Glycerol Effects on Protein Flexibility: A Tryptophan Phosphorescence Study

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ABSTRACT In exploring the dynamic properties of protein structure, numerous studies have focussed on the dependence of structural fluctuations on solvent viscosity, but the emerging picture is still not well defined. Exploiting the sensitivity of the phosphorescence lifetime of tryptophan to the viscosity of its environment we have used the delayed emission as an intrinsic probe of protein flexibility and investigated the effects of glycerol as a viscogenic cosolvent. The phosphorescence lifetime of alcohol dehydrogenase, alkaline phosphatase, apoazurin and RNase T_1, as a function of glycerol concentration was studied at various temperatures. Flexibility data, which refer to rather rigid sites of the globular structures, point out that, for some concentration ranges glycerol, effects on the rate of structural fluctuations of alcohol dehydrogenase and RNase T_1 do not obey Kramers' a power law on solvent viscosity and emphasize that cosolvent-induced structural changes can be important, even for inner cores of the macromolecule. When the data is analyzed in terms of Kramers' model, for the temperature range 0–30°C one derives frictional coefficients that are relatively large (0.6–0.7) for RNase T_1, where the probe is in a flexible region near the surface of the macromolecule and much smaller, less than 0.2, for the rigid sites of the other proteins. For the latter sites the frictional coefficient rises sharply between 40 and 60°C, and its value correlates weakly with molecular parameters such as the depth of burial or the rigidity of a particular site. For RNase T_1, coupling to solvent viscosity increases at subzero temperatures, with the coefficient becoming as large as 1 at −20°C. Temperature effects were interpreted by proposing that solvent damping of internal protein motions is particularly effective for low frequency, large amplitude, structural fluctuations yielding highly flexible conformers of the macromolecule.

INTRODUCTION

A range of experimental approaches have established the importance of conformational dynamics in proteins both as an intrinsic physical property of the macromolecule and in enzyme-catalyzed chemical reactions. A wealth of structural (1–6) and dynamical (7–12) informations have revealed a hierarchy of atomic displacement amplitudes and frequencies of structural fluctuations. The nature of these motions range from local vibrations of atoms and groups of atoms to global changes of relative positions of whole domains and their characteristic time scale stretch from fractions of a picosecond to seconds. Either type of motions depend in some way on environmental factors such as solvent composition, pH, ionic strength, and viscosity.

A popular approach for investigating the importance of protein dynamics in chemical reactions is to study the effect of the medium viscosity. Kramers' theory (13) models the kinetics of chemical reactions and relates the reaction rate to the viscosity, η, of the reactants environment as follows:

$$\text{Rate constant} = (B/\eta^k) \exp(-E^*/RT) \qquad (1)$$

where B is a constant, E^* is the height of the potential energy barrier and $k \leq 1$ is the frictional coefficient which is equal to 1 in the high viscosity limit. Useful extentions of the theory to intramolecular activated processes in proteins were given by Gavish (14, 15), Doster (16), and Schlitter (17). In treating the damping of structural fluctuations in proteins by the viscous drag of the solvent, they show that the degree of coupling of protein and solvent motions decreases the more internal and rigid is the region of the macromolecule concerned.

In recent years a number of experiments have confirmed the viscosity dependence of a variety of intramolecular processes. The validity of Kramer's law has been tested experimentally for polymer dynamics (18) and by computer simulations (19, 20). With proteins, increased solvent viscosity was shown to slow down rotational motions of intrinsic aromatic amino acid side-chains (21, 22) and attached spin-label probes (23) and to reduce kinetic coefficients of ligand binding (24–27), of hydrogen isotope exchange (28–30), and of a number of enzymatic reaction (14, 31, 32). Generally, these studies have concerned rather superficial or solvent/ligand-accessible sites of the macromolecule, which, being in more or less intimate contact with the solvent, represent rather flexible regions of the globular structure. Within certain solvent viscosity ranges the rate is often found to obey a power law with a frictional coefficient, k, less than 1; smaller values were obtained when more internal regions of the macromolecule are concerned (23). However, a notable exception to this pattern is represented by the slow exchanging protons in lysozyme for which k is actually larger than 1 (29, 30). Since slow exchange rates refer to rather tight cores of the protein structure, the large frictional coefficient found in glycerol/water solutions suggested (30) that either Gavish description of position-dependent viscosity effects is not correct or solvent viscosity is not the parameter responsible for the dramatic reduction in exchange rates. A different interpretation of this phenomenon was offered by Calhoun

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and Englander (28) who, by assuming that isotope exchange requires exposure of internal residues to the aqueous phase, infer that large amplitude fluctuations, leading to partially unfolded states of the protein, are strongly inhibited by the viscous drag of the solvent. Still a different view on the selectivity of coupling to particular motions of the polypeptide emerges from recent results on the ultrasonic absorption of bovine serum albumin (33). These data are believed to indicate that increased solvent viscosity dampens rapid chain movements which, in turn, would cause an enhancement of low frequency, large amplitude fluctuations in the polypeptide structure.

Solvent viscosity is increased with the addition of a viscogenic cosolvent, mainly polyols. The experimental difficulty of changing nothing but the viscosity is generally recognized, but there seems to be no satisfactory solution to the problem. Protein structure is sensitive not only to solvent viscosity, dielectric constant, and pH, but also to subtle, cosolvent specific thermodynamic effects (34, 35). Therefore, the usual assumption that the latter parameters are negligible should always be tested. Among viscogenic cosolvents glycerol is the most widely used. One reason is that it affords large increments in solvent viscosity with relatively small changes of pH and dielectric constant; another reason is that, being a stabilizing agent, even large concentrations do not usually alter the configuration of the native fold. However, increased protein stability by glycerol is achieved by inducing preferential hydration of the protein particularly of hydrophobic patches (34). To minimize the increased chemical potential, the protein reduces its surface area by adopting more compact conformations. As a result of this compactness or reduced free volume, the structure is expected to become also more rigid and large amplitude fluctuations in particular to be more inhibited. The objective of the present study is to determine the effects of glycerol on the flexibility of four well characterized globular proteins. Attention will be focussed mainly on the flexibility of, until now little studied, deeply buried, sites of the macromolecules both to test the hypothesis of weak coupling to solvent motions (14, 15) and to verify whether thermodynamic effects are important or not.

The intrinsic phosphorescence lifetime of tryptophan is markedly affected by the viscosity of the surrounding medium, τ, varying by more than 5 orders of magnitude on going from a glass to a fluid solution (36, 37). In the past decade a number of experiments have emphasized the exquisite sensitivity of τ to probe dynamical features of protein structure (12, 38, 39). In this work we monitor the phosphorescence lifetime of alcohol dehydrogenase, alkaline phosphatase, apoazurin, and RNase T_1, four single tryptophan-emitting proteins, as a function of glycerol content and at various temperatures. Flexibility data point out that, for some proteins and concentration ranges, glycerol effects on the rate of structural fluctuations do not follow a power law on solvent viscosity implying that glycerol-induced structural changes, even in the inner core of the globular structure, can be serious. Furthermore, where solvent kinetic effects appear to be dominating, the transmission coefficient is small at ambient temperature, rises sharply above 40°C, and since it does not simply correlate with either the depth of burial or the rigidity of the region, coupling seems to be strongly site specific.

MATERIALS AND METHODS

Liver alcohol dehydrogenase from horse (LADH) was obtained as a crystalline suspension from Boehringer (Mannheim, Germany). Electrophoretically purified alkaline phosphatase (AP) type III-R from *Escherichia coli* was obtained from Sigma Chemical Co. (St. Louis, MO). Lys^{25}-ribonuclease T_1 (RNase T_1) was purchased from Calbiochem Corp. (San Diego, CA). Apoazurin, obtained by removing copper from holoazurin (*Pseudomonas aeruginosa*) was a gift of Dr. N. Rosato, Department of Biochemistry-II, University of Rome. Spectroscopic grade glycerol from Merck (Darmstadt) was used without further purification. Water, doubly distilled over quartz, was further purified by Millipore system.

All proteins were dialyzed for at least 24 h under nitrogen against the chosen buffer (0.2 M sodium cacodylate, pH 5.5, for RNase T_1, and 0.2 M Tris-HCl, pH 8, for the other proteins). Any remaining insoluble precipitate was removed by centrifugation. Fresh preparations were made weekly, and, for the enzymes, no loss of activity was found during that time. The protein concentration was determined by absorbance measurements employing extinction coefficients at 280 nm of 3.53×10^4, 6.80×10^4, 8.4×10^3, and $1.74 \times 10^4\ M^{-1}\ cm^{-1}$ for LADH, AP, apoazurin, and RNase T_1, respectively. The glycerol content of the samples was increased up to 80% (w/w) keeping protein concentration constant and ionic strength fixed at 0.04 M. Even if glycerol-induced pH variations are believed not to be appreciable (24, 28, 40) we have chosen buffers and pH values that would further minimize their effects. For LADH, AP, and apoazurin, the phosphorescence lifetime at pH 8 is practically unaffected by one pH unit change either way. For RNase T_1 the choice of sodium cacodylate, pH 5.5, as buffer was dictated by the consideration that its pH has the smallest temperature dependence below 0°C.

All luminescence experiments were carried out at a protein concentration of about 1 mg/ml except for RNase T_1 which was 0.01 mg/ml. Prior to phosphorescence measurements oxygen was thoroughly removed from the sample by a procedure described elsewhere (38).

Routine fluorescence spectra were obtained with a Jasco model fluorometer. A conventional home-made instrument was employed for all phosphorescence intensity and spectra measurements (41). The excitation provided by a Cermax xenon lamp (LX 150 UV; ILC Technology, Sunnyvale, CA) was selected by a 250-mm grating monochromator (Jobin-Yvon, H25), and the emission was detected with an EMI 9635 QB photomultiplier. Phosphorescence decays in fluid solution were obtained following pulsed excitation by a frequency-doubled flash-pumped dye laser (UV 500 M-Candela) with a pulse duration of 1 μs and an energy per pulse typically of 1–10 mJ. The decay of tryptophan phosphorescence was monitored at 430 nm by an electronic shutter arrangement permitting the emission to be detected 1 ms after the excitation pulse. The decaying signal was digitized by an Applescope system (HR-14; RC Electronics) and then transferred to an Apple II computer for averaging. Subsequent analysis of decay curves in terms of a sum of exponential components was carried out by a nonlinear least-square fitting algorithm, implemented by the program Global Analysis (Global Unlimited, LFD University of Illinois, Urbana). All decay data reported here are averages obtained from three or more independent measurements.

The temperature of the sample, regulated by a conventional thermostat and by the flow of cold nitrogen through the sample holder, was maintained within ±0.2°C of nominal value.

All viscosities of the glycerol/buffer mixtures at the temperature of these experiments were taken from Miner and Dalton (42).

RESULTS

In this study we have measured phosphorescence lifetimes of Trp up to 60°C, a temperature well beyond 40°C, the highest

temperature ever reported in protein phosphorescence studies. Since we wish to employ τ as a monitor of the local protein viscosity, it is important to ascertain that the pronounced decrease in τ at the highest temperatures (Table 1) is not due to the onset of thermally activated, but viscosity-independent triplet relaxation pathways such as delayed fluorescence or triplet state ionization. Ideally, to test the viscosity dependence of τ one should monitor the lifetime of Trp, or model indole compounds, in media forming rigid matrices of known viscosity or in glasses in which case τ would be expected to approach 5–6 s. Two matrices forming glasses at room temperature, polymerized methylmethacrylate and 1,5-decandiol, were both found, despite rigorous purification, to be strongly phosphorescent (upon excitation at 300 nm) with long-lived emission (up to 1 s) overlapping the entire phosphorescence spectrum of the indole. As an alternative to glasses, then, we have chosen dry protein powders, a state of the protein which, according to several biophysical studies (43, 44), gives rather rigid if heterogenous molecular aggregates.

Trp phosphorescence from thoroughly dehydrated and deoxygenated samples of alkaline phosphatase, alcohol dehydrogenase, and subtilisin Carlsberg confirms that, relative to samples in aqueous solutions, the decay in dry samples is markedly slower and always nonexponential. Decays obtained between 40 and 70°C were fitted to a biexponential law and the average lifetime, $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$, is reported in Table 1. At the highest temperatures for which τ in solution was obtained, 60°C, (Table 2), τ_{av} of dry samples is twofold and 16-fold larger for AP and LADH, respectively. Subtilisin Carlsberg at 70°C has a lifetime of 0.71 s, which is four times greater than the longest phosphorescence lifetime measured in this study for a protein in solution at 60°C. From these data it is evident that autoquenching of the triplet state by thermally activated processes is negligible. Thus, even in the upper range of the temperature excursion in solution the small values of the phosphorescence lifetimes will reflect predominantly if not exclusively the increased flexibility of the protein structure embedding the aromatic side-chain.

RNase T_1 and azurin are monomeric proteins with a single Trp residue while, alcohol dehydrogenase, and alkaline phosphatase, are dimeric and possess two and three Trp residues per subunit, respectively. In fluid solutions, however, only internal Trp^{314} of LADH (45) and Trp^{109} of AP (46) are detectably phosphorescent, and thus all four proteins represents single Trp emission. Except for RNase T_1, the phosphorescence decay was found to be well approximated to an exponential law at all temperature and solvent compositions examined. Below 10°C or at high glycerol content fitting the decay of RNase T_1 requires two components, the short-lived one having an amplitude between 5 and 30% of the total intensity. The lifetime we report for this protein is the average value, $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$ where the α_s are the amplitudes of the preexponential terms.

The phosphorescence decay of each protein was measured at various temperatures below their thermal unfolding and at different weight percentage of glycerol as cosolvent. In Table 2 we report the phosphorescence lifetimes in buffer (τ_0) as a function of temperature while in Fig. 1 *a–d*, we plot the change in τ, brought about by the addition of glycerol as a function solvent viscosity. For each isotherm the values of τ/τ_0 refer to glycerol contents (w/w) that vary between 0 and 80%, and for which the solvent viscosity is known.

TABLE 1 Phosphorescence lifetimes* of dry protein powders as a function of temperature

Sample	40°C	50°C	60°C	70°C
		s		
Alcohol dehydrogenase	0.76	0.56	0.39	0.23
Alkaline phosphatase	0.66	0.55	0.47	0.30
Subtilisin Carlsberg	1.6	1.34	1.07	0.71

* These are average values, $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$, obtained from a biexponential fitting of the decay. The standard deviation of τ_{av} is typically around 5%.

TABLE 2 Trp phosphorescence lifetimes*, τ_0, of proteins in buffer as a function of temperature

Temperature	RNase T_1	Apoazurin	LADH	AP
°C		s		
−20	0.116§¶			
−10	0.051§¶			
1	0.093¶	1.65	1.40	3.30
10	0.05¶	0.99	0.97	2.61
20	0.031	0.54	0.59	1.76
30	0.025	0.267	0.29	1.22
40		0.129	0.15	0.75
50		0.053	0.060	0.38
60		0.025	0.025	0.16
70		0.017		0.10

* The error in the mean lifetime is typically around 5%.

§ At subzero temperatures τ_0 refers to 10 and 50% glycerol content for −10 and −20°C, respectively.

¶ This lifetime is the average value, $\alpha_1\tau_1 + \alpha_2\tau_2$, obtained from a biexponential fitting of the decay.

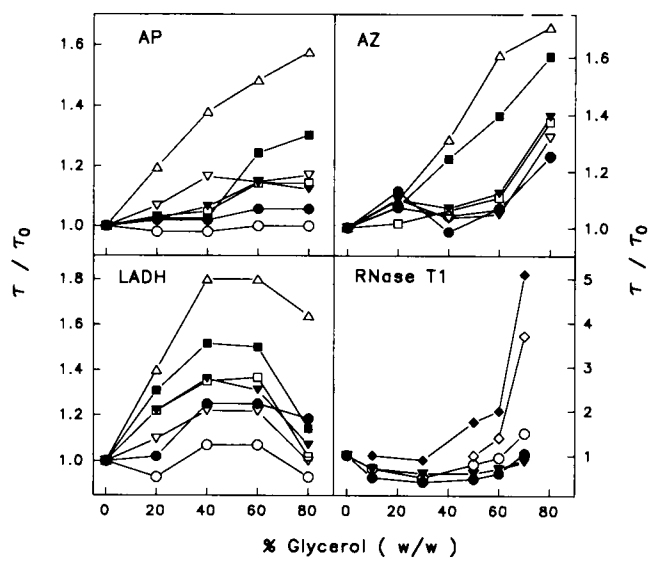

FIGURE 1 Relative Trp phosphorescence lifetimes of four proteins as a function of glycerol concentration. The isotherms refer to temperatures of −20 (◇), −10 (◆), 0 (○), 10 (●), 20 (▽), 30 (▼), 40 (□), 50 (■), and 60°C (△), respectively. Lifetime values are averages of three independent determinations; the error in the mean is typically 5%.

Inspection of Fig. 1 shows that solvent composition/viscosity has invariably a considerable influence on the phosphorescence lifetime of each protein and an increase in glycerol content generally leads to larger values of τ. One may discern some patterns on the behavior of τ/τ_0 but these are not common to all four proteins. For example, the increase in τ/τ_0 with solvent viscosity is markedly temperature-dependent. For proteins like AP, LADH and apoazurin τ is little affected below 30°C the largest increments in τ/τ_0 obtained at 50–60°C.

RNase T_1 is a case apart. First, we note that in the temperature range 0–30°C τ initially decreases with the addition of cosolvent reaching minimum values at intermediate, 30–50%, glycerol contents. Second, lifetimes above 30°C are not available, because the emission decays in a time comparable or shorter than the resolution of the apparatus (~1 ms). For this reason the temperature range was expanded by including subzero temperatures, namely −10 and −20°C (τ_0 for these temperatures is the lifetime in 10 and 50% glycerol/buffer, respectively, the minimum glycerol content to avoid freezing). At these temperatures we observe a dramatic increase in τ with solvent viscosity, a behavior that contrasts with the almost constant lifetime observed with the other three proteins (data not shown).

There are other peculiarities worth noting in Fig. 1. The increase in τ with solvent viscosity is not always monotonic. Particularly striking is the general decrease in τ with LADH for solvent mixtures with more than 50% glycerol. A possible explanation for this behavior might be found in a concomitant loss of stability of the LADH dimer, which by either increasing the rate of subunit dissociation or loosening the contact of the subunit interface would partially expose Trp^{314} to the mobile aqueous phase.

DISCUSSION

Increasing solvent viscosity has led in general to longer Trp phosphorescence lifetimes. Because long-lived emissions are susceptible to η-dependent, dynamic quenching by impurities it is important to emphasize that such reactions are not important in determining the observed change in τ. Among possible quenchers, O_2 with quenching rate constant, k_q, ranging between 10^6 and 10^9 M^{-1} s^{-1}, is the most effective (46, 47). Fortunately its presence can be revealed by photobleaching experiments (41). Other quenching impurities could be associated with buffering salts or with cosolvent. The former can be tested for by varying the buffer concentration. With glycerol possible quenchers are to be sought among aromatic or polyunsaturated impurities. Since for these molecules $k_q \leq 10^5$ (in buffer, 20°C), relatively large concentrations, $>10^{-4}$ M, are needed in order to influence a lifetime of 100 ms. In pure glycerol their concentration will be higher and hardly escape detection by absorption and chromatographic controls.

Furthermore, at low viscosity, i.e., within 0–40% glycerol content, quenching falls in the rapid diffusion limit regime (47, 48) where the rate is proportional to the quencher concentration but is independent of solvent viscosity. Within this range, increasing glycerol content should cause a reduction in τ lifetime data show that for three of the four proteins τ increases sharply in this range of solvent composition. If quenching is not important at low (0–40%) glycerol content, it is even less so at higher contents/viscosities when the reaction will eventually become diffusion controlled and k_q become proportional to $1/\eta$. On these grounds we excluded dynamic quenching of protein phosphorescence and emphasize that the lifetime reported is intrinsic.

The phosphorescence lifetime of the four proteins investigated shows a sometimes pronounced influence of glycerol on the flexibility of their structure. In the following discussion we attempt to analyze protein flexibility data in terms of Kramer's theory to establish whether or not the influence of the viscogenic cosolvent on structural fluctuations can be adequately described in terms of kinetic (solvent viscosity effects), with no need for calling on concomitant thermodynamic (protein conformation) effects. In Fig. 2 we report τ-derived (36) effective protein viscosities, η_p, at the site of the triplet probe, as a function of solvent viscosity, η_s. Kramer's theory adapted to the rate of structural fluctuations in protein (14, 15) predicts that for each isotherm η_p is related to η_s by the expression

$$\eta_p \propto \eta_s^k \qquad (2)$$

where k, the transmission or frictional coefficient, ranges between 0 and 1 according to the degree of coupling between solvent and protein motions. It is apparent from Fig. 2 that the protein motions examined by the triplet probe do not obey a simple power law on solvent viscosity. Large deviations over certain ranges of solvent composition are found especially with LADH (above 50% glycerol content) and RNase

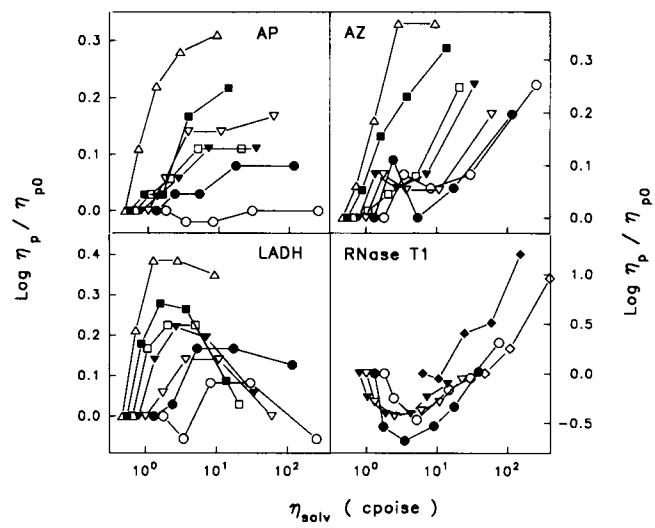

FIGURE 2 Change in lifetime-derived internal protein viscosity as a function of the viscosity of glycerol/buffer solvent mixtures of Fig. 1. η_{po} refers to the viscosity at the site of the triplet probe when the protein is in buffer, except for RNase T_1 at subzero temperatures for which it refers to 10 and 50% glycerol/buffer for −10 and −20°C, respectively. The isotherms have the same symbols as in Fig. 1.

T_1 (below 30% glycerol content) for which, k is actually negative. Clearly within these glycerol concentration ranges, changes in protein conformation, that is thermodynamic effects of cosolvent, must override any frictional damping of structural fluctuations. Although we presently have no idea on the nature of the conformational changes in question it is tempting to advance some speculative suggestions. With LADH, even if glycerol induced preferential hydration should in general stabilize the dimeric state of a protein, specific interactions with charged groups at the subunit interface might favor subunit dissociation; a process that by exposing Trp^{314} to the solvent would result in an artificial and dramatic shortening of τ. With RNase T_1 glycerol could bind in place of the 5-water molecules chain connecting the indole ring nitrogen of Trp^{59} to the aqueous interface and cause a substantial alternation of its environment (49).

To test the extent of a possible coupling between solvent motions and the flexibility of a protein site Kramer's plots were constructed for each isotherm and the coefficient k of the power law was obtained by linear regression analysis. From these plots the anomalous points (those of negative slope) of LADH and RNase T_1 were omitted on the assumption that specific glycerol effects on the protein conformation were confined to the above-mentioned concentration ranges.

According to Gavish's position-dependent internal protein viscosity (15) the deeper buried and the more rigid is a protein region the weaker is the transmission of solvent viscosity effects. In the present study we have examined internal regions of proteins whose effective viscosity ranges between approximately 10^2 and 10^7 poise the wide range covered both by analyzing different polypeptides and by altering the flexibility of a given site with changes in temperature. In Fig. 3 *A* the frictional coefficient is plotted as a function of the local viscosity, η_{po}, of the proteins in buffer, except for RNase T_1 where η_{po} refers to 50% glycerol concentration. It is evident that for rigid domains, $\eta_p > 10^5$ poise, the frictional coefficient is modest, $k \leq 0.2$, and in agreement with theoretical expectations the flexibility of these rigid sites exhibits weak coupling to solvent motions. More flexible regions of the protein, $\eta_p < 10^5$ poise, have in general larger values of k. However, the trend is not a smooth one, and k is not a single valued function of η_p. For example, around $\eta_p \sim 10^4$ poise k varies among the four proteins between 0.2 and 1. Furthermore, the trend is actually inverted with RNase T_1, whose frictional coefficient decreases as the Trp site becomes more mobile. If such discrepancies emphasize that there is no direct relationship between k and η_p of general validity the data of Fig. 3 does nevertheless suggest some guidelines along which theoretical expectation are in part fulfilled: (*a*) For each protein site, with the exception of RNase T_1, k does increase smoothly when η_p is decreased by raising the temperature. (*b*) Some correlation is also apparent between k and the depth of burial, the distance of the reporter chromophore from the solvent. Comparing the value of k of the four proteins at $\eta_p \sim 10^4$ poise we observe that the frictional coefficient decreases in the order RNase T_1 > LADH > AP > apoazurin, while the closest distance from Trp to the protein surface is about 2, 5, 15, and 8 Å, respectively.

Damping of structural fluctuations in proteins by the viscous drag of the solvent is believed to alter the frequency spectrum of protein motions. For example, Almagor et al. (33) interpreting ultrasonic absorption data on serum albumin propose a model which contemplates a strong attenuation of high frequency modes of peripheral side chains accompanied by an enhancement of large amplitude, low frequency, segmental motions of the polypeptide chain. The results of H-exchange studies on lysozyme (29, 30), on the other hand, show that the addition of glycerol slows down mostly the exchange of protons in rigid segments of the structure as to indicate that large amplitude fluctuations are preferentially hindered by the viscous drag. The temperature dependence of k can provide a rough indication on the kind of structural fluctuations that are principally affected by the viscous drag of the solvent. In Fig. 3 *B*, where k is plotted as a function of temperature, we note that for apoazurin, LADH, and AP there is a rather sharp rise in the value of k in the upper temperature range, between 40 and 60°C. As the rise in k is not commensurate with the temperature-induced decrease in η_p (Fig. 3 *A*) and occurs at roughly the same temperature for all three proteins, we must conclude that structural fluctuations characterized by large activation barriers are more effectively damped. We propose that glycerol is active in contrasting low frequency, large amplitude fluctuations, which lead to partially unfolded, rather flexible con-

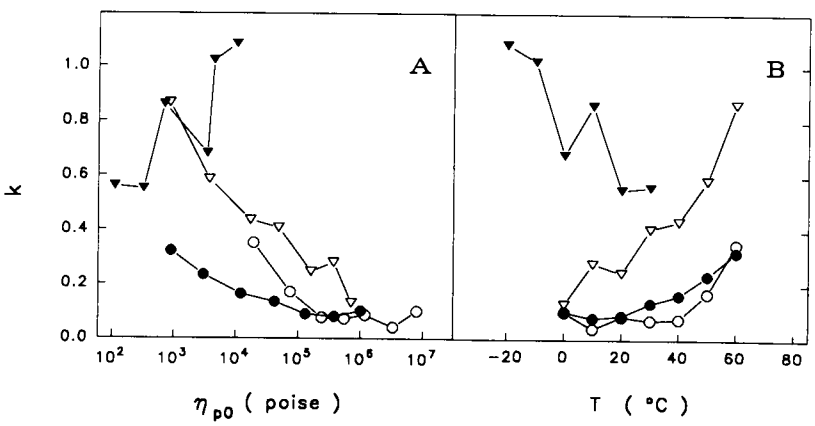

FIGURE 3 Dependence of the frictional coefficient, k, on the viscosity, η_{po}, of the protein site (*A*) and on temperature (*B*). The proteins are RNase T_1 (▼), LADH (▽), apoazurin (●) and alkaline phosphatase (○).

formers of the macromolecule. Support for this point of view comes also from the observation that k gets unexpectedly large, almost as large as anticipated for solvent exposed segments of the polypeptide ($k \sim 0.9$ for LADH), on approaching the temperature of protein unfolding. Also in keeping with this notion, is the observation that the frictional coefficient is larger for LADH which thermally denatures around 60°C (50) as opposed to apoazurin and AP whose melting temperatures are 86 (51) and 93°C (52), respectively. Finally, the above proposal is also consistent with the effects of glycerol on the thermal stabilization of lysozyme (35). Glycerol was found to raise the thermal unfolding temperature of lysozyme but, more importantly, this study established that stabilization of the native fold occurs mainly above 40°C and is greater the higher the temperature.

Among the four proteins examined in the present study RNase T_1 has the most flexible and superficial Trp site, and, in keeping with theoretical expectations, the frictional coefficient has the largest value. However, the temperature dependence of k is in apparent contrast with the hypothesis advance above, since it would predict that transitions to more flexible conformers of RNase T_1 are actually favored at subzero temperatures. First we note that because only three points were considered for each isotherm the derived frictional coefficients are probably the least reliable. Second, thermal stability studies in buffer (53) indicate that the protein is most stable near 0°C, and so there is a propensity for this protein to undergo cold denaturation. Whether the thermal unfolding profile is similar in 50% glycerol solution, the state of the protein for which we have derived frictional coefficients, and whether solvent viscosity effects are wholly responsible for the anomalous temperature behavior is premature to say.

In the present study we probed glycerol effects on the flexibility of four proteins focussing attention on internal rigid sites of the macromolecule for which both theory and molecular dynamic simulations predict little if any influence of solvent motions on their dynamic make up. It was found that glycerol has an important influence on the flexibility of these sites. For some proteins and glycerol concentration ranges large deviations form Kramer's theory indicate unequivocably that thermodynamic effects of the cosolvent on the conformation of the native fold are important and may even override concomitant kinetic effects. In glycerol ranges where changes in flexibility with solvent viscosity could be fitted to a power law, the derived frictional coefficient was found to be both site-specific and strongly temperature-dependent. If site specificity probably reflects particular H-bonding network to the solvent and/or free volume distribution in the macromolecule, the temperature dependence of k is believed to underlie the damping of large amplitude fluctuations. Consequently, glycerol effects on the flexibility of internal protein regions are predicted to be large under conditions favoring unfolding transitions; a proposal totally consistent with the mechanism by which glycerol stabilizes the native fold of a protein (34). Finally, if the present findings caution against the prevailing attitude of assuming little or no influence of cosolvent on internal protein motions (24, 25), they also do not support the proposal (33) that large amplitude segmental motions are actually enhanced by the viscous drag.

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