Pressure−Temperature Effects on Oxygen Quenching of Protein Phosphorescence

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Received May 10, 1999. Revised Manuscript Received July 7, 1999

Abstract: Oxygen quenching of protein phosphorescence was determined for the buried Trp residues of apoazurin, liver alcohol dehydrogenase, and alkaline phosphatase as a function of temperature (0−50 °C) and applied pressure (up to 3 kbar). Accurate control of the oxygen concentration in solution, by a method that employs an internal protein reference, largely confirms the small bimolecular quenching rate constants (k_q) reported previously for these proteins. Wide variations in flexibility of the globular fold, as attained from protein to protein or by changing external conditions of temperature and pressure, establish that the magnitude of k_q is directly correlated to the rigidity of the protein matrix surrounding the chromophore and demonstrates that the quenching rate constant is limited by hindered migration of oxygen through compact regions of the polypeptide. The magnitude of k_q implies that O_2 diffusion in proteins can be slowed over 1000-fold relative to water and much more than was inferred from the corresponding fluorescence quenching rate. The activation enthalpy for the structural fluctuations underlying O_2 diffusion in proteins ranges between 9 and 12 kcal mol^{-1}, similar among the three proteins but larger than the 3 kcal mol^{-1} for O_2 diffusion in water. The activation volumes, obtained from the pressure dependence of k_q, are largest and positive at 50 °C and below 2 kbar, but decrease monotonically at higher pressure and at lower temperature. This behavior, together with a similar magnitude of the activation volumes among proteins with different internal mobility are interpreted as to indicate an essential role of internal water molecules in conferring flexibility to protein structure.

Introduction

Much can be learned about the frequency and the amplitude of structural fluctuations in globular proteins by monitoring the diffusion of solutes of variable size through a generally compact globular fold. One method to measure the permeability of the protein matrix to small molecules is based on the rate by which the fluorescence[1−3] or phosphorescence[4−7] emission of internal tryptophan (Trp) residues is quenched by solutes that come into their proximity. Quenching experiments determine the excited-state lifetime (τ) as a function of the quencher concentration in solution, [Q], and evaluate the bimolecular quenching rate constant, k_q, from the gradient of the Stern−Volmer plot, $1/\tau - 1/\tau_0 + k_q[Q]$, where τ_0 is the unperturbed lifetime. For reactions that are under diffusion control $k_q \propto D_Q$, where D_Q is essentially the diffusion coefficient of Q inside the protein matrix. It is customary to estimate D_Q from the decrease of k_q relative to solvent exposed chromophores. In the seminal work of Lakowicz and Weber[2] on O_2 quenching of protein fluorescence, the modest, at most 5-fold reduction of ^Fk_q found for internal Trp residues in a large series of proteins led to the conclusion that structural fluctuations in the nanosecond time scale must occur to allow free diffusion of O_2 through the protein matrix. However, because there are globular proteins with tightly packed rigid cores, regions that appear to behave more like crystalline or glassy matrices rather than fluids, the conclusion has not been generally accepted. It was argued that there may be intrinsic limitations in the method for detecting markedly hindered diffusion of solutes and/or that the interpretation of fluorescence quenching data be inadequate. Even after careful elimination of potential artifacts deriving from inter-tryptophan energy transfer toward more exposed chromophores as well as relatively large degrees of static quenching by O_2 molecules already inside the protein, the smallest ^Fk_q values reported to date are around $^1/_{10}$ of that found for the free chromophore.[7]

O_2 is also an effective quencher of Trp phosphorescence.[20] It has been pointed out that because the lifetime of the delayed emission is 6−9 orders of magnitude longer, phosphorescence is better suited than fluorescence for monitoring potentially small quenching rate constants and characterize compact rigid cores of the globular structure. All comparisons between ^Fk_q and ^Pk_q obtained for the same Trp residue, however, have yielded discrepant estimates of oxygen diffusion in proteins.[3,5,6] Generally, phosphorescence quenching rates imply more hindered O_2 diffusion, as in the case of W109 of alkaline phosphatase for which internal O_2 migration would be slowed by over a factor of thousand, relative to diffusion in water.[5,6] The causes of this disagreement are not understood. It has been suggested that the indole-O_2 quenching reaction may not require physical contact between the partners and be longer-range for the singlet state than for the triplet state.[5] Thus, fluorescence quenching could be efficient without O_2 penetration of inner rigid domains. Further, at the 0.1−1 M [O_2] needed to quench the fluorescence of inaccessible Trp residues, several O_2 molecules are likely to

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10.1021/ja9915596 CCC: $18.00
Published on Web 08/31/1999

be always inside the protein, already near the chromophore or within its interaction range. On the other hand, according to a dynamical model advanced to account for fluorescence quenching of chromophores in protein interiors, O_2 migration inside the macromolecule is rapid and occurs freely in the nanosecond time scale, whereas crossing of the water−protein interface is generally opposed by a sizable activation barrier.[10,11] The model predicts that ${}^{F}k_q > {}^{P}k_q$ because Trp fluorescence quenching would report on fast internal O_2 diffusion, whereas phosphorescence quenching, which is conducted at μM $[O_2]$, would refer to the slow, O_2 entry step. Still another aspect tied to the much smaller $[O_2]$ needed to quench phosphorescence is accurate control of $[O_2]$ in solution. In these experiments, protein samples are first thoroughly deoxygenated, and subsequently micromolar quantities of O_2 are introduced, usually by equilibrating the solution with an appropriate O_2 partial pressure. At this level, O_2 leakage during sample manipulation may be prohibitively large and hard to detect. Further, O_2 depletion by photoinduced reactions with the triplet state[12] can also alter its concentration during phosphorescence measurements. It is now agreed that poor $[O_2]$ control has been the main reason for discrepant ${}^{P}k_q$ values among laboratories in the past.[5,13] Also, because of this uncertainty the difference between ${}^{F}k_q$ and ${}^{P}k_q$ has not received due consideration.[3]

Interpretation of k_q in terms of D_{O2} inside proteins is based on the assumption that the reaction is under diffusion control. However, there are inefficient reactions, or reactions that become inefficient when reactants are separated by an impermeable spacer, for which several collisions are required before the reaction takes place. Eventually a condition is attained where k_q is insensitive to the matrix viscosity. Such regime is known as the rapid diffusion limit[14] and is believed to apply to phosphorescence quenching by various solutes.[15] Should this regime apply to O_2 quenching of protein phosphorescence ${}^{P}k_q$ would provide an underestimate of D_{O2} and would also be largely unaffected by changes in protein flexibility.

Recently, a study on acrylamide quenching of Trp phosphorescence in proteins[7] showed that in the long, millisecond−second time scale of the delayed emission acrylamide has access to innermost regions of globular proteins and that the magnitude of k_q correlates with the flexibility of the macromolecule. In a subsequent investigation[16] k_q(acrylamide) served to demonstrate that applied pressure of a few kbar exerts a characteristic modulation of conformational dynamics. In this work a new method is introduced for practical and accurate $[O_2]$ control and, oxygen quenching of phosphorescence is examined in three proteins as a function of temperature and applied pressure. By comparing ${}^{P}k_q$ to other independent indicators of structural flexibility (${}^{P}k_q$(acrylamide) and the intrinsic phosphorescence lifetime, τ_0) of the same region of the macromolecule, we inquire on whether there exists a correlation between ${}^{P}k_q$ and the internal protein viscosity.

Protein systems and experimental conditions of temperature and pressure were carefully selected to satisfy the following criteria: (1) Full stability with respect to possible collateral effects of pressurization such as partial unfolding, release of metal ions or prosthetic groups, and subunit dissociation. (2) Phosphorescence emission from a single and structurally identified Trp residue per subunit[19] exhibiting a lifetime at least 5-fold larger than the 5 ms detection limit of the high-pressure apparatus. 3) Protein systems providing varying degrees of Trp burial within the globular structure in order to compare k_q between superficial regions and deep cores of the macromolecule. The proteins chosen are monomeric copper free azurin (AZ) and dimeric alkaline phosphatase (AP) and alcohol dehydrogenase (LADH). The latter are known not to dissociate under the conditions of this study.[17] The temperature range of pressure studies is 10−50 °C. Above 50 °C AZ is thermally unstable whereas below 10 °C, both AZ and LADH undergo partial unfolding at 3 kbar. The results largely confirm the small magnitude of ${}^{P}k_q$ reported earlier and, more importantly, establish a strong, direct correlation between k_q and the fluidity of the protein region embedding the chromophore.

Materials and Methods

Horse liver alcohol dehydrogenase was supplied by Boheringer (Mannheim, Germany). Alkaline phosphatase from *Escherichia coli* was obtained from Sigma. Copper-free azurin from *Pseudomonas aeruginosa* was a gift of Professor Finazzi-Agrò, University of Roma (Tor Vergata, Italy). Water, doubly distilled over quartz, was purified by a Milli-Q Plus system (Millipore Corporation, Bedford, MA). All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl suprapur (Merck, Darmstadt).

Luminescence Measurements. Fluorescence and phosphorescence spectra, intensities and phosphorescence decay kinetics were obtained with a homemade apparatus described before.[18,19] Briefly, continuous excitation for fluorescence and phosphorescence spectra was provided by Cermax xenon lamp (LX 150 UV, ILC), and the excitation wavelength, typically 290 nm, was selected by a 0.25 m grating monochromator (model 82−410, Jarrel-Ash) with a 10 nm band-pass. The emission collected through another 0.25 m grating monochromator (Jobin-Yvon, H25) with 7 nm bandwidth was detected by an EMI9635QB photomultiplier. Phosphorescence decays were obtained with pulsed excitation as provided by a frequency-doubled flash-pumped dye laser (UV500M Candela) tuned at 292 nm. The pulse duration was 1 μs and the light energy per pulse was typically 1−10 mJ. The phosphorescence signal collected at a right angle from the excitation beam was filtered (420−460 nm band-pass) and detected by an R928 photomultiplier. An electronic shutter arrangement protected the photomultiplier from the intense fluorescence pulse and permitted the delayed emission to be detected 4 ms after the excitation pulse high pressure apparatus. Alternatively, for lifetimes shorter than 5 ms, the photomultiplier was protected from the intense fluorescence pulse by a chopper blade that closes the emission slit during the excitation. The time resolution of this apparatus is typically 10 μs.[19] 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 was typically better than 5%.

Luminescence measurements under pressure were carried out by placing the sample cuvette in a pressure cell (ISS-NOVA, ISS Inc.) provided with sapphire windows and employing highly pure ethanol

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as pressurizing fluid. Details of the sample cuvette and procedure to avoid O_2 inlet during pressure cycles have been reported before.[18] The reversibility of phosphorescence lifetimes was checked at the end of each pressure cycle. Particular care was taken to ensure temperature equilibration of the sample after each pressure variation, which required at least 5 min.

Sample Preparation for Phosphorescence Measurements. Prior to phosphorescence measurements all proteins were extensively dialyzed in Tris-HCl (10 mM, pH 7.5) whose pH is one of the least sensitive to pressure. For measurements of the intrinsic phosphorescence lifetime it is paramount to rid the solution of all O_2 traces. Deoxygenation of protein samples was carried out by repeated cycles of mild evacuation followed by inlet of pure nitrogen as described before.[5] O_2 quenching experiments with AP, the protein that also served as internal reference for experiments with AZ and LADH, were carried out by equilibrating about 1 mL of the protein solution for 20 min with air or with known partial pressure of O_2, at every temperature examined. Partial pressures were determined from the overhead pressure (digital pressure meter 04713 Officine Galileo, Florence, Italy) and the composition of appositely prepared O_2/N_2 gas mixtures (SIO, Florence, Italy). Final concentrations of O_2 at each temperature were calculated using Henry's law and the solubility of O_2 in water at that temperature (Handbook of Chemistry and Physics, 1959). In O_2 quenching experiments with samples of AZ and LADH, containing about 20% AP (on a chromophore basis), various O_2 contents were simply attained by interrupting the initial de-oxygenation of the solution at various stages before completion. The bimolecular quenching rate constant (k_q) was obtained from measurements of the phosphorescence decay at various [O_2] according to the equation

$$1/\tau = (1/\tau_0) + k_q[O_2] \quad (1)$$

where τ_0 and τ are the phosphorescence lifetime in the absence and in the presence of a given [O_2], respectively. In the case of AZ and LADH, [O_2] was determined from its effect on the phosphorescence lifetime of AP in the same solution (see below). For the pressure dependence of k_q partially degassed solutions of individual proteins were transferred to the high-pressure cuvette inside a N_2 box. For each [O_2], τ was measured as a function of pressure up to 3 kbar. Stern−Volmer plots were then constructed at various pressures from parallel determinations of τ_0 and knowledge of [O_2], whose value at 1 atm was corrected for the small (8% at 3 kbar) volume contraction at higher pressure. At least 5 different [O_2] were employed in the construction of the Stern−Volmer plots. Analogous results on the pressure dependence of k_q were obtained at a single [O_2] from the relationship

$$k_q(p)/k_q(1\ \text{atm}) = \{[(1/\tau - 1/\tau_0)(p)]/[(1/\tau - 1/\tau_0)(1\ \text{atm})]\} \times [O_2](1\ \text{atm})/[O_2](p)$$

Determination of O_2 Quenching Rate Constants Utilizing AP Phosphorescence as an Internal Monitor of [O_2] in Solution. Here, a method is introduced for the simultaneous determination of both τ and [O_2] in the same excitation pulse by adding to the sample a reference protein whose k_q is already known. For a wide applicability of the method and an accurate determination of [O_2] the ideal reference protein should be stable over a broad range of experimental conditions, exhibit a phosphorescence lifetime that is quite distinct from that of most proteins and, finally, its k_q should be known to a great accuracy. These requisites are met satisfactorily by alkaline phosphatase from *E. coli*. AP is stable up to 90 °C and over a wide pH range. It is even impervious to 8 M urea and up to 5 M guanidinium chloride (unpublished results). Among the proteins whose phosphorescence in buffer at ambient temperature has been reported, it exhibits one of the longest intrinsic lifetime ($\tau_0 \approx 2$ s at 20 °C) and the smallest O_2 quenching rate constant. An interesting consequence of these features is that AP phosphorescence can be readily measured even in air saturated solutions ($\tau = 3.15$ ms at 20 °C), a convenient standard state for which [O_2] is known accurately. Further, because AP phosphorescence is considerably longer-lived than that of most proteins the phosphorescence decay from binary mixtures with other proteins will be characterized by quite distinct lifetimes a condition that permits precise evaluation of the individual components.

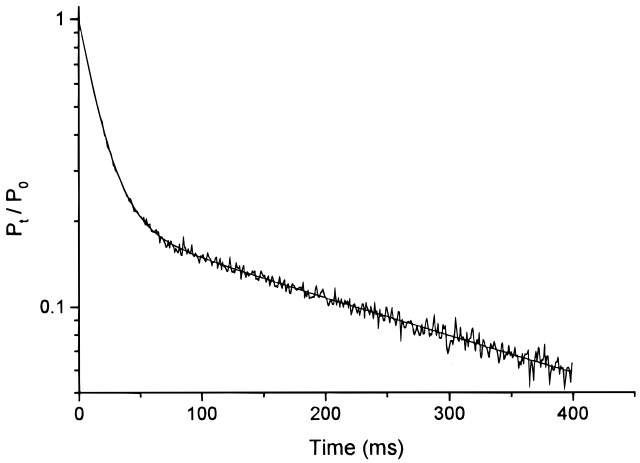

Figure 1. Trp phosphorescence decay from a sample containing 0.2 μM AP and 0.9 μM LADH in 10 mM Tris-HCl buffer, pH 7.5. The oxygen concentration is 2.2 μM, and the temperature is 20 °C. The data are from a single excitation pulse.

An example of raw data obtained with a solution of AP (0.2 μM) and LADH (0.9 μM) in the presence of 2.2 μM O_2 is given in Figure 1. The data are adequately fitted in terms of two exponential components yielding τ (AP) = 321 ms and τ (LADH) = 16 ms. Practically identical fitting parameters are obtained by an alternative two-steps fitting procedure in which first τ (AP) is derived from the long tail of the decay and subsequently τ (LADH) is estimated from a double exponential fit of the entire decay with τ (AP) as a fixed parameter.

A major cause of irreproducibility in the determination of O_2 phosphorescence quenching rate constants is the uncertainty in [O_2] in solution. Traditionally, O_2 levels were controlled by equilibrating the sample with a known O_2 partial pressure. Errors in the final O_2 concentration can derive from inaccurate gas composition, pressure measurements, incomplete equilibration and especially from O_2 leakage from the atmosphere during sample manipulation. Further, a certain amount of O_2 is also depleted during phosphorescence measurements, a problem particularly serious at low [O_2] and in viscous solutions.[12,13] O_2 photodepletion, which takes place within the excitation beam volume, results in a progressive increase in τ on repetitive excitation. All these problems are overcome by the reference protein method. An additional advantage of simultaneous τ and [O_2] measurements is that lengthy sample preparation and O_2 equilibration are no longer required, making thus O_2 quenching experiments rapid and straightforward. With the proteins examined in this study, the reproducibility of k_q (O_2) between independent data sets was excellent the error being typically less than 10%. Calhoun et al.[13] have used palladium coproporphyrin phosphorescence as an internal [O_2] monitor. The procedure involves separate excitation at two distinct wavelengths by different light sources. As the light beams coincide neither in time nor in space, uncertainties in [O_2] due to photodepletion or O_2 gradients cannot be avoided by this method. Further, because O_2 quenching of palladium coproporphyrin phosphorescence is less efficient in comparison to protein phosphorescence, the method is precise only at relatively large [O_2].

Results

O_2 Quenching of Protein Phosphorescence. In the presence of O_2, the phosphorescence of AZ, LADH and AP is short-lived, and the decay is strictly exponential even for LADH whose intrinsic emission is notoriously heterogeneous. A uniform decay contrasts with O_2 quenching of fluorescence where lifetime heterogeneity has been found to increase at higher quenching efficiency.[10,11] Throughout, $1/\tau$ increases linearly with [O_2] as should be expected for a dynamic quenching process. Representative lifetime Stern−Volmer plots at 20 °C are shown in Figure 2, and the bimolecular quenching rate constants, $^{P}k_q$,

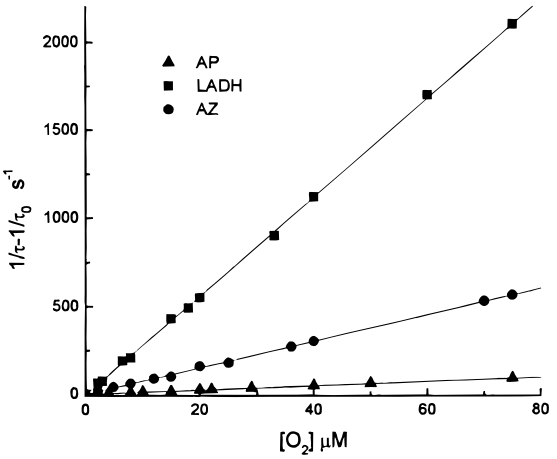

Figure 2. Typical lifetime Stern−Volmer plots for the quenching of protein phosphorescence by O_2. The protein samples are in 10 mM Tris-HCl buffer, pH 7.5, at 20 °C. The variation of the slope among independent sets of experiments is less than 10%.

derived from their slopes are given in Table 1. ${}^{P}k_q$ ranges from 1.2×10^6 M^{-1} s^{-1} for AP to 7.5×10^6 M^{-1} s^{-1} for AZ and 2.8×10^7 M^{-1} s^{-1} for LADH. Compared to previous reports, in which smaller $[O_2]$ ranges were employed, the agreement is good for AP and LADH[5,13] whereas, for AZ, ${}^{P}k_q$ is about 3 times smaller than that found by Calhoun et al.[13] Relative to quenching of free Trp ($k_q = 5 \times 10^9$ M^{-1} s^{-1})[20] the rate for buried Trp residues is 2−3 orders of magnitude smaller, and implies that O_2 diffusion to these internal sites is slowed relative to diffusion in water by at least the same factor.

An independent and direct monitor of the protein "microviscosity" ($\eta_{\tau 0}$) in the region surrounding the chromophore, is provided by the intrinsic phosphorescence lifetime, τ_0, through the empirical relationship linking τ_0 to the solvent viscosity in model indole compounds.[21] Table 1 lists both τ_0 and $\eta_{\tau 0}$, together with degree of burial of the chromophore in terms of the closest distance separating it from the protein−water interface (r_p). From these data we note that the decrease in k_q is directly correlated to the increase in internal protein viscosity which, in turn, is larger as one moves deeper inside the core of the globular fold.

O_2 quenching data with LADH and AZ complexes caution, however, that this correlation between k_q and the rigidity of the globular fold is not so strong as might at first appear. Formation of the LADH−NAD^+−pyrazole ternary complex or binding of Cd^{2+} to apoazurin raises the thermal stability of these proteins.[22,23] At the same time, it reduces acrylamide quenching of their phosphorescence by a factor of 13 and 37, respectively.[7] Although both indications are consistent with a decreased flexibility of the polypeptide, O_2 quenching of these complexes yield relatively unchanged k_q values (Table 1).

The temperature dependence of k_q in the range of 0−50 °C is shown in the Arrhenius plots of Figure 3. Small deviations from straight line behavior are evident with AZ and CdAZ. The slope of the best straight line through the data yield activation enthalpies that are similar among all the proteins, $\Delta H^{\ddagger}$ varying from 9 kcal mol^{-1} for AZ to 12.5 kcal $mole^{-1}$ for LADH. These values of $\Delta H^{\ddagger}$ are at least 3-fold larger than for O_2 diffusion in water (3.1 kcal mol^{-1})[2] but similar to that found for O_2 diffusion in polystyrene (7.1 kcal mol^{-1}) and polycarbonate (9.5 kcal mol^{-1}) plastics.[24]

Figure 3 displays also the effects of temperature on the protein microviscosity in the region surrounding the chromophore, $\eta_{\tau 0}$. Again, between 0 and 50 °C, $\ln(1/\eta_{\tau 0})$ vs $1/T$ plots are essentially linear. The activation enthalpies derived for this parameter are 2−3 times larger than the corresponding $\Delta H^{\ddagger}$ (k_q) implying that O_2 migration/quenching is governed by lower energy/smaller amplitude structural fluctuations than those involved in the relaxation of the excited chromophore.

Pressure Effects on O_2 Quenching of Protein Phosphorescence. Although in the 2−3 kbar range globular proteins generally preserve the native fold, they usually undergo appreciable variations in structural flexibility; an elastic response to pressure elicited by a combined reduction in internal free volume and increased hydration of the polypeptide.[16] The pressure dependence of k_q (up to 3 kbar) for AZ, LADH and AP at 50 °C is reported in Figure 4. In each case O_2 quenching is inhibited by applied pressure, k_q decreasing by at least 2-fold at 3 kbar. The k_q pressure profiles show a progressive increase in rigidity of the protein structure that occurs mostly below 2 kbar. A similar pressure modulation of protein flexibility is confirmed by the concomitant variation of τ_0. As illustrated in Figure 4 the reduced quenchability of these proteins is accompanied by a roughly mirror image increase in their τ_0.

Within the framework of transition state theory, the pressure dependence of k_q may be expressed in terms of an effective activation volume, $\Delta V^{\ddagger}$, $[k_q(p)/k_q(0) = \exp(-\Delta V^{\ddagger}/RT)]$, which, in turn, should represent the activation volume for O_2 diffusion within the protein matrix. At constant temperature, $k_q \propto D \propto 1/\eta$, and therefore the slope of $\ln[k_q(0)/k_q(p)]$ vs pressure plots, yields the activation volume of the protein viscous drag (η_{kq}) on O_2 migration. The logarithmic plots of both $k_q(0)/k_q(p)$ and $\eta_{\tau 0}(p)/\eta_{\tau 0}(0)$ relative to the data of Figure 4 are shown in Figure 5. A salient feature of these plots is their nonlinearity, the decline of the gradient above 2 kbar, implying that $\Delta V^{\ddagger}$ is pressure dependent, a behavior observed also with acrylamide quenching.[16] The largest values of $\Delta V^{\ddagger}$ are obtained for O_2 quenching in the lower pressure range and amount to 9, 16, and 10 mL mol^{-1} for AZ, LADH and AP, respectively. The corresponding values of $\Delta V^{\ddagger}$ (τ_0) rank in the same order but, as in the case of $\Delta H^{\ddagger}$, they are generally larger than $\Delta V^{\ddagger}(k_q)$ (Table 2).

Lowering the temperature to 30 and 10 °C causes a progressive loss in segmental flexibility of these polypeptides (as inferred from their τ_0 and k_q) as well as an appreciable attenuation of pressure effects. The pressure profiles of both $\ln[k_q(0)/k_q(p)]$ and $\ln[\eta_{\tau 0}(p)/\eta_{\tau 0}(0)]$ at 10 and 30 °C are compared to those at 50 °C in Figure 5. The general trend emerging from $\eta_{\tau 0}$ is that at lower temperature pressure is less effective at tightening the protein structure. In agreement with smaller pressure variations of $\eta_{\tau 0}$, $\Delta V^{\ddagger}$ (k_q) decreases monotonically upon lowering the temperature (Table 2). For AZ, $\Delta V^{\ddagger}$ (k_q) is actually negative at 30 °C and becomes more so at 10 °C, a behavior found also with acrylamide quenching.[16]

Discussion

Accurate determination of O_2 quenching rate constants over an unprecedentedly large range of $[O_2]$ confirm that for buried Trp residues k_q can be over 1000-fold smaller than for residues that are on the protein surface, exposed to the solvent. Larger

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Table 1. Oxygen Bimolecular Phosphorescence Quenching Rate Constant for NATA and for Internal Trp Residues in Proteins, at 20 °C in Tris-HCl 10 mM, pH 7.5

sample	Trp[a]	r_p (Å)[b]	$\bar{\tau}_0$ (ms)[c]	$\eta(\tau_0)$ (cP)	$^{P}k_q$ ($M^{-1}\,s^{-1}$)	$^{F}k_q$ ($M^{-1}\,s^{-1}$)	$^{F}k_q/^{P}k_q$
NATA[d]			1		5.0×10^9	12.3×10^9 (ref 2)	
LADH	314	4.5	628	4.9×10^4	2.8×10^7	0.6×10^9 (ref 3)	21
LADH−NAD^+−pyrazole	314	4.5	1020	1.6×10^5	3.0×10^7		
AZ	48	8.0	600	4.6×10^4	7.5×10^6	0.6×10^9 (ref 8)	80
Cd-AZ	48	8.0	540	3.6×10^4	1.0×10^7		
AP	109	11.0	2060	7.5×10^5	1.2×10^6	1.0×10^9 (ref 3)	880

[a] See ref 19. [b] r_p is the smallest thickness of the protein spacer separating the indole ring from the aqueous phase as determined from the X-ray structures obtained from the Brookhaven Protein Data Bank. [c] The intrinsic phosphorescence decay of LADH is heterogeneous and requires two exponential components with lifetimes $\tau_1 = 260$ ms and $\tau_2 = 702$ ms to adequately fit the data. $\bar{\tau}_0 = \alpha_1\tau_1 + \alpha_2\tau_2$ represents the average lifetime. [d] *N*-acetyl-tryptophanamide.

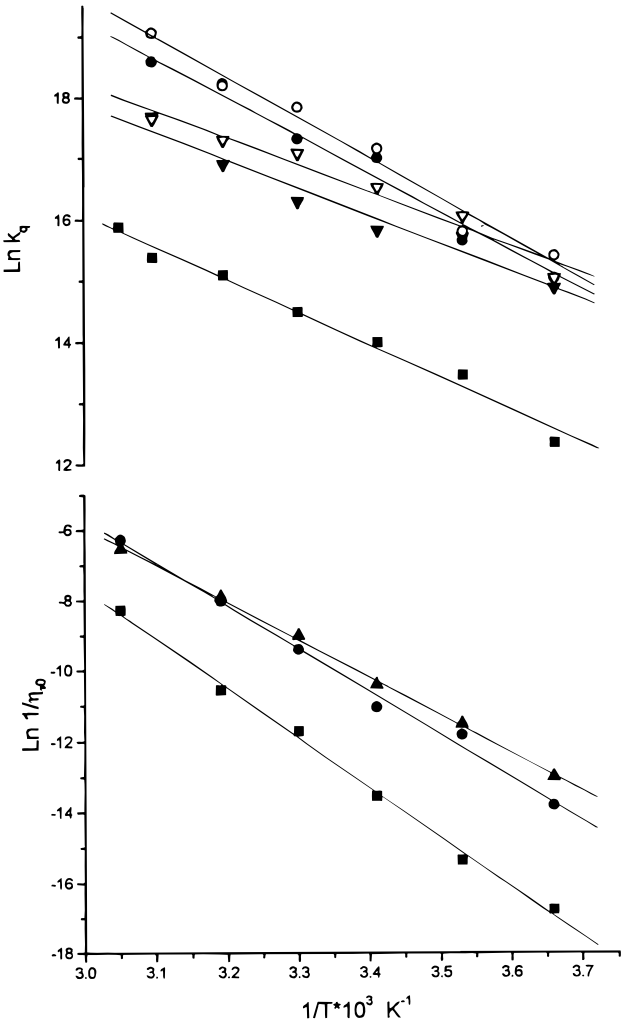

Figure 3. Arrhenius plots of the O_2 quenching rate constant (k_q) and of $1/\eta_{\tau 0}$, obtained at atmospheric pressure over the 0−50 °C temperature interval. The proteins samples are: (■) AP, (●) LADH, (○) LADH−NAD^+−pyrazole, (▼) Az, and (▽) CdAZ.

k_q values, reported in the past for these proteins,[26,25] are clearly imputable to inadequate $[O_2]$ control, a task that, in the sub μM concentration range used in phosphorescence studies, is arduous without a reliable internal O_2 monitor.

***k_q* and O_2 Diffusion through the Protein Matrix.** Quenching of protein luminescence by solutes in solutions is generally depicted as a two step process involving first the diffusion of the quencher to the chromophore, followed by the quenching reaction at a rate that depends, among other factors, on the distance of closest approach between reactants. For reactions involving electron exchange, electron transfer, or energy transfer, physical contact between reactants is not needed, and quenching can be efficient over separations as large as 10−50 Å. In these cases the overall bimolecular reaction need not to be limited by the penetration of quenchers through rigid chromophore's sites and k_q could be larger than would be anticipated from the protein permeability.

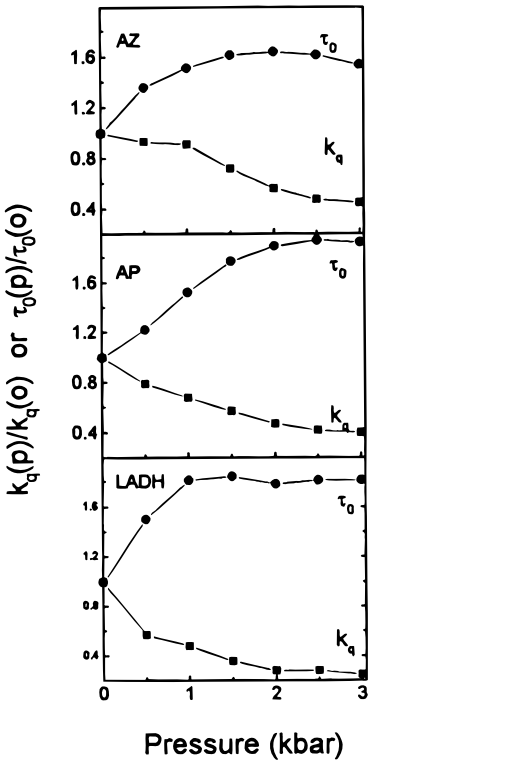

Figure 4. Pressure dependence of k_q(■) and of τ_0 (●), normalized for their values at 1 bar, at 50 °C.

The present study has established that globular proteins may oppose a considerable barrier to the reaction between O_2 and the triplet state of Trp, k_q decreasing monotonically with both the degree of burial of the chromophore, r_p, and the rigidity of the embedding protein structure ($\eta_{\tau 0}$) (Table 1). Reduced k_q's could in principle result from either hindered O_2 migration through the protein or from inefficient reaction rates, owing to relatively large quencher−chromophore separations with inaccessible cores, or a mixture of both. However, because large variations of k_q among proteins, and upon changing external conditions of temperature and pressure, are directly correlated to the flexibility of the globular fold it appears that long-range, through-space quenching makes little or no contribution to the overall rate. Significant in this respect is the 2−3-fold reduction of k_q as pressure is raised to 3 kbar. Quenching of LADH, AZ,

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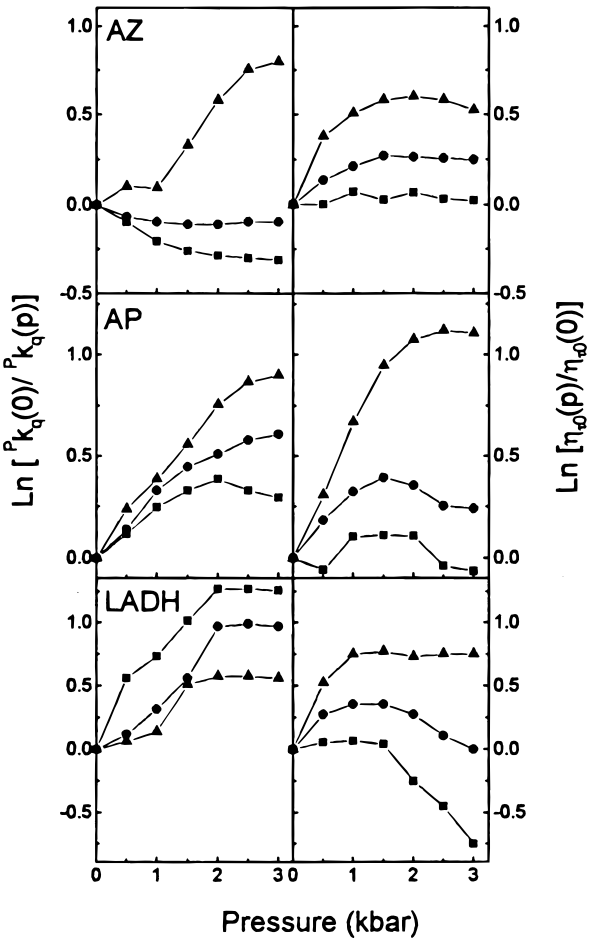

Figure 5. Logarithmic plots of pressure dependence of $1/k_q$ and of $\eta_{\tau 0}$ at 10 (■), 30 (●), and 50 (▲) °C. $\eta_{\tau 0}$ is calculated from τ_0 using its dependence on solvent viscosity (21).

Table 2. Activation Enthalpy ($\Delta H^{\ddagger}$) and Free Volume ($\Delta V^{\ddagger}$) Relative to the O_2 Quenching Rate Constant (k_q) and the Internal Microviscosity ($\eta_{\tau 0}$) Inferred from τ_0

protein	T (°C)	τ_0 (ms)	$\Delta H^{\ddagger}(k_q)$ (kcal)	$\Delta H^{\ddagger}(\tau_0)$ (kcal)	$\Delta V^{\ddagger}_{kq(O_2)}$ (mL)	$\Delta V^{\ddagger}_{\eta 0}$ (mL)
AZ	10	1100	9.1 ± 0.9	24 ± 2.1	−3.5 ± 0.3	2 ± 0.5
	30	209			−1.2 ± 0.2	5 ± 1.0
	50	39			9.0 ± 0.6	13 ± 0.7
AP	10	2843	10.3 ± 1.2	32 ± 3.1	4.6 ± 0.5	2 ± 0.5
	30	981			6.7 ± 0.5	8 ± 0.9
	50	161			9.8 ± 0.8	18 ± 1.1
LADH	10	1150	12.5 ± 1.5	26 ± 2.9	7.4 ± 0.6	2 ± 1.0
	30	270			11.9 ± 0.9	9 ± 0.8
	50	32			16.0 ± 1.1	20 ± 1.2

and AP phosphorescence by nitrite, a charged quencher relegated to the aqueous phase which therefore acts exclusively through-space, showed that the rate is not affected by pressure, an indication that r_p remains constant under these conditions.[16] Both τ_0 and acrylamide quenching rates,[16] on the other hand, reveal pressure modulations of the protein flexibility which are followed by a roughly parallel change in k_q. This strong correlation between k_q and conformational dynamics has two main implications: (1) The interaction between O_2 and the indole triplet state must be rather short-range as, even for LADH with an $r_p = 4.5$ Å, through-space interactions do not seem to compete with quenching by O_2 migration. (2) For LADH, AZ, and AP, O_2 quenching is under diffusion control and, therefore, the decrease in k_q relative to that of solvent exposed residues ($\sim 5 \times 10^9$ M^{-1} s^{-1}) provides a measure of the average diffusion coefficient (weighted in favor of low friction pathways), D_{O2}, inside these macromolecules. Hence, relative to water, D_{O2} is reduced by about 200 fold in LADH, 660 fold in AZ and 4200 fold in AP. By way of comparison, the factor is 100 and 300 for O_2 diffusion in polystyrene and polycarbonate plastics, respectively.[24]

Disparity in k_q between Fluorescence and Phosphorescence Quenching. It has been generally assumed that O_2 quenching of Trp fluorescence in proteins involve a direct collision or a very close encounter with the chromophore and that it be limited by D_{O2} in the protein interior. Should both $^{P}k_q$ and $^{F}k_q$ be determined by the slow diffusion of O_2 to the chromophore site, then for any Trp the ratio $^{F}k_q/{}^{P}k_q$ would be expected to be constant and roughly equal to 2, the spin statistical factor.[27] However, for W48 of AZ, W314 of LADH, and W109 of AP, the ratio $^{F}k_q/{}^{P}k_q$, even by considering the smallest estimates of $^{F}k_q$ reported in the literature, increases from about 2 for Trp exposed to the solvent to 21 for LADH, 80 for AZ, and 880 for AP (Table 1). As the chromophore moves deeper inside the macromolecule and the rigidity of the surrounding core increases, the disagreement between fluorescence and phosphorescence quenching becomes larger. Several reasons may account for this discrepancy: (1) With multi-tryptophan proteins, like LADH and AP, the fluorescence emission is composite, and quenching of specific Trp residues can only be estimated with great approximation. (2) More importantly, the model based on collisional quenching may be an oversimplification and a larger value of $^{F}k_q$ might simply reflect a Trp−O_2 interaction radius that is larger for the singlet state than for the triplet state. Recent evidence of photoinduced electron transfer in the indole-O_2 cluster in the gas phase lends support to this hypothesis as it suggests that an electron transfer reaction is most likely involved in O_2 quenching of Trp fluorescence and phosphorescence, both in solution and in proteins.[28] And, electron transfer rates are generally much shorter-range for the triplet state than for the singlet state; examples are electron transfer to Cu^{+} and Cu^{2+} [29,30] and to acrylamide.[7] (3) Another cause of discrepancy between $^{P}k_q$ and $^{F}k_q$ may be the vastly different [O_2] employed in fluorescence and phosphorescence studies. Owing to the low polarity of the protein interior and the propensity of O_2 to form weak complexes, the gas is expected to partition favorably inside proteins and, at ~1 M concentrations needed to quench the fluorescence of inaccessible residues ($^{F}k_q < 10^9$ M^{-1} s^{-1}), several molecules will constantly be within the globular fold. If the effective interaction radius with the singlet excited-state extends a few Å beyond van der Waals' contact, the presence of O_2 in proximity of the chromophores will result in large degrees of static or quasi-static quenching, a phenomenon recently recognized with some single Trp proteins.[8] Through-space quenching of chromophores placed in rigid cores would lead to the erroneous conclusion that even these regions are accessible to O_2 in the nanosecond time scale of fluorescence and modest reductions in $^{F}k_q$, due to the distance attenuation of the through-space rate, could instead be exchanged for relatively free O_2 diffusion within the protein matrix. By contrast, in the μM concentration range of phosphorescence quenching, O_2

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Table 3. Comparison of the Internal Protein Viscosity and the Related Activation Parameters, $\Delta H^{\ddagger}$ and $\Delta V^{\ddagger}$, Monitored by the Same Trp Probe According to k_q (O_2), k_q(acryl), and τ_0

	η_{prot} (cP)[a]			$\Delta H^{\ddagger}$ (kcal/mle			$\Delta V^{\ddagger}_{max}$ (mL)[b]		
protein	k_q (O_2)	k_q (acryl)	τ_0	k_q (O_2)	k_q (acryl)	τ_0	k_q (O_2)	k_q (acryl)	τ_0
LADH	2.0×10^2	1.2×10^5	4.9×10^4	12.5	10.5	26	16.0	16	20
AZ	6.7×10^2	4.6×10^7	4.6×10^4	9.1	22.0	24	9.0	10	13
AP	4.0×10^3	1.5×10^{10}	7.5×10^5	10.3	20.3	32	9.8	20	18

[a] Data at 20 °C. η_{prot} is obtained from either $k_{q(solv)}/k_{q(prot)}$ or from the empirical relationship between τ_0 and solvent viscosity in model studies (ref 21). [b] Data at 50 °C.

comes necessarily from the solvent; the overall process involving first its crossing of the water−protein boundary and then its diffusion through the macromolecule. In a dynamic model for quenching of fluorophores in protein interiors, Gratton et al.[10] have proposed that the protein−water interface offers a relatively large barrier to O_2 penetration, whereas its migration within the protein matrix is rapid and substantially unhindered. According to this hypothesis, ${}^{P}k_q$ would represent the entry step and for this reason should be substantially smaller than ${}^{F}k_q$ which, instead, would refer to rapid internal diffusion. This scenario, however, is totally inconsistent with the present phosphorescence results. As stated above, independent internal monitors of protein flexibility (τ_0 and k_q(acrylamide)) emphasize that there is a direct correlation between ${}^{P}k_q$ and the internal protein viscosity, irrespective of whether the latter is varied by changing protein or changing external conditions of temperature and pressure. Furthermore, orders of magnitude variations of ${}^{P}k_q$ among proteins are not contemplated by this model as the nature of the barrier to the penetration of small, neutral molecules, offered by the protein−water interface, is presumably similar in all polypeptides.

Protein Dynamics from Trp Phosphorescence. Flexibility of protein structure is inferred from a number of phenomena, most of which yield an "effective" microviscosity of a particular region of the macromolecule. It may be instructive to contrast the different appraisals of the local viscosity made by the same probe (Trp) that are based on the diffusion of different size quenchers (O_2 and acrylamide) and on τ_0. Table 3 compares the estimates of the protein viscosity about W48 of AZ, W314 of LADH and W109 of AP according to their τ_0 and k_q (assuming for the latter Stokes−Einstein behavior, i.e., $k_q \propto T/\eta_{protein}$). In the same table are also reported the corresponding activation parameters, $\Delta H^{\ddagger}$ and $\Delta V^{\ddagger}$, that characterize the underlying structural fluctuations. It is immediately apparent that the viscous drag for O_2 migration is 3−6.5 orders of magnitude smaller than that encountered by acrylamide, and the difference increases with deeper, more rigid cores. It should be recalled that even in simple fluids O_2 diffusion is rather insensitive to the medium viscosity, as evidenced by marked deviations from Stokes−Einstein behavior.[31,32] Clearly, the different appraisal of the internal protein viscosity by the two quenchers is imputable to the larger amplitude fluctuations that are required for the migration of bulkier acrylamide. This is also implicit in the greater activation barrier ($\Delta H^{\ddagger}$) to acrylamide diffusion, as it indicates that more intramolecular bonds have to be broken simultaneously in the diffusion process. An exception to this pattern is LADH for which $\Delta H^{\ddagger}(k_q(O_2)) \approx \Delta H^{\ddagger}$ (k_q(acryl)), and the reason for it may be found in the peculiar location of W314. The chromophore lies at the subunit-subunit interface and, therefore, transient breaks of subunit contacts will allow relatively easy access even to bulky solutes. Another distinction between O_2 and acrylamide quenching is the vastly different sensitivity to the flexibility of the globular fold. In fact, the variation of k_q between W109 of AP, which is one the least accessible residues presently known, and Trp residues exposed to the solvent, is roughly 3 orders of magnitude for O_2 and 10 orders for acrylamide. The relative insensitivity of O_2 quenching probably accounts for the negligible change in $k_q(O_2)$ observed upon formation of the LADH−NAD^+−pyrazole ternary complex and of Cd^{2+} binding to apoazurin, events that result in a substantial decrease of k_q(acryl).

The intrinsic lifetime provides a picture of structural fluidity in the immediate environment of the indole ring, and the magnitude of $\eta(\tau_0)$ is seen to be intermediate to that derived from O_2 and from acrylamide quenching constants. It refers to motions that are effective in deactivating the excited triplet state but until now no indication of their amplitude spectrum is available. Judging simply from the relative magnitude of $\eta(\tau_0)$ and of $\Delta H^{\ddagger}(\tau_0)$, these motions appear of more limited amplitude than those involved in acrylamide migration but can have comparable activation barriers. By comparison, indole ring flipping motions of W314 in LADH have much larger activation barriers and at ambient temperature are completely blocked.[33]

An unforeseen result of the present study is the rather indiscriminate influence of applied pressure in modulating small and large amplitude structural fluctuations. Intuitively, given the tight packing and compactness of these protein cores, migration of solutes should require transient formation of suitable, molecular size cavities and consequently the activation volume ($\Delta V^{\ddagger}$) of the process should be proportional to the bulkiness of the solute. However, the $\Delta V^{\ddagger}$ measured for $k_q(O_2)$ and k_q(acryl) are surprisingly similar. As summarized in Table 3, the maximum $\Delta V^{\ddagger}$ measured at 50 °C ranges from 9 to 16 mL mol^{-1} for $k_q(O_2)$ and 10 to 20 mL mol^{-1} for k_q(acryl), and neither the actual magnitudes nor the differences are commensurate with the size of O_2 and acrylamide. An analogous range of $\Delta V^{\ddagger}$, 13−20 mL mol^{-1}, is observed also for the variation in the intrinsic lifetime. This similarity among activation volumes is maintained also in the pressure profiles at 30 and 10 °C (Figure 5) where $\Delta V^{\ddagger}$ is smaller or even negative. Recently, to account for the small magnitude of $\Delta V^{\ddagger}$ for acrylamide quenching and its pressure−temperature dependence it has been proposed that the observed $\Delta V^{\ddagger}$ is the balance between a positive contribution due to cavity expansion ($\Delta V^{\ddagger}_{cavities}$) and a negative contribution arising from internal hydration of the polypeptide ($\Delta V_{hydration}$).[16] It was also concluded that transient, internal hydration of the polypeptide must play an important role on conformational dynamics and that it may even be the dominant factor at low temperature and at high pressure; a deduction confirmed also by molecular dynamics simulations of solvated proteins at high pressure.[34] The present findings on O_2 quenching extend this conclusion even to

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segmental motions of relatively small amplitude and reinforce the concept of water as plasticizer of protein structure.[35]

In conclusion, quenching of protein phosphorescence confirms that O_2 migration through well structured cores of globular proteins can be slowed by over 1000 fold relative to diffusion in H_2O, a result in net disagreement with estimates based on fluorescence quenching. In comparison to acrylamide, however, O_2 diffuses readily through these macromolecules and its rate represents a far less sensitive monitor of conformational dynamics than k_q(acryl). As a monitor of protein structure, O_2 quenching should therefore be preferred only for studying the dynamics of rigid matrices such as those of proteins in low temperature viscous solvents or of partially hydrated powders. The ease and accuracy of $k_q(O_2)$ determinations by the use of an internal reference protein should make these measurements applicable to any protein and to a variety of external conditions.

JA9915596

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