QUENCHING OF ALKALINE PHOSPHATASE PHOSPHORESCENCE BY O_2 AND NO

Evidence for Inflexible Regions of Protein Structure

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ABSTRACT The rate constant for quenching the phosphorescence of alkaline phosphatase by molecular oxygen was measured as a function of temperature. The results disagree with previous determinations and, contrary to fluorescence quenching, show that diffusion of O_2 to this region of the macromolecule is a highly hindered process. When nitric oxide is introduced as a quencher, similarly small rate constants were found. While the activation energy for this process is identical for both quenchers, it is much smaller than for structural fluctuations at the chromophore site as manifested by the intrinsic triplet-state lifetime. These findings are analyzed in terms of a mechanism that takes into account static quenching at large distances and does not require penetration of the quencher all the way to the chromophore.

INTRODUCTION

In recent years, a number of experimental approaches have addressed themselves to the question of the dynamical makeup of protein structures and its relationship to function. In this effort, the quenching of protein luminescence by small molecules has attracted considerable interest because of the possibility that they may penetrate and move about within proteins and thus shed light on the conformational flexibility of the protein.

The ability of molecular oxygen to quench the fluorescence of even deeply buried tryptophan residues has led to the conclusion that fluctuations on a nanosecond time scale are ubiquitous to protein structures (Lakowicz and Weber, 1973*a*,*b*; Calhoun et al., 1983; Hagaman and Eftink, 1984). Such a picture of rather uniform and flexible protein structures is not shared by the outcome of other methods of investigation. In particular, the evidence accumulated with H-exchange studies demonstrates that the rate at which various regions of the macromolecule come into contact with water may vary by several orders of magnitude (Gregory and Lumry, 1985). Interpretation of fluorescence quenching by O_2 and other small molecules may suffer from the relative insensitivity of the method. Owing to the short-lived fluorescence state of tryptophan, large concentrations of quenchers are needed to compete against the emission process. These difficulties are overcome with phosphorescence quenching as the triplet-state lifetime is five to eight orders of magnitude longer (Saviotti and Galley, 1974). Furthermore, as room-temperature phosphorescence is only detected from those tryptophan residues embedded in particularly rigid microenvironments (Strambini and Gonnelli, 1985), phosphorescence quenching should report selectively on conformational fluctuations of these compact regions of the macromolecule.

Alkaline phosphatase (AP) has the longest room-temperature phosphorescence lifetime detected to date, a remarkable thermal stability (no sulfur bridges present), and an imperviousness to urea (unpublished data), all attributes of stable compact tertiary structures. On these grounds, the macromolecule should be impermeable to quenchers. It is remarkable that, although diffusion by a number of small molecules is highly restricted, evidence has been presented that oxygen quenches both the singlet and triplet states of the internal tryptophan with a rate constant ($kq = 10^9\ M^{-1}\ s^{-1}$) not significantly different from that of the other solvent exposed residues (Calhoun et al., 1983). For this singular behavior, O_2 was deemed a peculiar quencher that, perhaps because of its small size and nonpolar nature, has unique permeability properties.

Contrary to fluorescence, phosphorescence quenching by O_2 is prone to misinterpretation because of the small concentration of O_2 required and the absence of analytical methods to control it. Relatively large errors may arise from less than perfect degassing, the method of its introduction, and photodepletion (Strambini, 1983). Thus, independent confirmation of its peculiar diffusion behavior by another quencher with similar molecular characteristics would be invaluable.

A neutral quencher the size of O_2 and with comparable quenching properties in model studies in solution is nitric oxide (Birks, 1970). The present work deals with the introduction of NO as a quencher of the room-temperature phosphorescence of AP. NO, as opposed to O_2, was found

not to undergo photodepletion and to quench the phosphorescence at a rate constant very close to that of oxygen. In disagreement with previous reports, such rate constants are the smallest ever found in proteins.

MATERIALS AND METHODS

Alkaline phosphatase (type III) from *Escherichia coli* was obtained as a suspension in 2.5 M ammonium sulfate from Sigma Chemical Co. (St. Louis, MO). Horse liver alcohol dehydrogenase (LADH) was the crystalline suspension supplied by Boehringer Mannheim Diagnostics, Inc. (Houston, TX). Both enzymes were dialyzed for at least 24 h against 0.03 M pyrophosphate buffer, pH 8.6. Fresh preparations were made weekly and no loss of activity was found during that time. Enzyme activities were assayed according to the method of Garen and Levinthal (1960) for AP and the method of Dalziel (1962) for LADH. In both cases, the activity agrees with the maximum value given for these preparations.

Sample Preparation

In both absorption and emission studies, the final concentration of AP and LADH in 0.03 M pyrophosphate buffer, pH 8.6, was typically 10^{-5} M. For satisfactory deoxygenation of the sample before phosphorescence measurements or the introduction of a given quantity of O_2 or NO, the following procedure was adopted. About 0.5 ml of protein solution was placed in a L-shaped quartz cell made of a thick short arm containing a small stirrer in which gas exchange and equilibration was to take place. Afterwards, the sample was transferred to the thin arm (4-mm inner diam) for emission measurements. The short arm was provided with a vacuum-tight steel cap by means of which the cell could be connected to a steel vacuum line for gas exchange and be removed without danger of air leakage (Swagelock patent D-316). Complete removal of O_2 from the protein solution was obtained in ~10 min by repeated application of a moderate vacuum followed by the inlet of very pure nitrogen (0.1 ppm in O_2, SIO, Florence, Italy) at a pressure of 3 atm and gentle stirring. A check on the thoroughness of deoxygenation was provided by the dependence of phosphorescence lifetimes on the amount of excitation absorbed by the sample (Strambini, 1983). At 25°C, lifetimes of 0.4 and 1.57 s for LADH and AP, respectively, compare favorably with the values of 0.25 and 1.4 s reported previously (Saviotti and Galley, 1974; Calhoun et al., 1983).

Various O_2 and NO concentrations at a given temperature were introduced by equilibrating the thermostated solution for ~15 min with known partial pressures of O_2 and NO. Partial pressures were determined from the overhead pressure (digital pressure meter models OG 713 and OG 973; Officine Galileo, Florence, Italy) and the composition of appositely prepared mixtures of these gases with N_2 (SIO). The same results were obtained by either varying the overall pressure of a given gas mixture or maintaining the pressure at a constant level and changing the mixture composition. Final concentrations of O_2 and NO at each temperature were calculated using Henry's law and the solubility of these gases in water at that temperature (Handbook of Chemistry and Physics, 1959). Measurements of enzyme activity before degassing and after phosphorescence measurements showed no deterioration of the sample.

Spectroscopic Measurements

Fluorescence and phosphorescence spectra were obtained with a conventionally designed instrument (Strambini, 1983). The excitation centered at 297 nm was selected by a 250-mm grating monochromator (Jarrel-Ash) employing a band pass of 2 nm for fluorescence and 10 nm for phosphorescence. The emission was dispersed by a 250-mm grating monochromator (model H25; Jobin-Yvon) and detected with a photomultiplier (model 9635 QB; EMI). Phosphorescence decays were monitored at 440 nm by a double shutter arrangement permitting the emission to be detected 2 ms after the excitation cut off. The decaying signal was stored, and upon occurrence averaged in a time averaging computer (model C-1024; Varian Associates, Inc., Palo Alto, CA) and successively transferred to an Apple II computer for data analysis. For the weakest intensities, 20–30 decays were sufficient to give a good signal-to-noise ratio. Throughout these experiments, the phosphorescence decay is well represented by a single exponential function and, since the signal-to-noise ratio is good, the lifetime was obtained directly from the slope of a straight line drawn through a semilogarithmic plot of the data. Adopting this procedure, the error between successive lifetime determinations in the same sample is typically of 1–2%. The bimolecular quenching rate constant was obtained from measurements of the decay of phosphorescence according to the equation $\tau_0/\tau = 1 + \tau_0 kq\,[Q]$, where τ_0 and τ are the phosphorescence lifetimes in the absence and in the presence of a given quencher at concentration [Q], respectively.

Circular dichroism measurements were carried out in a recording spectropolarimeter (model J-500A; Jasco, Inc., Easton, MD) employing a cell of 1-mm path length.

RESULTS

The rate constant for the phosphorescence quenching of alkaline phosphatase by O_2 was measured in buffer as a function of temperature. The data from a typical experiment reporting the decay of the phosphorescence of this protein in the deoxygenated preparation and after addition of O_2 are shown in Fig. 1. The emission decays with time in an exponential fashion and in O_2-free solution has a lifetime of 1.57 ± 0.02 s at 25°C. This value is somewhat larger than 1.4 s reported previously (Saviotti and Galley, 1974; Calhoun et al., 1983) and may reflect a more thorough removal of O_2 from the solution. The dependence of the triplet-state lifetime on the O_2 concentration is given in Fig. 2. The rate of quenching in different runs was observed to be quite reproducible. It obeys a strictly linear relationship on the concentration of O_2 over a wide range of concentrations and temperatures. At 25°C, the biomolecular rate constant, $kq(T)$, was found to be $1.2(\pm 0.1) \times 10^6$ $M^{-1}\,s^{-1}$. This is by far the smallest value published in the literature for O_2 diffusion in any protein and contrasts with

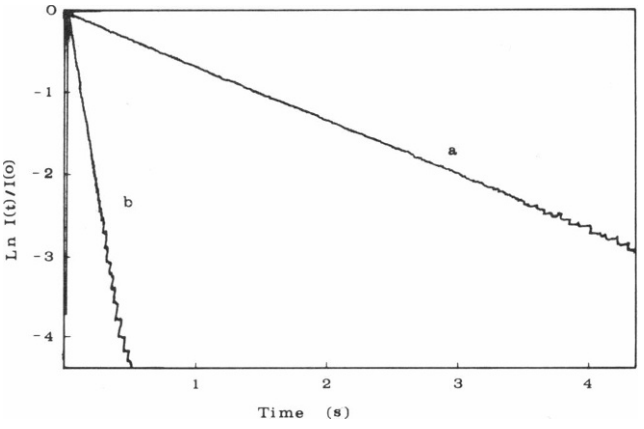

FIGURE 1 Decay of the phosphorescence intensity at 440 nm with time beginning 2 ms after cut off of the excitation. The sample is 10^{-5} M alkaline phosphatase in 30 mM pyrophosphate buffer, pH 8.6 at 25°C. (*a*) Oxygen free sample, single sweep. (*b*) O_2 = 7.8 μM. The trace is the average of 20 sweeps.

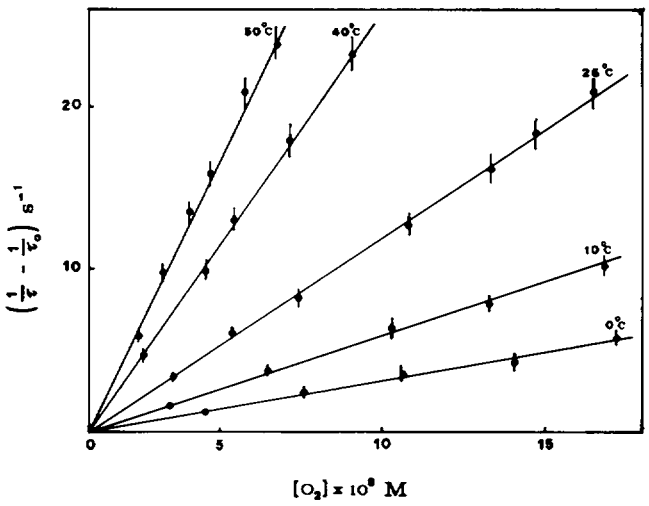

FIGURE 2 Oxygen quenching of alkaline phosphatase phosphorescence in 30 mM pyrophosphate buffer, pH 8.6, as a function of temperature. Error bars, the range in the values obtained with three independent experiments.

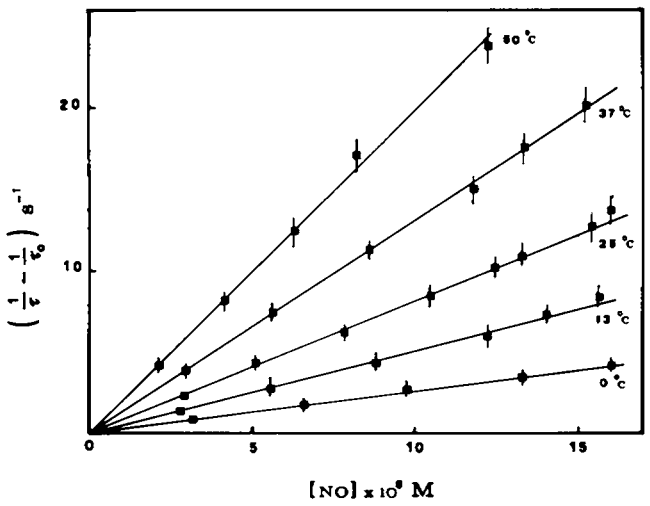

FIGURE 3 Nitric oxide quenching of alkaline phosphatase phosphorescence in 30 mM pyrophosphate, pH 8.6, as a function of temperature. Error bars, the range in the values obtained with three independent experiments.

the value of $\sim 10^9$ M^{-1} s^{-1} reported previously (Calhoun et al., 1983).

When parallel O_2 quenching experiments were carried out with LADH, the other protein studied in that publication, $kq(T)$ was found to be 3.6×10^7 M^{-1}, s^{-1}, again much smaller than the reported value of 6×10^8 M^{-1} s^{-1}. A smaller value of $kq(T)$, 1.4×10^8 M^{-1} s^{-1}, was observed for this enzyme by Barboy and Feitelson (1985), although a direct comparison may not be justified owing to the presence of 25% glycerol in solution. When separate experiments were carried out adopting the procedure of introducing O_2 described by Calhoun et al. (1983), the results were not reproducible.

The ability of O_2 to quench the emission from internal tryptophan residues might be mimicked by nitric oxide, a neutral quencher of roughly the same size of O_2. NO is reactive towards O_2 and possibly some oxidizing functional groups in proteins. It is imperative, therefore, to free the solution of all O_2 before NO addition, to demonstrate the reversibility of NO quenching, and to check the integrity of the macromolecule. These precautions were taken by the use of spectroscopic controls such as absorption and emission spectra and phosphorescence lifetime and circular dichroism spectra in addition to enzyme activity measurements taken after storage for several hours at the highest concentration of NO used.

The dependence of the triplet-state lifetime of AP on the concentration of NO at various temperatures is given in Fig. 3. The rate of quenching is again strictly linear on NO concentration. Further, as the straight lines extrapolate to the origin at zero concentration, any NO depletion by side reactions must be insignificant. The value of $kq(T)$ is $8.2 \pm 0.2 \times 10^5$ M^{-1} s^{-1} at 25°C, only ~30% less than the corresponding value for O_2. Taking into account the accuracy of solubility data, such a difference may well be of the magnitude of the overall experimental error.

The temperature dependence of $kq(T)$ for O_2 and NO quenching is shown in Fig. 4 to be practically identical with activation energies of 7.36 and 7.95 kcal mol^{-1}, respectively. The activation energy is not sensitive to the polarity of the quencher molecule suggesting that the migration of these small molecules through the protein matrix is determined by structural fluctuations of the macromolecule, which is similar in nature.

DISCUSSION

Alkaline phosphatase is a dimeric enzyme containing three tryptophan residues per subunit. Of these, only Trp-109 is not accessible to the solvent, being enclosed within a highly structured region of the macromolecule formed by α-helics and β-plated sheets (Wyckoff et al., 1983). The earlier observation that only a single residue contributes to the room temperature phosphorescence of AP (Domanus et al., 1979), together with the requirement of a rigid microenvironment in order for tryptophan to phosphoresce at room temperature assigns this emission exclusively to Trp-109.

Oxygen quenches both excited singlet and triplet states of aromatic molecules. For model indole compounds in aqueous solution, the ratio of the corresponding rate constants, $kq(S)/kq(T)$, is ~2 (Lakowicz and Weber, 1973*a*; Bent and Hayon, 1975). The quenching of phosphorescence from Trp-109 in AP by O_2, at a rate $\sim 5 \times 10^3$ times smaller than in water (Bent and Hayon, 1975) can only be interpreted in terms of hindered diffusion through the protein matrix. This conclusion is at odds with the supposed ability of O_2 to quench indiscriminately the fluorescence of this chromophore in proteins. In particular, $kq(S)$

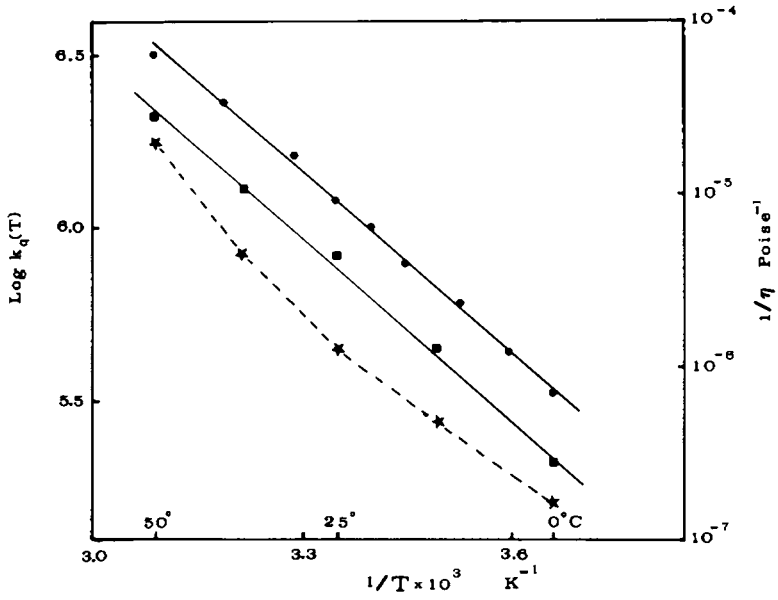

FIGURE 4 Arrhenius plot of the triplet quenching rate constant of Trp-109 in alkaline phosphatase by oxygen (●) and nitric oxide (■). The temperature dependence of the effective viscosity η(★) at the site of Trp-109 as obtained from the intrinsic phosphorescence lifetime (Strambini and Gonnelli, 1985) is shown for comparison.

for Trp-109 in AP was estimated to be $\sim 10^9$ M^{-1} s^{-1} (Calhoun et al., 1983), essentially the same as for the exposed residues. Analogously, for Trp-314 of LADH, the only other case for which singlet and triplet data is available, $kq(S) = 0.5 - 0.6 \times 10^9$ M^{-1} s^{-1} (Calhoun et al., 1983; Hagaman and Eftink, 1984) against a $kq(T) = 3.6 \times 10^7$ determined in the present experiments.

Possible causes that invalidate a comparison between $kq(S)$ and $kq(T)$, assuming that the efficiency of the interaction between oxygen and the indole nucleus remains the same as in water, are outlined below.

(*a*) Owing to the great difference in lifetime between excited singlet and triplet states, a concentration of O_2 about eight orders of magnitude larger is required to quench fluorescence in comparison to phosphorescence. With the former, the concentration of oxygen reaches prohibitively large values as soon as kq is 10^8 M^{-1} s^{-1} or smaller. A certain degree of static quenching is now commonly recognized in these studies (Hagaman and Eftink, 1984) and subtle perturbations in the dynamical makeup of inner regions of protein structure due to O_2 binding and the high pressures employed cannot be ruled out.

(*b*) The diffusion pathway monitored in triplet quenching may be different from singlet quenching. The quencher in the former case must come from the solvent, whereas for the latter it is on average already within the macromolecule. A model based on this realization has recently been put forward (Gratton et al., 1984) to account for the smaller rate of O_2 quenching found for a long-lived fluorescence probe bound to a protein in comparison to the fluorescence of tryptophan (Vaughan and Weber, 1970).

(*c*) In proteins with more than one tryptophan residue, the analysis of fluorescence quenching data is often complex. It may be intrinsically difficult to distinguish the quenching of each residue and the problem may be further complicated by the possibility of singlet energy transfer among residues. A case in point is AP. There is much evidence that Trp-109 transfers to the other solvent-accessible residues (Domanus et al., 1979). The extent of transfer may even increase as O_2 selectively shortens the acceptor's lifetime and reduces the possibility of back transfer. Because of these difficulties and the possibility of quenching at distances greater than Van der Waals contact (Birks, 1970), it is felt that quenching of protein fluorescence, particularly of well protected tryptophans, is a process whose interpretation in terms of structural fluctuations may not always be straightforward.

A completely independent assessment of fluidity in protein structure is obtained from the remarkable shortening of the indole triplet-state lifetime upon lowering the viscosity of its microenvironment (Strambini and Gonnelli, 1985). Such dependence was found to provide unique information on the local dynamics of protein structures as modulated by the degree of hydration (Strambini and Gabellieri, 1984) or by interactions with classical denaturants (Strambini and Gonnelli, 1986).

Trp-314 of LADH and Trp-109 of AP phosphoresce at room temperature with lifetimes of 0.4 and 1.57 s, the longest reported to date for globular proteins in solution.

Indeed, both residues are enclosed within a shell formed of β-pleated sheets and α-helical rods (Brändén et al., 1975; Wyckoff et al., 1983) an agglomerate that in H-exchange terminology has been classified as a "knot" for its extraordinary rigidity (Gregory and Lumry, 1985). If the migration of small molecules requires local disruption of this structure, a correlation might be anticipated between phosphorescence lifetimes and quenching rate constants.

The present experimental results point out that the relationship between the two parameters, $kq(T)$ and τ_0, is not a simple one. If the τ_0 value of 1.57 s at 25°C is used to

estimate the local viscosity (Strambini and Gonnelli 1985), value of $\eta = 8 \times 10^5$ poise is obtained. This would imply that the quenching constant should be reduced from the value of 5×10^9 M^{-1} s^{-1} found in aqueous solutions by a factor of $0.01/8 \times 10^5$ resulting in a quenching constant of 62 M^{-1} s^{-1}. The observed value for $kq(T)$ of 1.2×10^6 M^{-1} s^{-1} is much larger than what would be estimated if diffusion were governed by the local fluidity. That the two quantities intrinsic lifetime and rate of quenching in AP are determined by structural fluctuations of a different nature is evidenced also by the approximately three times smaller activation energy for the diffusion process (a straight line fit of the points in Fig. 4 gives $Ea = 20.5$ kcal mol^{-1}).

Two hypotheses are advanced to account for this discrepancy: (*a*) O_2 and NO, being small uncharged molecules, may require for migration relatively small openings that in part may already exist or need minor atomic displacements. The Stokes-Einstein relation, ($D\alpha\ T/\eta$), is not obeyed by O_2 at high viscosity, η, (Ware, 1962), and deviations can be large with big solvent molecules where O_2 diffusion may be even two orders of magnitude larger than predicted (Subczynski and Hyde, 1984). The discrepancy to be accounted for at 25°C is between a $kq(T) = 62^2$ M^{-1} s^{-1} predicted from the triplet lifetime and 1.2×10^6 M^{-1} s^{-1} actually measured, a gap too wide to be attributed entirely to deviations from the Stokes-Einstein equation.

(*b*) Alternatively, one must consider the possibility that quenching may occur without penetration of the structure surrounding the chromophore. Siegel and Judeikis (1968) have shown clearly that it is not necessary for O_2 and NO to contact the chromophore as they are able to quench naphthalene phosphorescence at up to distances of 10.5 Å or greater (Birks, 1970).

Dynamic quenching of the phosphorescence of a tryptophan residue within an impermeable core of the macromolecule may thus be pictured as follows:

$$Q + \text{core} \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} Q - \text{core} \xrightarrow{k_t},$$

where k_d is the rate at which Q diffuses to the surface of the core region, k_{-d} is the probability that it dissociates from it, and k_t is the probability with which the excited triplet state is statically quenched by Q on the surface. If k_0 is the inverse of the intrinsic triplet lifetime, then the quenching efficiency per encounter of Q with the surface of the core is given by $k_t/(k_t + k_0 + k_{-d})$. In this scheme, the rate of quenching is linear on the concentration of Q and $kq(T) = k_d \cdot k_t/(k_t + k_0 + k_{-d})$ (Gijzeman et al., 1973).

A comparison between the rate constants for oxygen and nitroxide quenching suggests that the efficiency of the quenching process is close to one. The molecules NO and O_2 are both uncharged and roughly of the same size and it is therefore reasonable to assume that $k_d(O_2) \simeq k_d(NO)$, if anything, the polar nitroxide might be migrating more slowly owing to the dragging effect of polar groups in the protein. Thus, the common value of $kq(T)$ found for these quenchers implies that they display the same efficiency. Since $k_t(O_2)/k_t(NO) = 20$–30 (Birks, 1970; Kearns and Stone, 1971), this is possible only if in both cases the efficiency is large and $kq(T) \simeq k_d$.

For this model to be compatible with the three-dimensional structure of AP, the distance between Trp-109 and the outer surface of the β-pleated sheet surrounding it must be sufficiently small to allow the quenching step of the process to be efficient, that is, $k_t \geq 10\ k_{-d}$. The rate constant for static quenching in a process mediated by an exchange interaction is of the order of 10^{12} s^{-1} when the partners are in Van der Waals contact and it is expected to decrease exponentially with the distance that separates them. A fair estimate of the distance dependence can be obtained by analogy to triplet–triplet energy transfer where the rate decreases by about a factor of 10 for each increment of 1 Å in separation (Strambini and Galley, 1975). A more difficult task is to estimate the value of k_{-d} because serious solvent cage effects within the protein and migration on a surface may prolong considerably the average dwelling time of the quencher about the core. Studies with chromophores free in solution show k_{-d} to be dependent on the solvent viscosity and its magnitude is usually taken to be equal to 2 k_d (Gijzeman et al., 1973). For the present quenching data, one may then consider the value of $k_{-d} = 2 \times 10^6$ s^{-1} to probably be an upper bound. As a result, efficient quenching requires $k_t \geq 2 \times 10^7$ s^{-1}, which using the above mentioned distance dependence for k_t corresponds to a maximum separation of 4–5 Å beyond Van der Waals contact. This is larger than the thickness of a β-pleated sheet, the distance of Trp-109 to the outer surface of the structured region seen in x-ray.

In summary, the mechanism of quenching proposed here departs from the usual interpretation of colliding partners stressing that the effective static interaction radius depends both on the local viscosity and the excited-state lifetime. Owing to interaction at relatively large distances, it contemplates rate constants that are larger than predicted by migration and allows for the possibility that there exist sufficiently compact regions of protein structure through which even the smallest molecules are unable to penetrate in the micro-millisecond time scale.

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