

Effect of Heavy Water on Protein Flexibility

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ABSTRACT The effects of heavy water (D_2O) on internal dynamics of proteins were assessed by both the intrinsic phosphorescence lifetime of deeply buried Trp residues, which reports on the local structure about the triplet probe, and the bimolecular acrylamide phosphorescence quenching rate constant that is a measure of the average acrylamide diffusion coefficient through the macromolecule. The results obtained with several protein systems (ribonuclease T1, superoxide dismutase, β -lactoglobulin, liver alcohol dehydrogenase, alkaline phosphatase, and apo- and Cd-azurin) demonstrate that in most cases D_2O does significantly increase the rigidity of the native structure. With the exception of alkaline phosphatase, the kinetics of the structure tightening effect of deuteration are rapid compared with the rate of H/D exchange of internal protons, which would then assign the dampening of structural fluctuations in D_2O to a solvent effect, rather than to stronger intramolecular D bonding. Structure tightening by heavy water is generally amplified at higher temperatures, supporting a mostly hydrophobic nature of the underlying interaction, and under conditions that destabilize the globular fold.

INTRODUCTION

The topic of deuterium isotope effects is usually concerned with the effects on the rate of cleavage of covalent bonds to hydrogen caused by the substitution with the heavier isotope. Deuterium isotope effects on other, noncovalent interactions are also known to occur but they are often considered to be small or insignificant, especially in biological experiments where deuterium substituted molecules are used as tracers or when heavy water (D_2O) is the solvent chosen for conducting biophysical studies (Jencks, 1969). Although on individual bonds the change in free energy is small the cumulative effect on a large macromolecule may be significant. With proteins, whose folded structure is the result of a delicate balance between intramolecular and hydration interactions, D_2O may alter their conformation in solution, the dynamics of the structure, and their function. At a macroscopic level, there is evidence suggesting that D_2O is a worse solvent than water and that polypeptides tend to reduce the surface area in contact with the solvent by adopting a more compact globular shape or associating into larger aggregates. This has been inferred mainly from the stabilizing effect of D_2O on thermal, GuHCl, and urea-induced denaturation of several proteins (Maybury and Katz, 1956; Hermans and Sheraga, 1959; Dong et al., 1998; Parker and Clarke, 1997; Verheul et al., 1998) and from the promotion of aggregated states of oligomeric proteins (Baghurst et al., 1972; Bonnetè et al., 1994; Omori et al., 1997; Chakrabarti et al., 1999). The consensus on the stabilizing influence of D_2O is not general, however, whereas the thermal stability of val-tRNA^{val} synthetase was unaffected

by it (Kern et al., 1980), a recent study based on the free energy of transfer of model compounds from H_2O to D_2O concluded that at ambient temperature the globular state is expected to be even less stable in heavy water (Makhatadze et al., 1995).

On the macroscopic level both experimental (Benjamin and Benson, 1962) and theoretical (Scheiner and Cuma, 1996) studies have demonstrated that in water, deuterium bonds are stronger than H bonds by ~ 0.1 to 0.2 kcal mol⁻¹. The increased strength of the deuterium bond is attributed to the higher mass of the deuteron lowering the zero-point vibrational energy of the intermolecular mode of highest frequency. This mode is associated with a bending motion of the proton donor molecule distorting the linearity of the H bond (Scheiner and Cuma, 1996). Concerning the stability of the native protein fold, a greater enthalpic D_2O - D_2O affinity is expected to lead to a commensurate increase in the hydrophobic interaction. In a number of cases (Parker and Clarke, 1997; Chakrabarti et al., 1999) the stabilizing effect of D_2O has indeed been attributed to the enhancement of hydrophobic interactions. Nemethy and Sheraga (1964) have pointed out, however, that intrapeptide deuterium bonds are presumably stronger than H bonds and proposed that the greater protein stability is partly due to stronger H bonding (Maybury and Katz, 1956; Baghurst et al., 1972). On the other hand, Schowen and Schowen (1982) studying the solvent isotope effect on hydrogen bonding found that enthalpic and entropic effects cancel each other.

In contrast to the wealth of thermodynamic data little is known about the effects of D_2O on the conformational flexibility of proteins. Knowledge of the influence of D_2O on structural fluctuations may be important both at a basic level, to identify the nature of the underlying interactions, and also for its possible implications on the catalytic efficiency of enzymatic proteins in this medium. The present report addresses the issue of D_2O effects on protein dynamics by means of sensitive parameters based on the phosphorescence emission of deeply buried Trp residues. In brief,

Submitted October 3, 2001, and accepted for publication February 22, 2002.

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0006-3495/02/06/3246/08 \$2.00

the magnitude of the intrinsic room-temperature phosphorescence lifetime (τ_0) of Trp reports on the local flexibility of the protein matrix around the chromophore (Strambini and Gonnelli, 1995; Gonnelli and Strambini, 1995), whereas the bimolecular rate constant (k_q), derived from quenching of protein phosphorescence by acrylamide, relates to the diffusion of the solute through the protein fold to the chromophore site and is correlated to the structural flexibility of the macromolecule (Cioni and Strambini, 1998). The influence of D₂O on the phosphorescence of alcohol dehydrogenase (Saviotti, 1975; Kishner et al., 1979; Vanderkooi et al., 1987) and alkaline phosphatase (Schlyer et al., 1996; Fischer et al., 2000) have reported an increase of τ_0 in D₂O, but the lengthening of the lifetime was not unanimously attributed to a decreased protein flexibility. The present report examines the influence of D₂O on both τ_0 and k_q on multiple protein systems and across a wide temperature range. The proteins chosen are monomeric ribonuclease T1, apo- and Cd-azurin (Az, CdAz), dimeric alcohol dehydrogenase (LADH), β -lactoglobulin (β -LG), superoxide dismutase (SOD), and alkaline phosphatase (AP). In each case the crystallographic structure is known and the phosphorescence emission in fluid solution is due to a single, well-identified Trp residue buried within the globular fold (Gonnelli and Strambini, 1995; Cioni et al., 2001). Further, the potentially quenching side-chains of Cys, His, and Tyr (Gonnelli and Strambini, 1995) are in each case removed from the chromophore environment assuring, thereby, that τ is not influenced intramolecular reactions. Both τ_0 and k_q concur in indicating that with most proteins D₂O increases the rigidity of the native structure. Whereas no apparent correlation is found between the magnitude of the change and the degree of burial of the probe or the actual flexibility of the polypeptide, the effect is generally enhanced at high temperature and by conditions that destabilize the folded state.

MATERIAL AND METHODS

All chemicals were of the highest purity grade available from commercial sources. *N*-acetyltryptophanamide (NATA) was purchased from Sigma (St. Louis, MO) and before use was recrystallized three times from ethanol/water. Acrylamide (>99.9% electrophoretic purity) was from Bio-Rad Laboratories (Richmond, CA). Water, doubly distilled over quartz, was purified by Milli-Q Plus system (Millipore Corporation, Bedford, MA). The D₂O was purchased from Sigma and was 99.9% pure. Deuterated buffers were titrated to the appropriate pD ($-\log[D^+]$) with DCl (99% pure) and NaOD (99% pure), both of which were purchased from Sigma. All pDs were determined at room temperature using the relationship pD = pH + 0.4. All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl suprapur (Merck, Darmstadt).

The protein horse liver alcohol dehydrogenase (LADH) was supplied by Boehringer (Mannheim, Germany). AP from *Escherichia coli*, and β -LG were obtained from Sigma. Monomeric ribonuclease T1 was purchased from Calbiochem Co. (San Diego, CA). Copper-free azurin (Az) from *Pseudomonas aeruginosa* was a gift of Prof. Finazzi-Agrò (University of Roma, Tor Vergata, Italy) and copper-free SOD from *Photobacterium leiognathi* was a gift of Prof. Desideri, (University of Roma, Tor Vergata,

Italy). CdAz was formed from apoazurin by the addition of CdCl₂ in the Cd:protein molar ratio of 2:1. Complex formation was verified by looking at the Trp fluorescence intensity in competition experiments with Cu²⁺ as Cu-azurin is strongly quenched (Strambini and Gabbellieri, 1991).

Fully deuterated proteins were prepared by mixing 10 μ L of concentrated protein stock in H₂O buffer with 990 μ L of the D₂O buffer (exchange solution). To complete the exchange process these mixtures were incubated overnight at different temperatures depending on the thermal stability of each protein: 30°C for SOD and Az + 0.5 M GuHCl, 40°C for LADH, β -LG, and Az, 60°C for AP, 75°C for CdAz. Longer incubation times, up to 36 h, did not modify the phosphorescence characteristics of the sample. Fully deuterated CdAz was also prepared by the addition of Cd²⁺ to deuterated apoazurin.

For phosphorescence measurements in fluid solutions, it is paramount to rid the solution of all O₂ traces. The samples were placed in 5 × 5 square quartz cuvette especially designed to allow thorough removal of O₂ by the alternative application of moderate vacuum and inlet of ultra pure N₂ (Strambini and Gonnelli, 1995). In all experiments the concentration of protein was ~5 μ M. Acrylamide quenching experiments were carried out as described before (Cioni and Strambini, 1998).

Luminescence measurements

Phosphorescence decays in fluid solutions were measured on an apparatus described before (Strambini and Gonnelli, 1995). Pulsed excitation is provided by a frequency-doubled, Nd:Yag-pumped dye laser (Quanta Systems, Milano, Italy) (λ_{ex} = 292 nm) with pulse duration of 5 ns and a typical energy per pulse of 0.1 mJ. Phosphorescence and delayed fluorescence, emitted at 90° from the excitation, are selected by filter combinations with a transmission windows 420 to 460 nm and 320 to 400 nm, respectively. A gating circuit (Kao and Verkman, 1996) that inverts the polarity of dynodes 1 and 3, for up to 1.5 ms after the laser pulse, protects the photomultiplier (EMI 9235QA, Middlesex, UK) from the intense prompt fluorescence light pulse. Alternatively, for lifetimes shorter than 5 ms the photomultiplier was protected by a chopper blade that closes the emission slit during the excitation pulse (Strambini and Gonnelli, 1995). The time resolution of this apparatus is typically 10 μ s. The photocurrent was amplified by a current to voltage converter (SR570, Stanford Research Systems) and digitized by a computerscope system (ISC-16, RC Electronics) capable of averaging multiple sweeps. All phosphorescence decays were analyzed in terms of a sum of exponential components by a nonlinear least squares fitting algorithm (Global Unlimited, LFD, University of Illinois). Lifetime data used in the analysis are averages of two or more independent measurements. The reproducibility of phosphorescence lifetimes between samples was typically better than 5%.

RESULTS

Phosphorescence lifetime of NATA and of proteins in D₂O

In ordinary phosphate buffer, 5 mM, pH 7, the phosphorescence lifetime (τ) of NATA, measured by its delayed fluorescence, is 1.0 to 1.1 ms, at 20°C (Strambini and Gonnelli, 1995). On replacement of H₂O with D₂O the mean lifetime, obtained with different D₂O stocks, is 0.95 ± 0.02 ms, ~10% smaller than in water. It would appear that neither D₂O as a solvent nor deuteration of the indole ring nitrogen has significant effects on the radiationless deactivation of the excited triplet state, the process responsible for the shortening of the phosphorescence lifetime in fluid media (Strambini and Gonnelli, 1995). Naturally, one cannot rule

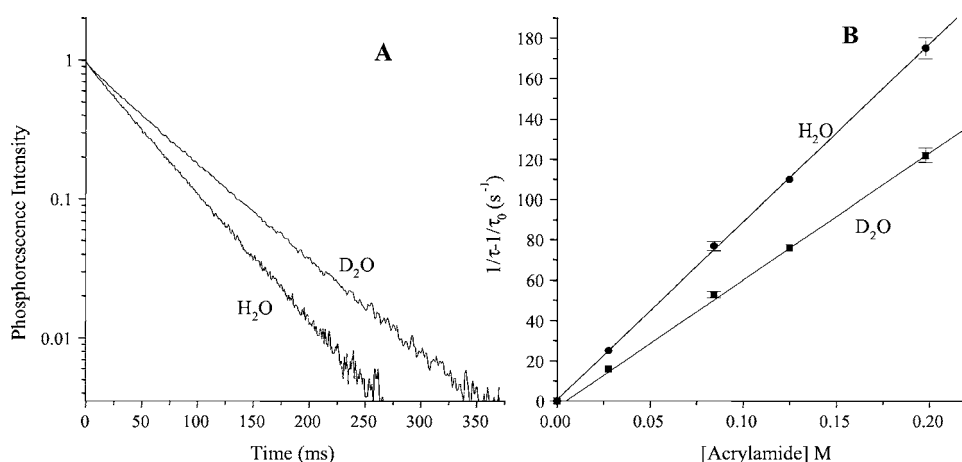


FIGURE 1 Effects of D₂O on the phosphorescence decay of azurin (*A*) and on the lifetime Stern-Volmer plot for the quenching of azurin phosphorescence by acrylamide (*B*). The sample is 3 μ M azurin in Tris-HCl (50 mM, pH 7.5) at 50°C. In *B* each point is the average of at least three independent experiments, and the error bars indicate the range of τ variations.

out the possibility that traces of quenching impurities in the D₂O supply mask a potentially larger intrinsic lifetime in heavy water. Phosphorescence decays measurements on NATA were also carried out in viscous propylene glycol/D₂O (50/50, v/v) mixtures, down to 140 K where τ reaches the limiting value of 6.5 s. The results indicate that also in viscous solutions, where bimolecular quenching reactions are inhibited, deuteration of the ring nitrogen does not affect the lifetime of NATA. The same conclusion was drawn from comparing τ_0 in ice formed by light and heavy water, at 140 K.

In contrast to the free chromophore, in the set of proteins examined the phosphorescence decay of internal Trps is generally slowed down in D₂O. An example of raw data and

of the typical lifetime variation in proteins is given in Fig. 1 *A* for Az at 50°C. The intrinsic lifetime of each protein in water (τ_H) and in D₂O (τ_D), at selected temperatures between 0°C and 50°C, is given Table 1, whereas its variation is shown by the lifetime ratio, τ_D/τ_H , in Fig. 2. The phosphorescence of LADH, SOD, and β -LG is intrinsically heterogeneous and the multiplicity of phosphorescence lifetimes reflects the presence of more than one stable conformation of the macromolecule, each with its own τ (Cioni et al., 1994). The lifetime heterogeneity is found to be preserved in D₂O, and the statistical analysis indicates that the fractional intensities of the lifetime components are largely unchanged relative to water, suggesting that heavy water does not alter the equilibrium between the different confor-

TABLE 1 Intrinsic phosphorescence lifetime (ms) of native proteins in H₂O/D₂O

Protein	r_p (Å)*	Buffer/pH	T (°C)					
			0	10	20	30	40	50
RNase T_1	2	Na phosph. 10 mM/6	100/101	55/60	28/36	15.3/22.2	7.14/12.11	3.12/5.92
SOD	3	Tris-HCl 10 mM/7.5	32/33	17/17	8.92/9.1	4.21/4.1	2.28/2.31	1.14/1.18
β -LG	6	Na phosph. 10 mM/6	53/61	44/55	30/44	19/30	8.5/14.5	2.5/4.5
LADH	4.5	Tris-HCl 10 mM/7.5	1520/2128	1150/1564	630/819	311/379	129/156	51.1/61.3
Az	8	Tris-HCl 10 mM/7.5	1550/1612	1182/1217	564/603	271/304	100/125	45.3/58.9
Az+0.5 M GuHCl	8	Tris-HCl 10 mM/7.5	1410/1495	1093/1170	501/571	213/253	77.3/111.3	17.2/28.4
CdAz	8	Tris-HCl 10 mM/7.5	1336/1330	954/960	479/476	204/208	100/101	57.9/56.8
AP	11	Tris-HCl 10 mM/7.5	3302/3300	2843/2900	2060/2142	1117/1218	600/732	270/540

*Shortest distance of phosphorescing Trp (indole ring) from the aqueous interface. The range of τ variations is typically of 2 to 3%.

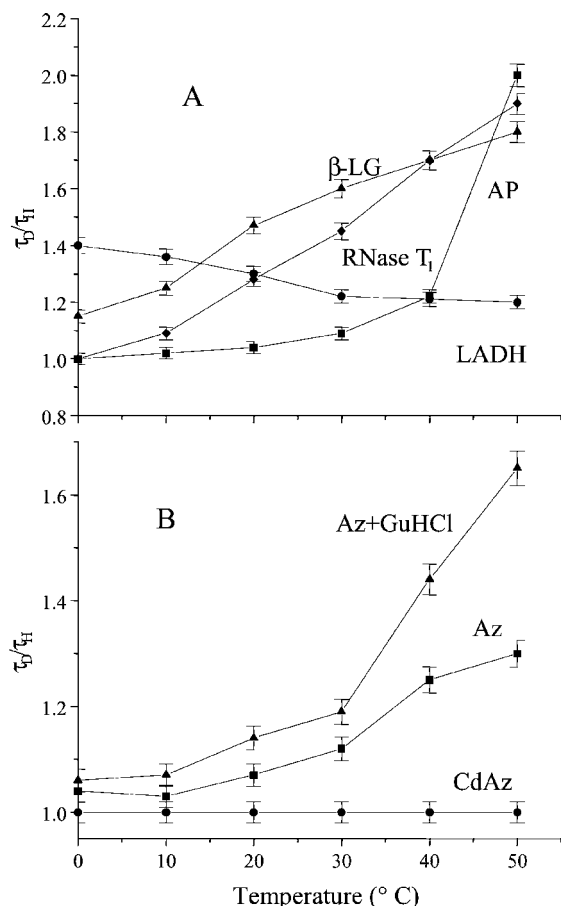


FIGURE 2 Temperature dependence of the phosphorescence lifetime ratio τ_D/τ_H for LADH, AP, RNase T₁, and β -LG (A) and for Az, CdAz, and Az + 0.5 M GuHCl (B). The buffers used and their pH are reported in Table 1.

mations. Throughout, heterogeneous decays were adequately fitted in terms of two lifetime components and the magnitude reported in Table 1 and Fig. 2 refer to the averaged lifetime ($\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$). Lastly, a comparison with previous studies, shows that both τ_H and τ_D are substantially longer for LADH (Saviotti, 1975; Kishner et al., 1979; Vanderkooi et al., 1987) but are similar, under equivalent experimental conditions, in the case of AP (Fischer et al., 2000). The shorter lifetimes reported previously for LADH presumably reflects incomplete sample deoxygenation.

With the exception of SOD, where the lifetime is practically unchanged in D₂O, for the other proteins the ratio τ_D/τ_H increases from 5% to over 50%, depending on the protein and temperature. From the empirical correlation between τ and solvent viscosity, observed with Trp derivatives in viscous solutions (Strambini and Gonnelli, 1995), the increment of τ attests to a greater rigidity of the protein structure in D₂O, corresponding to a roughly 10% to 100% reduction in local fluidity. In this respect the effect of D₂O

on protein dynamics is similar to that of cooling the solution, and one can estimate the equivalent temperature reduction from the temperature variation of τ in water (Table 1) and the difference $\delta\tau = \tau_D - \tau_H$. At 50°C, this amounts to a cooling of $\sim 7.5^\circ\text{C}$ for monomeric ribonuclease T₁, 3°C for β -LG, 2.5°C for Az, and 7°C for AP. For LADH at 10°C it is 3.5°C . Interestingly, these magnitudes are in the same range of the increase in the thermal unfolding temperature reported for some proteins in D₂O (Bonnetè et al., 1994; Verheul et al., 1998).

There is considerable variability of the D₂O effects among the proteins examined, and an analysis of the data based on crystallographic structures and τ (Table 1) indicates that the magnitude of the lifetime change in D₂O is not simply related to the degree of burial of the chromophore (r_p , the shortest distance of the indole ring from the aqueous phase) nor to the local fluidity of the protein structure as inferred by τ . The only emerging trend is for larger τ_D/τ_H ratios at higher temperatures, although the behavior is opposite in the case of LADH. Because of the marked protein/site specificity any enquiry on a possible correlation between the effects D₂O on τ and the thermodynamic stability of the globular fold need to refer to the same protein system. To this end Az was destabilized by the addition of 0.5 M GdnHCl and stabilized upon binding of Cd²⁺ to the copper-binding site (T_m increases from 65°C to 95°C (Engeseth and McMillin, 1986)). The results (Fig. 2 B) show that the change in Az stability does modulate the deuterium effect, the τ_D/τ_H ratio becoming significantly larger in the presence of denaturant as opposed to being practically abolished in the stable CdAz.

Acrylamide quenching of protein phosphorescence

The phosphorescence lifetime is a local probe reporting on the structure about the indole ring and, therefore, provides a limited, site specific picture of conformational dynamics. Moreover, there is the possibility, even if remote, that τ be affected by D₂O induced changes in protein conformation that bring intramolecular quenching residues (Cys, His, and Tyr) within interaction distance of Trp or that, as suggested recently by Fischer et al. (2000) for AP, H/D exchange at the ring nitrogen play a role in the τ increase in D₂O. An independent monitor of protein flexibility is the permeability of the macromolecule to small solutes like acrylamide that quench the phosphorescence emission (Cioni and Strambini, 1998). Quenching experiments determine the excited lifetime as a function of quencher concentration in solution, $[Q]$, and evaluate the bimolecular quenching rate constant from the gradient of the Stern-Volmer plot, $1/\tau = 1/\tau_0 + k_q[Q]$, in which τ_0 is the unperturbed lifetime. Measurements were conducted at 20°C for most proteins and at 50°C for Az and AP, which have relatively small quenching constants at ambient temperature. In these ex-

TABLE 2 Second order acrylamide-quenching constants in H₂O and in D₂O, at selected temperatures

Protein	T (°C)	k_q^H (M ⁻¹ s ⁻¹)	k_q^H/k_q^D	$\eta_\tau^D/\eta_\tau^{H*}$
RNase T ₁	20	$6.0 \pm 1 \times 10^4$	1.1	1.3
SOD	0	$1.4 \pm 0.3 \times 10^4$	1.0	1.0
β -LG	20	$4.2 \pm 1 \times 10^4$	1.5	1.5
LADH	20	$1.2 \pm 0.4 \times 10^4$	1.1	1.5
Az	50	$8.8 \pm 1 \times 10^2$	1.4	1.3
CdAz	50	0.86 ± 0.2	1.0	1.0
AP	50	7.2 ± 1	1.8	2.0

*Estimate of the local viscosity obtained from the empirical correlation between τ and solvent viscosity in model studies (Strambini and Gonnelli, 1995).

periments the acrylamide concentration was increased until the lifetime decreased by at least fivefold. The decays of SOD, β -LG, and LADH, which are intrinsically heterogeneous, were found to remain so even when the quencher considerably reduces the average τ . This implies that the lifetime conformers have distinct acrylamide quenching constants. For convenience, lifetime Stern-Volmer plots were all constructed from the average lifetime obtained, in general, from a biexponential fitting of phosphorescence decays. Thus, the value of k_q derived from the gradient of these plots is an average quantity.

For every protein, $1/\tau$ increased linearly with acrylamide concentration, as should be expected for a dynamic quenching reaction. Representative Stern-Volmer plots are shown in Fig. 1 B for acrylamide quenching of Az in H₂O and in D₂O at 50°C. The accessibility of acrylamide to the core of Az is clearly reduced when the protein is placed in heavy water, the bimolecular quenching rate constant, k_q , decreasing by 1.4-fold. The greater viscous drag to acrylamide migration to the site of W48 ($k_q \propto T/\eta$, η the frictional drag to acrylamide diffusion inside the protein) is consistent with a deuterium-induced stiffening of the globular structure. The same conclusion was reached from oxygen quenching of AP phosphorescence (Schlyer et al., 1996). In heavy water, k_q is smaller also for β -LG, AP, and LADH, but not for SOD and CdAz. The magnitude of k_q and its variation in D₂O is given in Table 2. In this table the ratio k_q^D/k_q^H , which is proportional to the change in frictional coefficient to acrylamide diffusion, is compared with the change in local "viscosity" inferred from τ , under the same conditions. Except for RNase T1 and LADH, the correlation between the two dynamic parameters is surprisingly good. It confirms that the τ increase in heavy water reflects a more rigid protein environment and also indicates that the dampening of structural fluctuations by D₂O extends to beyond the proximity of the phosphorescence probe. The mismatch noted for RNase T1 is probably due to the superficial location of W59 that makes acrylamide quenching possible directly from the solvent (Cioni and Strambini, 1998), whereas distinct structural fluctuations may govern the parameters τ and k_q in the case of LADH. Again, as for the

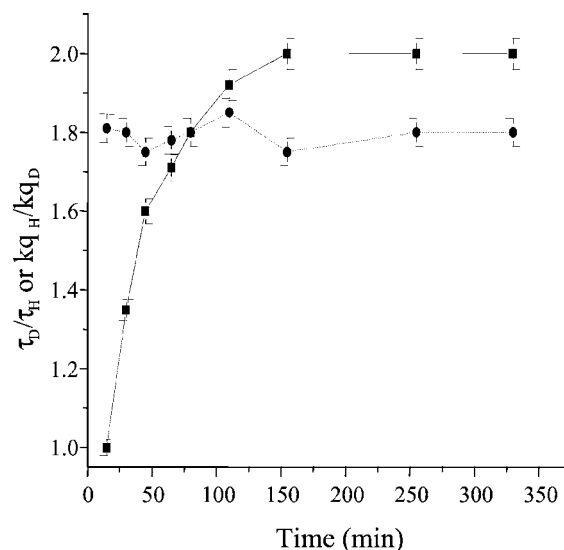


FIGURE 3 Time course of deuteration effects on τ (■) and k_q (●) of AP at 50°C. τ_D and k_{qD} represent average quantities.

intrinsic lifetime, there is no apparent relationship between the effectiveness of D₂O in tightening the structure and the actual flexibility of the polypeptide inferred from k_q .

Kinetics of τ and k_q variations in D₂O

The above phosphorescence results refer to samples equilibrated with D₂O for at least 18 h. To test how rapid is the onset of the τ and k_q variations H/D exchange was slowed by equilibrating the sample at ambient temperature for a period as short as 10 min, the minimal dead time for deoxygenation. Except for AP, equilibration times longer than 10 min did not change the phosphorescence results. For this set of proteins it can therefore be concluded that the H/D substitution of medium and slowly exchanging protons has no detectable influence on τ and k_q . Consequently, the observed tightening of the globular fold is due to either the substitution of superficial, rapidly exchanging protons or to a solvent effect of D₂O. To help discriminate between the two we note that exchange by the EX2 mechanism, which apparently applies to most protons (Bai et al., 1994), is sharply pH dependent the rate increasing by a factor of 10 per pH unit on either side of the minimum at approximately pH 5. For Az and RNase T1 the increase of τ after 10 min equilibration in D₂O (at 30°C) was found to be equivalent at pH 7 and 5. Thus, despite 100-fold reduction in exchange rate the influence of D₂O on τ is rapid and complete within the dead time of the measurement. Such pH invariance is more consistent with a solvation-based mechanism.

Among the proteins examined, AP is the only case of slow induction of deuterium effects on τ . The time course of τ and of k_q (Fig. 3) shows that contrary to the increase in k_q , which occurs within the dead time of the measurement, τ

reaches a steady value only after a couple of hours (at 50°C). Divergent kinetics indicate that, for this most deeply buried protein site, migration of acrylamide and τ are determined by distinct structural fluctuations not necessarily involving the same region of the protein. During the lifetime transition the phosphorescence decay is not exponential and can be fitted reasonably well in terms of two components having the lifetime of the protein in water and in D₂O, respectively. Both the kinetics of the process and the magnitude of the lifetime components are in accord with the results reported before by Fischer et al. (2000). The authors have proposed that H-D exchange at the indole ring nitrogen is responsible for the increase of τ but provided no evidence of concomitant ring deuteration. Alternative explanations for the slow increase of τ are: tightening of the structure about the probe (W109) as a result of slow deuteration of protected H bonds or, slow structural isomerization toward a more compact, D₂O stabilized conformation of the macromolecule.

DISCUSSION

Influence of D₂O on protein flexibility

The slowing down of acrylamide diffusion through the globular fold provides unequivocal evidence that for the majority of the proteins examined heavy water has the effect of stiffening the polypeptide structure. The conclusion is also strongly supported by the concomitant lengthening of the phosphorescence lifetime, which is interpreted in terms of a more rigid local structure about the triplet probe, in accord with the very first phosphorescence studies of Saviotti (1975) and Kishner et al. (1979). The correlation between τ and the local flexibility of the protein matrix is based on the sharp dependence of τ (Trp derivatives) on solvent viscosity (Strambini and Gonnelli, 1995) as well as on the numerous examples of protein systems and experimental conditions in which modulation of protein flexibility resulted in a corresponding variation of τ . It should be mentioned that, in principle, in deuterated matrices τ may also be affected proton exchange at the indole ring nitrogen or even by indirect effects of the deuterated protein matrix (Busel and Burshtein, 1970). Indeed, Fischer et al. (2000) have argued that, by analogy to the increase of τ with perdeuterated aromatic hydrocarbons (Lower and El-Sayed, 1966), the lengthening of τ of AP is probably caused by deuteration of the indole ring nitrogen, modifying the photophysics of the chromophore. The authors even foresee that the lifetime increase should be universal in proteins and that it may serve as a selective monitor of H-D exchange at the aromatic ring. Although Busel and Burshtein (1970) find a modest 10% increase of τ of Trp in a fully deuterated glass at 77 K, multiple evidence from this study does not support this hypothesis. We note that the magnitude of the lifetime increase on deuteration varies considerably among proteins

(the effect lacks completely in SOD and CdAz) and with temperature. Most significantly, however, the lifetime of the free chromophore (NATA) in fluid solutions was found to be similar between H₂O and D₂O. Even more stringent, the lifetime increment in D₂O was found to correlate closely with increased structural rigidity of the protein monitored independently by the rate of acrylamide diffusion to the site of the chromophore. Table 2 shows in fact that in five of seven cases the enhancement in internal protein viscosity estimated by k_q is quite similar to that derived by τ . Naturally, the conformational flexibility estimated by τ and k_q needs not match perfectly if the two parameters are dominated by different structural fluctuations (Cioni and Strambini, 1998). This appears to be the case with LADH and AP.

A lengthening of the RTP phosphorescence lifetime of proteins after equilibration with D₂O has been reported before for LADH (Saviotti, 1975; Kishner et al., 1979; Vanderkooi et al., 1987) and for AP (including two mutants) (Fischer et al., 2000). The present study, although confirming this trend for other proteins and for a wider range of experimental conditions, emphasizes also a marked variability among protein systems. Following the initial goal of enquiring on the effects of heavy water on protein dynamics, the present investigation also sought for potential correlations between the effectiveness of D₂O tightening and certain specific features of the polypeptide structure such as: the proximity of the region to the aqueous phase (r_p , Table 1), the tightness of the structure (τ , k_q), and the thermodynamic stability of the macromolecule to unfolding. Apparently, the disparity among proteins is related to neither the proximity of the probe to the water interface nor to the actual flexibility of the local structure (τ). Indeed, a similarly large flexibility reduction is found for superficial (Rnase T1, $r_p = 1-2 \text{ \AA}$) and very internal (AP, $r_p = 11.5 \text{ \AA}$) sites, as well as for regions characterized by either very loose ($\tau = 3 \text{ ms}$, for RNase T1) or tight ($\tau = 270 \text{ ms}$, for AP) local structures. It is instructive to note, however, that when differences in sequence and three-dimensional structure are largely taken into account, as in the internal comparison between stabilized/destabilized Az, the response to D₂O appears to be inversely proportional to the stability of the globular structure. Binding of Cd to Az increases its T_m from 65°C to 95°C (Engeseth and McMillin, 1986) and drastically reduces k_q (Cioni and Strambini, 1998), both responses implying a more compact and stable structure for the metal complex. An opposite effect on stability is expected from the addition of nondenaturing concentrations of GndHCl. Interestingly, the increase in intrinsic lifetime by D₂O was found to be largest for the looser state of azurin and to be negligible for the Cd complex. Altogether, these findings suggest that the influence of D₂O in dampening structural fluctuations may vary substantially with protein sequence/fold/site, but is likely to be enhanced by conditions, such as high temperature, that destabilize the globular structure. Because our monitor reports essentially on the

local structure we cannot distinguish the extent to which the variability is site-to-site or protein-to-protein. A degree of protein-to-protein variability of the D₂O effects has also been found with thermal unfolding equilibria (Hermans and Sheraga, 1959; Bonnetè et al., 1994; Dong et al., 1998; Verheul et al., 1998).

Nature of the interactions underlying the D₂O effect

It is generally accepted that segmental rigidity to polypeptides is conferred primarily by the intramolecular network of cooperatively formed H bonds and that changes in flexibility involve an alteration in either their number or strength. Thus, enhanced structural rigidity in D₂O can be accounted for by both stronger intrapeptide D bonds over H bonds or formation of extra intramolecular bonds at the expense of hydrogen bonds with the solvent, through additional folding/compaction of the structure. It has been proposed that the greater propensity of D₂O, relative to water, to form solvent-solvent H bonds (Scheiner and Cuma, 1996) should make an important contribution to the latter process. To help discriminate between these two possibilities, strength or number of intrapeptide H bonds, one criterion can be the time dependence of the flexibility changes after dilution in D₂O. Solvent effects are expected to be immediate, or as rapid as any eventual change in conformation that might be induced by the new solvent. By contrast, isotopic exchange of internal protons is generally slow and pH dependent, taking from hours to days, particularly for protons engaged in H bonds (Milne et al., 1998). Kinetic runs showed that, with the exception of AP, the structure tightening effect of deuteration was complete after the 10-min dead time of sample preparation. It remained short even when in two proteins the exchange rate has been slowed down 100-fold by lowering the pH from 7 to 5. Based on these observations, the dampening of structural fluctuations in D₂O should be assigned to a solvent effect, although a role of rapid deuteration in the periphery cannot be ruled out completely. Slow isotopic exchange, according to Fischer et al. (2000), or an unusually sluggish conformational isomerization could instead be implicated in tightening the inner core of AP where the increase of τ , after dilution with D₂O, requires hours to reach equilibrium. In this respect it should be recalled that during AP refolding the transition from quasative to native conformations monitored by τ was found to be extremely slow (days) (Subramanian et al., 1995).

Overall, the generally short induction time of the D₂O effects on protein dynamics is more consistent with a change in the solvation properties of heavy water, presumably leading to an increase in the number of intramolecular bonding interactions and additional compaction of the polypeptide. This conclusion was also reached by a molecular dynamics simulation study on plastocyanin where di-

minished protein hydration in D₂O promoted closer packing of the polypeptide and an increase in the number of intra-protein hydrogen bonds (Guzzi et al., 1999). Similarly, Kreshech et al. (1965), from the free energy of transfer of model compounds between the two solvents, have concluded that the increased strength of solvent-solvent hydrogen bonds in D₂O stabilizes the folded state of proteins predominantly through the enhancement of the hydrophobic interaction. In other words, the free energy gain in burying nonpolar amino acid side-chains is greater the larger the cohesive property of bulk solvent is. The observation that, for most of the proteins examined here, the tightening effect of heavy water increases significantly with temperature (Fig. 2) lends support to the hydrophobic nature of the underlying interaction (Parker and Clarke, 1997). On the other hand, an inverted temperature dependence with LADH as well as the slow induction of D₂O effects in AP could reflect stronger intramolecular deuterium bonds.

In summary, the present study demonstrates that D₂O significantly increases the rigidity of most protein structures, the effect being generally amplified by temperature as well as by the destabilization of the folded state. The commonly observed inverse correlation between structural flexibility and stability to thermal denaturation (Tang and Dill, 1998) would suggest that the folded state is also more stable in D₂O than in water. This is in accord with the observation that subunit aggregation is promoted in D₂O (Chakrabarti et al., 1999) but not with the prediction that at ambient temperature proteins will be less stable in D₂O than in water (Lopez and Makhatazde, 1998). Finally, these isotope effects on protein dynamics/stability have also practical implications for the interpretation of biophysical studies (nuclear magnetic resonance, H-D exchange of amide protons) conducted in heavy water, particularly when attempting to elucidate small changes in protein conformation. Likewise, in the use of solvent isotope effects on the kinetics of enzymatic reactions for mechanistic purposes, consideration should be given to the fact that the reduction in protein flexibility reported here, or changes in conformation, may in part account for the slowing down of catalytic rates.

The authors thank Prof. Finazzi-Agrò and Prof Desideri for the generous supply of protein stocks.

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