are inhomogeneous on a microscopic scale from measurements of water tracers in the presence of viscous cosolvents of different size (G. Barshtein, A. Almagor, B. Gavish, and S. Yedgar, unpublished data).

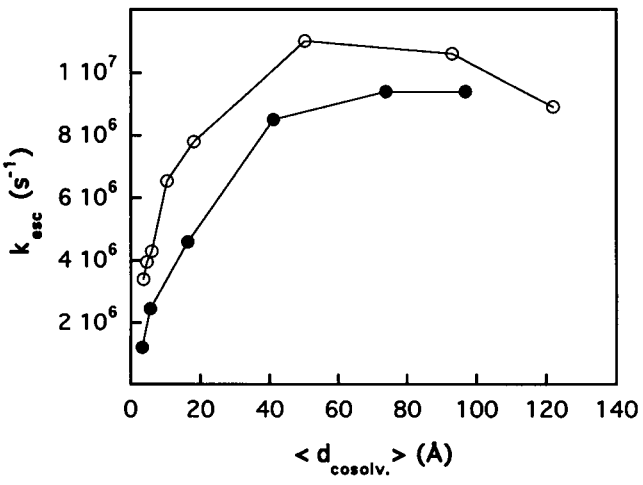

FIGURE 6 Plot of k_{esc} as a function of the average distance between cosolvent molecules. (○) Measurements at 15 cP. (●) Measurements at 56 cP. The rate reaches that measured in pure buffer (1×10^7 s^{-1}) at an intermolecular distance of about 40 Å, i.e., at a distance comparable to that of the protein size.

A determination of the rotational diffusion coefficient would provide an alternative way for probing the microviscosity of mixed solvents. An attempt to measure the rotational correlation time of hemerythrin using its absorption anisotropy decay was unsuccessful because of the low extinction coefficient and small intrinsic anisotropy of the characteristic charge transfer band of HrO_2. However, it should be possible to investigate proteins of various size, such as hemoproteins, that have been shown previously to give rise to a measurable anisotropy decay (Gros et al., 1984; Ansari et al., 1992).

The question whether viscosity induced by macromolecules affects protein reactions in general is of physiological relevance. The viscosity of body fluids is determined by macromolecules such as proteins, glycoconjugates, and lipoproteins, and is altered in pathological states. Previous studies have demonstrated that extracellular fluid microviscosity is a regulator of cellular functions and of membrane enzyme activity (Yedgar et al., 1987, 1982). In search for the mechanism of this phenomenon, the effect of solvent viscosity, modified by small (glycerol) and macromolecular (dextrans) cosolvents, on albumin internal friction has been studied by ultrasonic absorption spectroscopy. It has been found that the protein internal friction is increased as a function of the solvent viscosity when small cosolvents are used (Almagor et al., 1992). However, in accord with the present study, the cosolvent viscosity effect was diminished as the molecular weight of the cosolvent increased (Almagor et al., 1990).

In conclusion, understanding the fractional viscosity exponent and its cosolvent dependence probably requires that spatial dimensions and frequency of motion should be taken into account to determine the time interval and the volume over which protein-solvent interactions should be averaged in a consistent theory of protein reactions.

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