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Quenching of Protein Fluorescence by Oxygen. Detection of Structural Fluctuations in Proteins on the Nanosecond Time Scale

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Abstract

Quenching of the tryptophan fluorescence of native proteins was studied using oxygen concentrations up to 0.13 M, corresponding to equilibration with oxygen at a pressure of 1500 psi. Measurement of absorption spectra and enzymic activities of protein solutions under these conditions reveal no significant perturbation of the protein structure. The oxygen quenching constant (k_+*) for a variety of proteins indicates that the apparent oxygen diffusion rate through the protein matrix is 20–50% of its diffusion rate in water. No tryptophan residues appear to be excluded from quenching, and no correlation of the fluorescence emission maxima with k_+* was found, indicating that the rapid oxygen diffusion is present in all regions of the protein, even those normally considered inaccessible to solvent. Energy transfer among tryptophans was excluded as a possible mechanism for the rapid quenching by studies using 305-nm excitation, where energy transfer is known to fail. The dynamic character of the observed quenching was proven by the proportional decrease of the fluorescence lifetimes and yields measured under the same conditions. We conclude that proteins, in general, undergo rapid structural fluctuations on the nanosecond time scale which permit diffusion of oxygen.

The previous paper (Lakowicz and Weber, 1973) described the methodology and presented experimental data for the quenching of small molecules, and some linear biopolymers, by oxygen. Here we examine the quenching of the fluorescence of proteins by oxygen. X-Ray determined structures and solvent perturbation studies of many proteins have shown that tryptophan residues are often situated in the interior of the protein matrix and appear inaccessible to solvent. It must be pointed out, however, that both of these techniques yield information about the average conformation and solvation of the amino acid residues, but no information about the existence of the structural fluctuations which may occur. Since quenching of fluorescence by oxygen depends on the collisional rate between oxygen and fluorophore, we expected oxygen quenching of tryptophan fluorescence in proteins to yield information on the dynamics of those structural changes in the nanosecond time scale that would allow diffusion of oxygen through the protein matrix. Such local fluctuations must be indispensable for the effective quenching of a fluorophore shown by X-ray structural studies to be out of direct contact with water.

The quenching of the fluorescence of fluorophore residues forming part of a protein, or in general of a compact macromolecule, presents many complexities that are absent in the case of an isolated fluorophore in solution. These complexities can best be examined by reference to the modified Stern–Volmer equation discussed in the previous paper

$$F_0/F = \left(1 + K_{eq}[Q]\right)\left(1 + k_+ * \tau_0[Q]\right) \quad (1)$$

F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. The first factor on the right-hand side describes the "static" quenching, that is quenching by formation of a complex between quencher and fluorophore, with the equilibrium constant K_{eq} predating the excitation. The second factor describes the dynamic quenching, resulting from encounters of quencher and fluorophore during the fluorescence lifetime. The rate of quenching encounters is given by the product of the bimolecular quenching rate constant k_+*, the unquenched fluorescence lifetime τ_0, and the quencher concentration [Q]. The existence of two modes of quenching *for the same fluorophore*, static and dynamic, is revealed by a positive curvature in the plot of F_0/F against [Q]; we have already commented on the small degrees of static quenching that may be revealed by this means (Lakowicz and Weber, 1973). Additionally, the existence of dark complexes between fluorophore and quencher may be detected by the changes in the absorption spectrum of the fluorophore as the oxygen pressure is raised. The definition of [Q], the effective value of the concentration of quencher responsible for the effects, presents a difficulty when macromolecules are studied. The quenching by iodide of the fluorescence of lysine or glutamic acid copolymers containing tryptophan (Lehrer, 1971) is an example of the extreme way in which charges in the macromolecule may alter the effective concentration of quencher in the vicinity of the fluorophore. Fortunately, such an effect is absent in the case of oxygen quenching as dramatically shown by the absence of differences in the quenching of the cited copolymers by oxygen (Lakowicz and Weber, 1973). On the other hand, the accumulation of hydrophobic residues in the interior of the protein, so clearly shown by crystallographic investigations, provides a medium which can preferentially dissolve oxygen as compared with water. The local concentration of oxygen will perhaps differ inside the protein from the nominal concentration in water, but this internal concentration does not appear to be definable in any simple way since it is likely to vary from one region of the protein to another. As a result, the bimolecular quenching rate constants, k_+*, include an unknown but not a large factor resulting from identification of the effective local concentration with the solvent concentration [Q]. This identification can change quantitatively, though in no case qualitatively, the conclusions about the efficiency of the dynamic quenching process.

Effects of the Heterogeneity of the Fluorophores.

Unless there is a single fluorophore in the macromolecule, placed in a unique environment, both k_+* and τ_0 must be considered average values, resulting from weighted contributions from the several fluorophores present.

If τ_i is the fluorescence lifetime of the ith component of the population, q_i its relative fluorescence efficiency after quenching, and f_i the fractional contribution of each group to the unquenched fluorescence intensity, the observed fluorescence lifetime $\bar{\tau}$ is given by

$$\bar{\tau} = \sum_i f_i q_i \tau_i / \sum_i f_i q_i \tag{2}$$

If each member of the population is subjected to a *single mode of quenching*, as shown by the absence of convexity toward the [Q] axis in the plot of F_0/F against [Q], we can set

$$\tau_i = \tau_{0i} q_i \tag{3}$$

with τ_{0i} = lifetime of the unquenched fluorescence, for those members of the fluorophore population for which quenching is dynamic, and

$$\tau_i = \tau_{0i} \tag{4}$$

for those in which quenching is only by the static mechanism. The experimental findings on the several proteins studied are unequivocal in the absence of indications of static quenching. Accordingly, we assume that the case of eq 4 is not represented, and introducing eq 3 into 2 we obtain

$$\bar{\tau} = \sum_i f_i q_i^2 \tau_{0i} / \sum_i f_i q_i = (1/\bar{q}) \sum_i f_i q_i^2 \tau_{0i} \tag{5}$$

where $\bar{q} = F/F_0$ is the relative fluorescence efficiency of the quenched solution. Writing

$$q_i^2 = (\bar{q} + \delta q_i)^2 = \bar{q}^2 + 2\bar{q}\delta q_i + \delta q_i^2 \tag{6}$$

we find

$$\bar{\tau} = \bar{\tau}_0 \bar{q} + 2 \sum_i f_i \tau_{0i} \delta q_i + (1/\bar{q}) \sum_i f_i \tau_{0i} \delta q_i^2 \tag{7}$$

with

$$\bar{\tau}_0 = \sum_i f_i \tau_{0i} \tag{8}$$

Equation 7 shows that for a homogeneous population ($\delta q_i = 0$) the relations

$$\bar{\tau} = \tau_0 \bar{q} \quad \text{or} \quad F_0/F = \tau_0/\tau \tag{9}$$

obtain, but that, in general

$$\tau_0/\tau \gtrless F_0/F \tag{10}$$

according to whether

$$S = \sum_i 2f_i\tau_{0i}\delta q_i + \sum_i f_i\tau_{0i}\left(\delta q_i^2/\bar{q}\right) \gtrless 0 \tag{11}$$

Evidently, if $\tau_{0i} = \tau_0$ and $\delta q_i \neq 0$, that is, in the case in which there is a single unquenched lifetime, $\sum f_i \delta q_i = 0$, from the definition of $\bar{q}$, and the second term in eq 11 being always positive, it follows that

$$S \geq 0; \tau_0/\tau \leq F_0/F \tag{12}$$

The opposite case requires therefore that the lifetimes of the unquenched fluorophores be appreciably different. The requirements of this case are best appreciated by assuming two types of fluorophores with quenching rate constants k_1 and k_2 and unquenched lifetimes τ_{01} and τ_{02}, respectively, in equal numbers. For this case

$$\begin{aligned} &f_1 = f_2 = 1/2 \\ &\delta q_i = -\delta q_2; \delta {q_1}^2 = \delta {q_2}^2 \\ &\Delta\tau_1 = \tau_{01} - \bar{\tau}_0 = -\Delta\tau_2 \\ &S = 2\Delta\tau_1\delta q_1 + \left(\bar{\tau}_0/\bar{q}\right)\delta {q_1}^2 < 0 \end{aligned} \tag{13}$$

The last condition implies

$$\Delta\tau_1\delta q_1 < 0 \tag{I}$$

and also

$$2\left|\Delta\tau_1\right|/\tau_0 > |\delta q|/\bar{q} \tag{II}$$

For the case of two populations contributing equally to the unquenched intensity

$$\begin{aligned} q_1 &= 1/\left(1 + k_1\tau_{01}[\mathrm{Q}]\right) \\ q_2 &= 1/\left(1 + k_2\tau_{02}[\mathrm{Q}]\right) \end{aligned} \tag{14}$$

$$\frac{\delta q_1}{\bar{q}} = \frac{|\delta q|}{\bar{q}} = \frac{\left(k_2\tau_{02} - k_1\tau_{01}\right)[\mathrm{Q}]/2}{1 + \left(\frac{\left(k_2\tau_{02} - k_1\tau_{01}\right)}{2}\right)[\mathrm{Q}]} < \frac{2\left|\Delta\tau_1\right|}{\tau_0} \tag{15}$$

Evidently the inequality (15) is satisfied if we set $k_1 = k_2$ so that we have the general rule that identity of bimolecular quenching rate constants and heterogeneity of unquenched lifetimes give rise to $\tau_0/\tau > F_0/F$, while heterogeneity of bimolecular quenching constants and identity of unquenched lifetimes (eq 11) result in the opposite case: $\tau_0/\tau < F_0/F$. Thus, for proteins containing a heterogeneous population of fluorophores, precise agreement between τ_0/τ and F_0/F would in general not be expected. However, comparison of these quantities can be used to confirm the dynamic character of the observed quenching.

Experimental Procedures

Dynamic quenching of fluorescence is described by eq 1 with $K_{eq} = 0$. Quenching of tryptophan or protein fluorescence by dissolved oxygen at 1 atm of oxygen pressure is insignificant. For fluorophores in aqueous solutions k_+* is ~1×10^{10} M^{-1} sec^{-1}, which is the diffusion-controlled value. We would expect the tryptophans of proteins to be somewhat protected from collisions with oxygen, and thus k_+* to be decreased. If we assume the diffusion of oxygen to a tryptophan residue in a protein to be decreased by a factor of four, for a typical protein fluorescence lifetime of 4 nsec, an oxygen concentration of 0.13 M would reduce the fluorescence to 40% of its initial value. This oxygen concentration requires equilibration with 100 atm of oxygen pressure.

Our experiments involved measurement of absorption and emission spectra, fluorescence intensities and lifetimes, and enzyme activities in solutions equilibrated with oxygen pressures up to 1500 psi. Two stainless steel cells provided with quartz windows were used, one for the fluorescence measurements, the other for absorption spectra and enzyme assays. A detailed description of these cells and the techniques used for these measurements is given in the previous paper (Lakowicz and Weber, 1973). The cell for enzyme assays allowed preequilibration of the enzyme and substrate solutions with the increased oxygen pressure prior to initiation of the reaction, which was followed spectrophotometrically.

Ultra Pure guanidine hydrochloride[1] (Gdn·HCl) was obtained from Schwarz/Mann. Azurin from *Pseudomonas aeruginosa* (Wharton, 1973) was a generous gift from Dr. D. Wharton, Cornell University. α-Chymotrypsin, trypsin, ribonuclease, carboxypeptidase A (all from bovine pancreas), pepsin (swine stomach mucosa), and aldolase (rabbit muscle) were all obtained from Worthington Biochemical. Edestin (hemp seed), carbonic anhydrase (bovine erythrocytes), and lysozyme (egg white) were obtained from Sigma Chemical Co. Bovine serum albumin lot D71309 was a product of Armour Pharmaceutical Co. Human serum albumin was from Pentex, lot 24. Bovine-γ-globulin was from Calbiochem, lot 53588. Trypsinogen was obtained from Mannheim-Boehringer. *N*-Benzoyl-L-tyrosine ethyl ester, *N*-benzoylglycyl-L-phenylalanine, and *p*-tosyl-L-arginine methyl ester hydrochloride were purchased from Sigma. Di-*N*-acetyl-D-glucosamine ((N-Ac-GlcN)$_2$) was a generous gift from Dr. John A. Rupley, University of Arizona. This β-1,4-glucosamine dimer was isolated by the method described by Rupley (1964).

Enzyme Assays.

α-Chymotrypsin and trypsin were assayed according to Hummel (1959), carboxypeptidase A according to Folk and Schirmer (1963), lysozyme according to Shugar (1952), and carbonic anhydrase according to Armstrong et al. (1966). For assays at atmospheric pressure the volumes appropriate for the 1-cm^2 cuvets were used. For comparison of activities at 0 and 1500 psi 21 ml of total solution was needed for observations in the high-pressure absorption cell. Substrate (20 ml) was placed in the variable light path internal cell of the

[1]Abbreviations used are: Gdn·HCl, guanidine hydrochloride; N-Ac-Trp-NH_2, *N*-acetyl-L-tryptophanamide; (N-Ac-GlcN)$_2$, di-*N*-acetyl-D-glucosamine.

high-pressure absorbance cell and 1 ml of enzyme solution was added following the procedure described in the previous paper.

Fluorescence Lifetimes.

Fluorescence lifetimes were measured using the cross-correlation phase fluorometer constructed by Spencer and Weber (1969). Lifetimes may be calculated from either the phase shift or the demodulation of the emission relative to the exciting light. Unless otherwise stated, lifetimes were measured from the phase shift which is more accurate for lifetimes below 10 nsec. The frequency of light modulation was 14.2 MHz.

Unless otherwise stated, all experiments were performed at 25 ± 0.2°.

Results and Discussion

Effects of Oxygen Pressure on the Properties of Proteins.

The use of any type of probe to investigate macromolecular structure requires that the probe does not significantly alter the structure. Interpretation of our data thus requires knowledge of the effect of the increased pressure and oxygen concentration on the solution structure of proteins.

Aqueous solutions, in equilibrium with 100 atm of oxygen pressure, are 0.13 M in O_2. Since oxygen is a small, nonionic species, little change in solvent structure and no change in ionic strength occurs. Hence, the structure of proteins, even those sensitive to salt, should be unperturbed. Proteins are known to denature at high hydrostatic pressures, but in most cases no effect is seen below 3000 atm (Miyagawa and Suzuki, 1962a,b; Brandts et al., 1970). Thus, it appears quite certain that the pressures used in our experiments would not appreciably perturb the protein structure. In spite of the preceding arguments, that proteins would not be affected by our experimental conditions, we felt it necessary to investigate this possibility more fully.

The ultraviolet absorption spectra of proteins are known to be sensitive to changes in tertiary structure. In the previous paper we reported the lack of significant perturbations in the absorption spectra of indole or tryptophan at the oxygen concentrations employed. Similarly, the absorption spectra of solutions of bovine serum albumin and lysozyme, in 0.1 M sodium phosphate, pH 7.0, are unperturbed by equilibration with 1500 psi of O_2 indicating the absence of significant structural changes in the proximity of the absorbing chromophores.

The enzymic activity is probably a more sensitive criterion of changes in structure than the absorption spectrum. It is well known that loss of enzymic activity without serious spectroscopic changes is often observed, while the opposite behavior is exceptional. The apparatus for the spectrophotometric measurement of enzyme activity after preequilibration of enzyme and substrate under 1500 psi of O_2 has been described. Table I compares the specific activities of enzymes measured under identical conditions except for the oxygen pressure. Within experimental error there is no change in activity in any of the five proteins investigated. Trypsin and α-chymotrypsin, subjected to 1500 psi of O_2 for 12 hr and assayed after release of pressure, showed no change in activity. Thus, we conclude that by

spectroscopic or enzymic criteria, neither the increased pressure nor the elevated oxygen concentration used in these experiments produced significant, or even detectable, changes in protein structure.

Oxygen Quenching Constants for Proteins.

Table II contains Stern–Volmer quenching constants and independently measured unquenched lifetimes (τ_0) for a number of proteins. From these data the bimolecular quenching constant $k_+^* = K/\tau_0$ may be determined. The previous paper shows that indole derivatives and tyrosine in solution are quenched with a rate of $k_+^* = 1.2 \times 10^{10}$ M^{-1} sec^{-1}, which is the diffusion-controlled value. It is evident from Table II that the tryptophan residues of these proteins are quenched at no less than 20–50 % of the diffusion-controlled rate. The wavelength of the fluorescence emission maximum is generally thought to be an indication of the polarity of the regions where the indole groups are located (Lippert, 1957; Weber, 1961). Solvent studies with indole and tryptophan indicate that contact with water causes a red shift (longer wavelength) of the emission maximum (Weber and Teale, 1965). Proteins with the shorter wavelength emission maxima are thus thought to have their indole moieties protected from the solvent. This proposition has been broadly confirmed for those proteins (like chymotrypsin, carboxypeptidase, or lysozyme) for which detailed crystallographic structures are available. If the protein structure offers an appreciable barrier to O_2 diffusion one would expect the “bluer” tryptophans to be protected from collisions with oxygen as compared with the “redder” kind. Table II demonstrates that there is no correlation between these two parameters, indicating that oxygen diffused readily into regions of the protein matrix which are generally considered inaccessible to solvent. These data imply that the observed quenching is not determined to any degree by the amount of contact the tryptophan residues have with the solvent.

Figure 1 shows some typical Stern–Volmer plots for proteins. If the tryptophans of a given protein have significantly different Stern–Volmer quenching constants, reflecting varying degrees of protection from collisions with oxygen, a downward curvature in the Stern–Volmer plots would be observed. None of the proteins studied gave evident nonlinear plots. Assuming equal fluorescence intensities from two tryptophan populations, the quenching constants K for each population would have to differ by about a factor of 4 for the nonlinearity to become noticeable. Although the data do not indicate therefore that all tryptophan residues are quenched at the same rate, they do indicate that there is no significant fraction of tryptophans which is inaccessible to oxygen. Moreover, we see in Table II that the bimolecular quenching constants k_+^* are within a factor of four for all the cases studied. The previous paper demonstrates that actual collisions between oxygen and fluorophore are required for quenching. Thus we conclude that oxygen diffuses through the protein matrix at 20–50 % of the diffusion rate in water.

Effect of Oxygen Pressure on the Fluorescence Emission Spectra of Proteins.

Most of the proteins used in these experiments contain more than a single tryptophan as well as several tyrosine residues. When 280-nm excitation is used some tyrosine fluorescence may be observed. Since each tryptophan has an emission maximum and fluorescence lifetime depending on its particular environment, we expect changes in the emission spectra

due to preferential quenching of certain tryptophans. The previous paper reports that tryptophan itself displays only a small change in its emission spectrum when 80 % of its fluoresence is quenched by oxygen.

The fluoresence emission spectra of human serum albumin equilibrated with air and 1468 psi of O_2 are shown in Figure 2a. The large increase in the relative tyrosine contribution is understandable since tyrosine generally has a shorter lifetime than tryptophan, and is thus quenched to a smaller extent. The fluorescent lifetimes of tyrosine in 0.1 and 0.005 M sodium phosphate, pH 7, are 2.0 and 3.2 nsec, respectively. Moreover, the tyrosine fluorescence in bovine and human serum albumin is approximately 90% quenched (Weber and Young, 1964) and although, due to technical difficulties, its lifetime has not been measured, it is unlikely to be as long as 1 nsec. On the other hand the tryptophan fluorescence in human serum albumin is one of the longest measured, 6 nsec. The observed spectrum therefore supports the claims that all fluorescent groups in the protein are quenched with similar bimolecular quenching constants (k_+*) and that selectivity of quenching is determined to a large extent by the fluorescence lifetime.

The dramatic changes in human serum albumin are rather unique and reflect the fact that, besides differences in fluorescence lifetime, the contribution of tyrosine is larger than in most proteins (Teale, 1960). Bovine serum albumin, with two tryptophans as opposed to the single one in human serum albumin, shows already a much smaller increase in the relative tyrosine contribution (Figure 2b). No increase in the relative tyrosine contribution or shift in the emission maximum was observed with α-chymotrypsin, carboxypeptidase A, or aldolase (Figure 2c). Trypsinogen displays a blue shift of approximately 2 nm at 100 atm of oxygen pressure. Carbonic anhydrase (Figure 2d) and lysozyme display blue shifts of about 5 nm at 100 atm, which are possibly due to selective quenching of the “redder” tryptophans. Pepsin shows a 10-nm blue shift under these conditions. Since removal of the oxygen results in the return of the original fluorescence spectrum and intensity we conclude that no significant photochemical degradation occurs during these experiments. Solutions were only illuminated for the time necessary to perform the required measurements.

The general blue shifts observed in proteins need not be due to selective quenching of the redder tryptophans alone. A blue shift was observed in pepsin even though all the tryptophans appear to be accessible to oxygen (see Figure 5 and the related discussion). Fluorophores in the excited state generally have an increased dipole moment relative to the ground state (Lippert, 1957). Reorientation of polar groups surrounding the increased dipole moment is responsible for the red shifts of fluorescence emission spectra observed in polar solvents. Bakhshiev (1964) has pointed out that a fraction of the excited molecules may emit before solvent relaxation is complete. Their fluorescence will thus appear at shorter wavelengths. Relaxation of the polar groups surrounding 6-*p*-toluidinylnaphthalene-2-sulfonate when complexed to bovine serum albumin was in fact observed by Brand and Gohlke (1971). When a solution is quenched by oxygen we selectively observe those molecules which emit at shorter times, since these molecules are less likely to collide with oxygen and be quenched. In our studies pepsin is quenched from 5.5 to 1.2 nsec, a greater relative change than any of the other proteins studied. Perhaps at 1.2 nsec relaxation of the polar groups surrounding the tryptophans is incomplete. This ability to observe directly the

unrelaxed emission spectrum provides an additional method to investigate short-term fluctuations of the protein matrix and other media (Weber and Lakowicz, 1973).

Effect of Excitation Wavelength.

For most of these experiments 280-nm excitation was used. Both tyrosine and tryptophan absorb energy of this wavelength. The percentages of light absorbed by the indole groups, relative to the total absorbance at 280, have been listed by Teale (1960). These percentages vary from 93% for lysozyme to 19% for human serum albumin. Generally tyrosyl residues are more exposed to the solvent than tryptophan (Herskovits and Sorensen, 1968b) and therefore, on excitation at 280 nm, one may expect increased quenching efficiency due to a rapid decrease in the tyrosine emission. The fluorescence emission spectra of proteins under oxygen pressure eliminate this possibility. In all cases where a tyrosine contribution could be observed, its intensity relative to tryptophan increased as the oxygen concentration increased. The shorter lifetime of tyrosine and the similar rate with which all residues in the protein collide with oxygen will cause the tyrosine fluorescence to be quenched less effectively than tryptophan regardless of exposure to solvent. The relative fluorescence intensity at each oxygen concentration was measured by the intensity at the emission maximum of the unquenched solution, rather than by the area under the emission spectrum. Therefore, the fluorescence intensity is almost entirely determined by the tryptophan fluorescence. Of the proteins studied here, human serum albumin has the most pronounced tyrosine contribution to the fluorescence emission spectrum, but even in this favorable case the relative tyrosine fluorescence at 342 nm, the wavelength which was used to collect the fluorescence intensity data for human serum albumin, could be no more than 4% of the total. For the other proteins this percentage is much less and therefore the excitation of tyrosine as well as tryptophan at 280 nm will have no effect on the present measurements.

One possible phenomenon upon excitation at 280 nm is energy transfer among tryptophans. If all the tryptophans of a protein transferred their energy to a single tryptophan easily quenched by oxygen, a larger average quenching constant may be observed than if they were indepenently quenched. For rapid energy transfer in both directions between two populations, having quenching constants K_1 and K_2, one would observe $K = 0.5(K_1 + K_2)$. If transfer occurs rapidly only from population 1 to 2, K_2 would be observed. We note that such a mechanism cannot be operative in human serum albumin or azurin which contains a single tryptophan. In order to exclude complications due to tyrosine absorption and energy transfer among tryptophans, quenching experiments were performed using both 280- and 305-nm excitation. Above 300 nm 100% of the absorbance is due to tryptophan (Teale, 1960). Energy transfer between fluorophores is known to fail when excitation is on the long wavelength edge of the absorption band (Weber and Shinitzky, 1970). For indole, transfer fails upon 305-nm excitation (Weber, 1960a) and proteins exhibit a very high polarization on excitation at this wavelength (Weber, 1960b) indicating the absence of inter-tryptophan transfer.

Table III lists quenching data and unquenched lifetimes for proteins excited at 305 nm. Variations in the bimolecular quenching constant of 15–34% were observed. We have not yet arrived at an explanation for these variations. Indole and tryptophan display identical oxygen

bimolecular quenching constants using 280- or 305-nm excitation. Quenching of tryptophan by iodide (0.1 M sodium phosphate, pH 7, and [KCl] + [KI] = 0.5 M) yields a Stern–Volmer constant of 10.4 M^{-1} regardless of the excitation wavelength (280, 290, 300, or 305 nm). The measurement of fluorescence lifetimes is rather difficult. In addition to the usual problem of low exciting light intensity in the ultraviolet, the absorbance of protein solutions is greatly reduced at 305 nm. Errors in the measured lifetimes could account for some of the differences. Selective excitation of buried residues could also account for some of the differences. Buried residues are expected to absorb at the longer wavelengths due to the well-known blue shift shown by indole in hydroxylic solvents. Unfortunately the degree of selection possible cannot be calculated from available solvent perturbation studies (Herskovits and Sorensen, 1968a).

Regardless of the source of these variations our conclusion concerning the free diffusion of oxygen through the protein matrix is not affected. If the variations in K/τ_0, with excitation wavelength are due to selective excitation of buried tryptophan residues, then even those residues most protected from solvent are accessible to collisions with oxygen at rates comparable to the diffusion-controlled value for a free fluorophore.

Proof of Dynamic Quenching by Lifetime Measurements.

In the previous paper we discussed that the dynamic nature of the observed quenching may be proven by the equivalence of F_0/F and τ_0/τ. Figure 3a compares these two parameters for the protein aldolase and thus proves the dynamic character of the observed quenching. Aldolase is an important example because its 328-nm fluorescence emission maximum and solvent perturbation studies (Herskovits and Sorensen, 1968b) indicate that the tryptophans are protected from the solvent. Thus, even those tryptophans commonly thought to be inaccessible to solvent are easily and dynamically quenched by oxygen. Figures 3b, c, and d show similar data for α-chymotrypsin, pepsin, and carbonic anhydrase. Both positive and negative deviations of τ_0/τ from F_0/F are observed. Such deviations are easily accounted for by small amounts of heterogeneity in the fluorescence lifetimes and bimolecular quenching constants of the tryptophans comprising the emitting population (eq 7 and 15). Table IV summarizes Stern–Volmer quenching constants observed by lifetime measurements. Bovine and human serum albumin have uniquely large negative deviations in τ_0/τ compared to F_0/F. These deviations may be related to a local increase in the oxygen concentration surrounding the fluorophores. The fluorescence intensity quenching data are not consistent with a static mode of quenching because positive curvature in the Stern–Volmer plots was not observed.

We conclude that the predominant mode of quenching is dynamic and that the bimolecular quenching constant is representative of the diffusional rate of oxygen through the protein matrix.

Lysozyme Effect of (N-Ac-GlcN)$_2$.

Lysozyme contains six tryptophans, three of which are near the active site (Phillips, 1967). Two of these latter tryptophans account for 80% of the fluorescence in native lysozyme (Imoto et al., 1971). One expects the presence of substrate to reduce the diffusion of oxygen to the active site, thus reducing the bimolecular quenching constant. Since each experiment

requires several hours the use of any readily hydrolyzable substrate was not practical. Rupley and Gates (1967) showed the dimer of (*N*-acetyl-D-glycosamine to be cleaved at a negligible rate. $(N\text{-Ac-GlcN})_2$ was used in a concentration sufficient to complex 80 % of the lysozyme (Rupley et al., 1967). Figure 4a shows a decrease in the bimolecular quenching constant of 40% due to $(N\text{-Ac-GlcN})_2$. $(N\text{-Ac-GlcN})_2$ causes a 10-nm blue shift in the emission spectrum, but no change in the fluorescence lifetime. Using the data in Figure 4a one can calculate the quenching constant for the 100% complexed solution to be 3.6 M^{-1}. The presence of $(N\text{-Ac-GlcN})_2$ reduces the number of effective collisions by 50%. We conclude that the diffusion of oxygen is sensitive to steric effects, but that even in the presence of saturating concentrations of $(N\text{-Ac-GlcN})_2$ the tryptophans of lysozyme are still quenched at 24% of the diffusion-controlled rate.

Comparison of O_2 and I^- Quenching of Proteins.

Iodide (I^-) is also known to be a general efficient quencher of fluorophores (Förster, 1951), as well as of the fluorescence of proteins (Lehrer, 1971). The previous paper demonstrates the dynamic nature of iodide quenching of indole. We also compared oxygen and iodide quenching of charged glutamate or lysine polymers, each containing a small percentage of tryptophan. These data demonstrated the complete insensitivity of oxygen quenching to charge effects, whereas previous data (Lehrer, 1971) showed iodide quenching of these copolymers to be extremely sensitive to charge effects. Figure 4b compares oxygen and iodide quenching of trypsinogen. Oxygen is at least an order of magnitude more effective than I^-. Trypsinogen contains 17 glutamic acids and 11 lysines (Travis and Liener, 1965) and has an isoelectric point of 9.3. Since these data were obtained at pH 3, the charge on the trypsinogen is positive. Thus, the charge effect is most likely to increase the amount of quenching observed with I^-. Less effective quenching by I^- is not unique to trypsinogen, but was also observed for carbonic anhydrase and carboxypeptidase A (Table V). For these proteins O_2 quenching is again more effective than I^- by an order of magnitude. We conclude that quenching by I^- is limited by charge and solvation effects, whereas oxygen quenching, being insensitive to these effects, can yield a more accurate picture of the fluctuations of the protein structure in solution.

Pepsin shows an exceptionally large iodide quenching constant and the Stern–Volmer plot shows a negative deviation. Such deviations may be present for the other proteins but are not as obvious because of the small fraction of the fluorescence quenched even at 0.4 M I^-. Downward curvature in a Stern–Volmer plot may be explained by two populations of tryptophans, one of which is inaccessible to quencher. Quenching data may then be described by the following equation

$$F_0/\Delta F = \left(1/[\mathrm{X}]f_\mathrm{a}K_\mathrm{q}\right) + \left(1/f_\mathrm{a}\right)$$

(Lehrer, 1971). ΔF is the change in fluorescence due to addition of a given concentration of quencher [X], K_q is the quenching constant of the accessible tryptophans, and f_a is the fraction of tryptophans which are accessible. A plot of $F_0/\Delta F$ *vs.* 1/[X] intercepts the *y* axis at $1/f_a$. Such a plot for oxygen and iodide quenching of pepsin is shown in Figure 5. For I^-, $f_a = 0.85$, consistent with one inaccessible tryptophan out of the six in pepsin (Arnon and

Perlmann, 1963). For oxygen quenching, however, we find $f_a = 1.03$, indicating that all the tryptophans are accessible to oxygen. We conclude that oxygen diffuses to all regions of the protein matrix, and that the inaccessibility of tryptophans, as observed by I^- quenching, is due to charge and solvation effects associated with the I^- ion.

Denaturation Studies.

We have already shown that proteins are quite permeable to oxygen. Even though we found no correlation between the fluorescence emission maxima and the bimolecular quenching constant the variations of the latter quantity should be representative of the tertiary structure of proteins. Thus, disruption of the tertiary structure should remove differences in k_+*. Guanidinium chloride (6 M) was used to denature the proteins. Such a drastic solvent change is expected to alter the oxygen solubility and the diffusion coefficients of oxygen and iodide. Rather than attempting to measure the changes in these two latter quantities, we decided it would be easier and more accurate to measure the apparent bimolecular quenching constant by quenching studies in the new solvent system using free fluorophores. In calculating the oxygen bimolecular quenching constant it was assumed that the oxygen solubility is unchanged. Table VI lists oxygen and iodide Stern–Volmer quenching constants and lifetimes for fluorophores and proteins in 6 M guanidinium chloride. Using these data and the data from Table I of the previous paper we constructed Table VII, which shows a decrease in k_+* for both O_2 and I^- quenching of free fluorophores. Assuming the efficiency of quenching by O_2 and I^- is unaltered by the solvent change, we conclude that the diffusion coefficient of I^- is reduced, and that the solubility and/or diffusion coefficient of oxygen is reduced in 6 M Gdn·HCl. This decrease is approximately the same for O_2 and I^-. In 6 M Gdn·HCl, on the average k is reduced to 0.52 of its water solution value. We can normalize k_+* values in 6 M Gdn·HCl to those in the absence of 6 M Gdn·HCl by division by 0.52, and thus correct for changes in solubility and diffusion coefficient. Table VIII contains "normalized" k_+* values for proteins. We note that for O_2 k_+* values increase by a factor of 2–3 and for I^- k_+* values increase by a factor of 10. The oxygen values are now close to the diffusion-controlled limit and the range of this parameter is reduced. Thus, the k_+* values for native proteins, though not far below the diffusion-controlled limit, are below this rate due to the tertiary structure.

Conclusions

(1) The quenching of the fluorescence of tryptophan in proteins is undoubtedly a time-dependent process brought about by the collisions of the oxygen molecules with the excited tryptophan residues, during the few nanoseconds of the fluorescence lifetime.

(2) The oxygen pressures required for these studies (up to 1500 psi) do not appreciably change either the enzymic activity or the absorption spectra of the proteins studied.

(3) The absolute values of the bimolecular quenching constants for various proteins are only two to five times smaller than those for free tryptophan, and over an order of magnitude greater than the quenching constants of protein fluorescence by iodide, although both iodide and oxygen have comparable quenching efficiencies on free fluorophores and on the proteins denatured by guanidine hydrochloride. The only reasonable explanation for these facts is

that oxygen diffuses to the neighborhood of the tryptophans with much higher efficiency than iodide. Size, charge, and liposolubility must all play a part in this difference.

(4) No evidence was found that any tryptophan residues were excluded from the quenching action of oxygen or that the relatively unimportant differences among the various proteins were related to the accessibility of the tryptophans to the solvent. The tryptophans of aldolase or the unique tryptophan residue in azurin are among the bluer emitters, yet they are quenched with an efficiency wholly comparable to those of other proteins with much redder emission. These results indicate that protein structures are stabilized by a large number of small energy interactions, each of which is easily and reversibly disrupted by thermal energy, independently of the others.

(5) The steric inhibition of the quenching of tryptophan by the protein structure is much smaller than that of fluorophores like pyrene butyrate, 8-anilinonaphthalene-l-sulfonic acid, and 6-diethylaminonaphthalene-1-sulfonic acid adsorbed on serum albumin. These differences indicate that the binding energies between the surrounding protein residues and these polycyclic aromatics are conspicuously larger than those between tryptophan and its surroundings. By the same token the latter energies must be much weaker than those operating between ethidium bromide and the purine and pyrimidine bases in DNA.

(6) The general conclusion to be derived from all the points mentioned above is that the functional properties of protein molecules are not properly represented by rigid models that do not include the rapid structural fluctuations necessary to explain the phenomena that we have observed. Our experimental findings are fully consistent with the ideas on the character of protein conformation put forward by one of us (Weber, 1972) but not with the often expressed belief that proteins exist in a very small number of permissible conformations. Such models are, in our opinion, inconsistent with the weak forces that determine protein structure.

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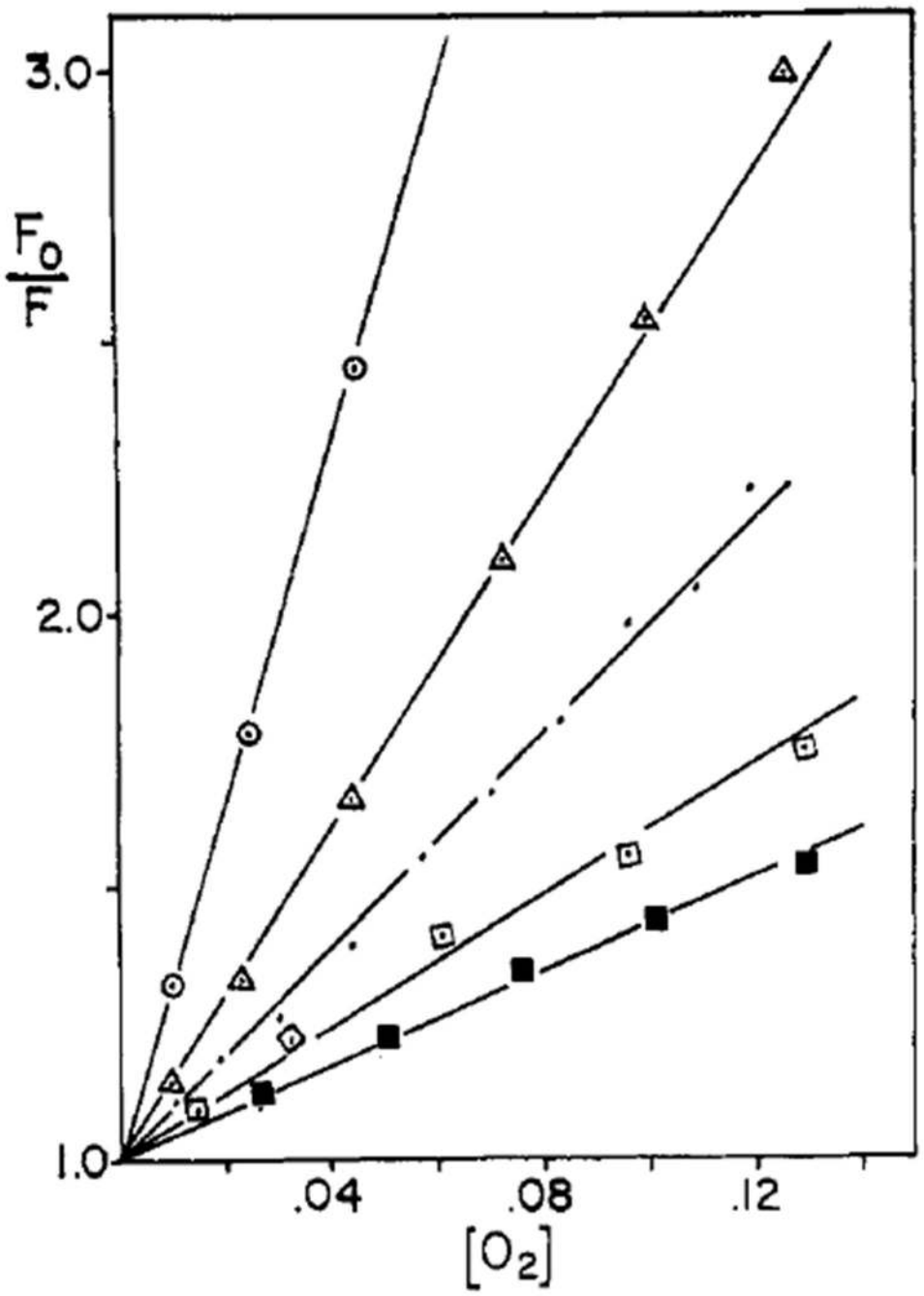

FIGURE 1:

Typical Stern–Volmer plots for proteins. Instrumental conditions used are listed in footnote *a* of Table II: tryptophan (◯); bovine serum albumin (Δ); IgG (·); aldolase (□); and α-chymotrypsin(■).

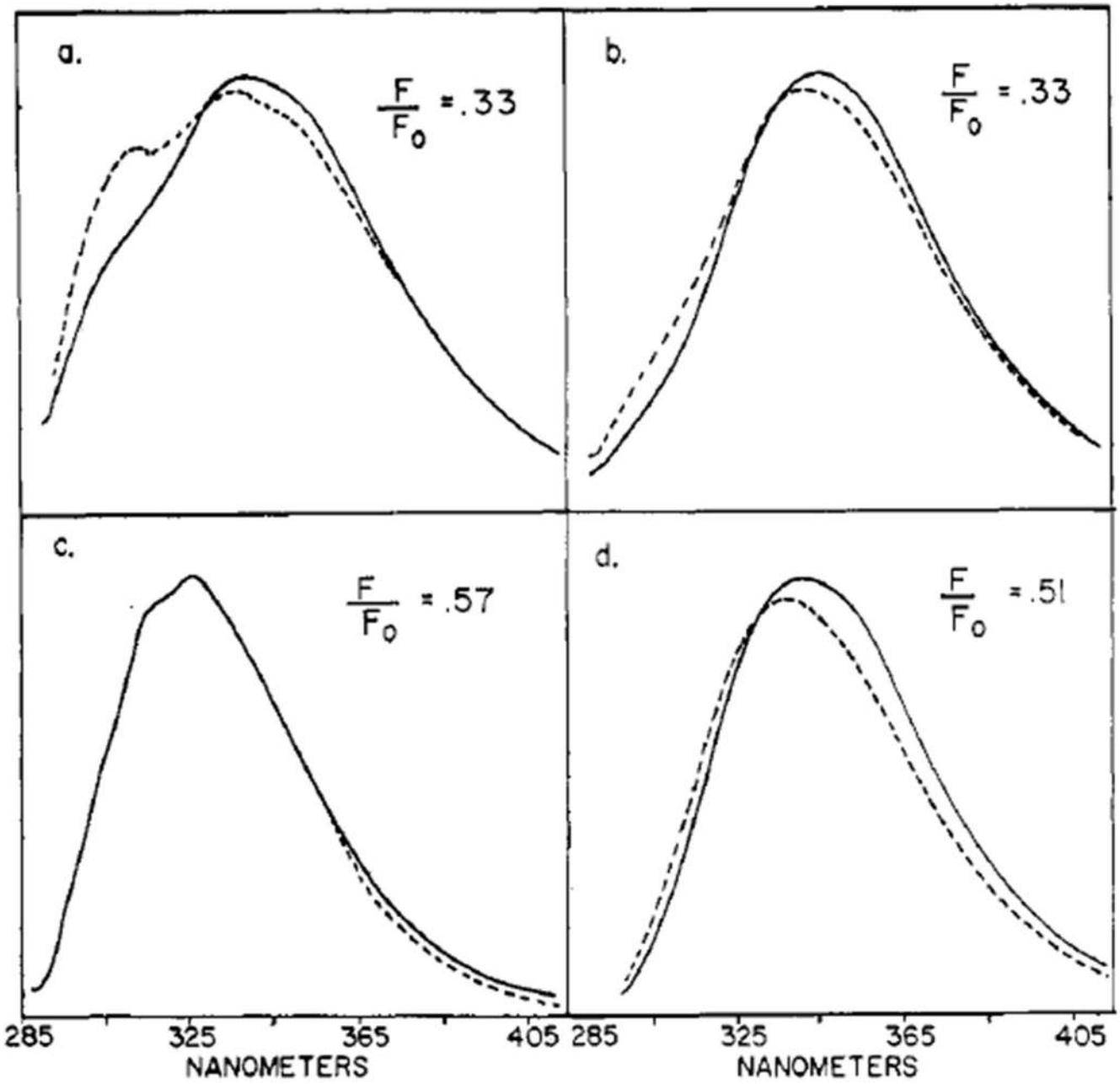

FIGURE 2:

Technical fluorescence emission spectra of proteins equilibrated with air and increased oxygen pressure. The fraction of the original fluorescence remaining is listed as F/F_0. Experimental conditions are listed in the footnotes to Table II: (a) human serum albumin; (b) bovine serum albumin; (c) aldolase; (d) carbonic anhydrase.

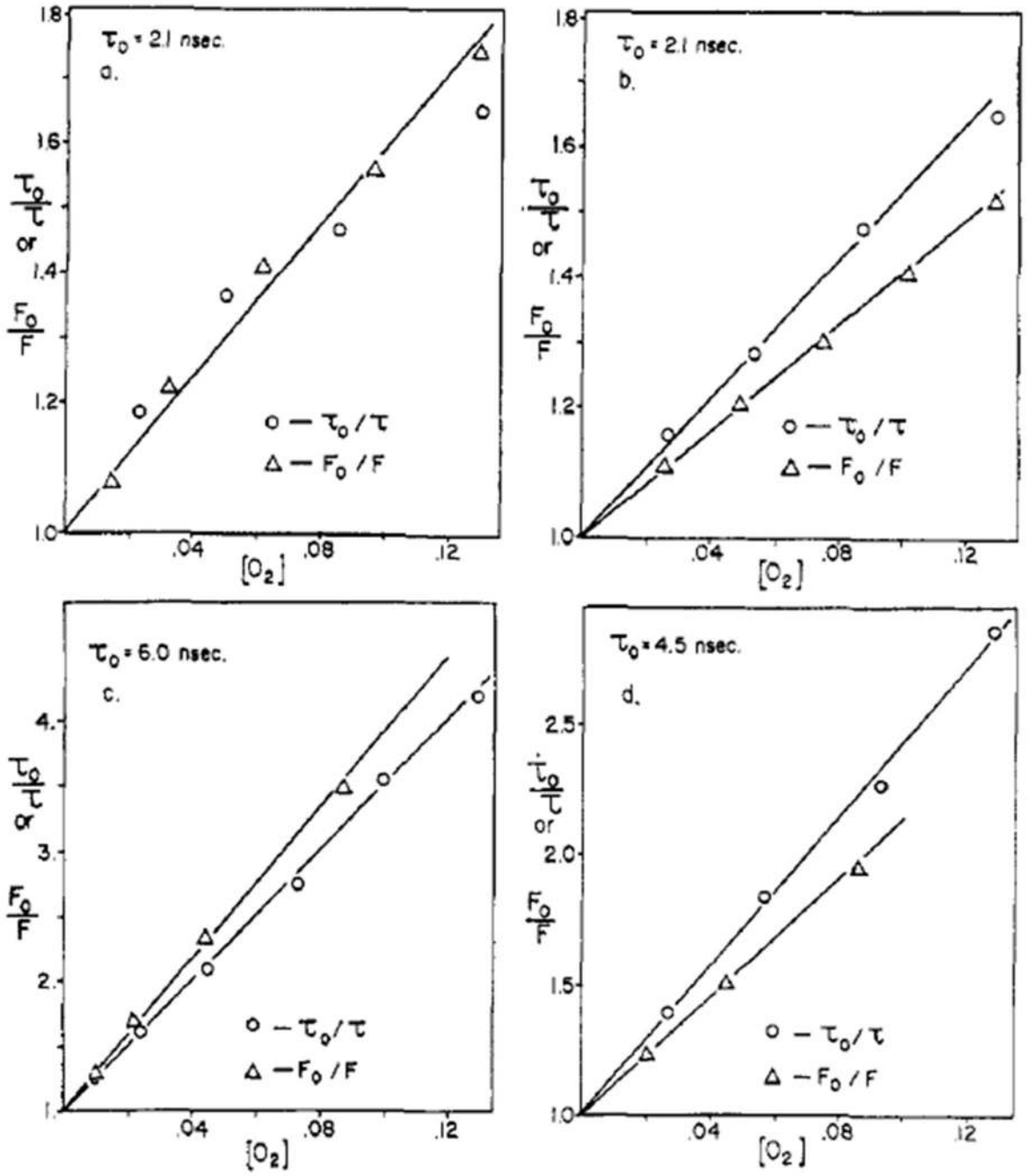

FIGURE 3:

Oxygen quenching of protein fluorescence as observed by fluorescence lifetimes (◯) and yields (Δ). Experimental conditions are listed in the footnotes to Table II and under Experimental Procedures: (a) aldolase; (b) α-chymotrypsin; (c) pepsin; (d) carbonic anhydrase.

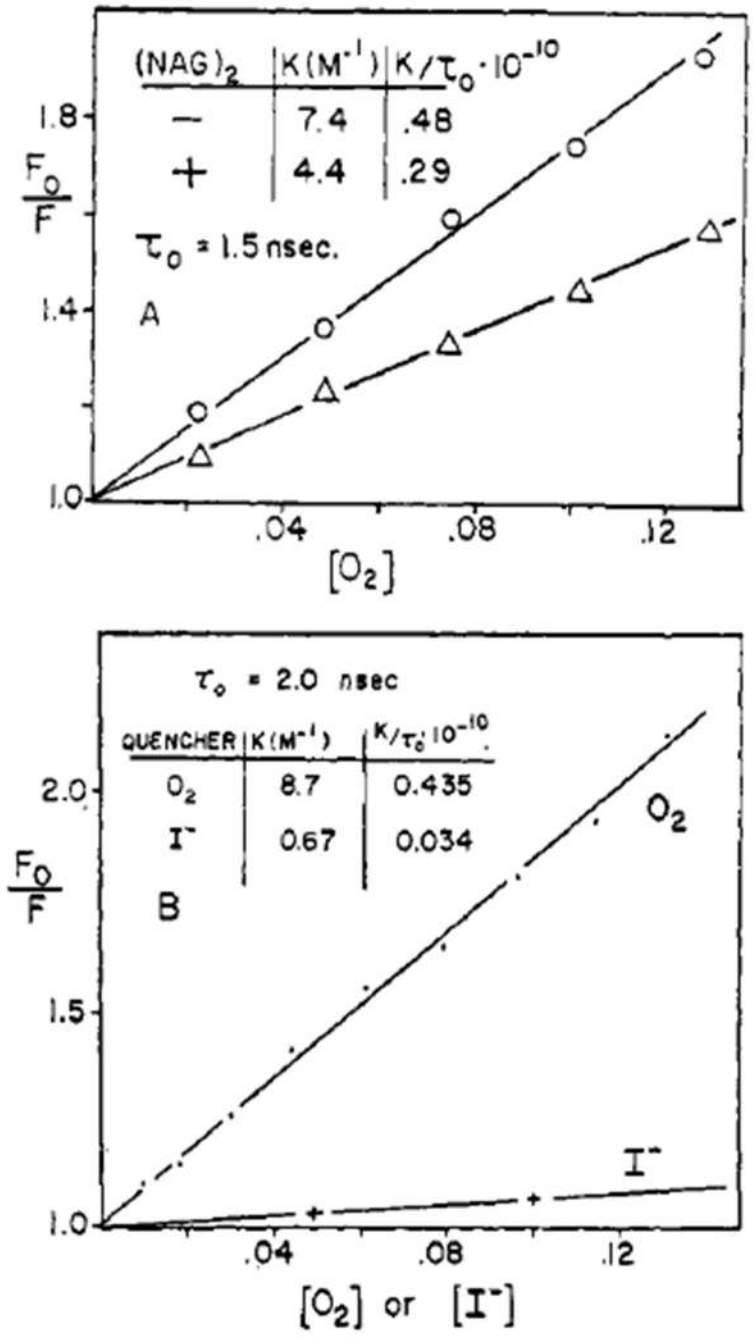

FIGURE 4:

(a) Oxygen quenching of lysozyme in the presence (Δ) and absence (◯) of $(N\text{-Ac-GlcN})_2$; 0.1 M sodium phosphate, pH 7; 280-nm excitation, 340- (no $(N\text{-Ac-GlcN})_2$) or 330-nm (with $(N\text{-Ac-GlcN})_2$ emission; [lysozyme] = 7.6×10^{-5} M, $[(N\text{-Ac-GlcN})_2] = 3.2 \times 10^{-3}$ M. (b) Comparison of oxygen and iodide quenching of trypsinogen; O_2 quench: 0.001 M HC1, 280-nm excitation; I^- quench: 0.001 M HC1, 290-nm excitation, [KCl] + [KI] = 0.50 M, *ca.* 10^{-4} M $Na_2S_2O_3$; 334-nm emission was used for both samples.

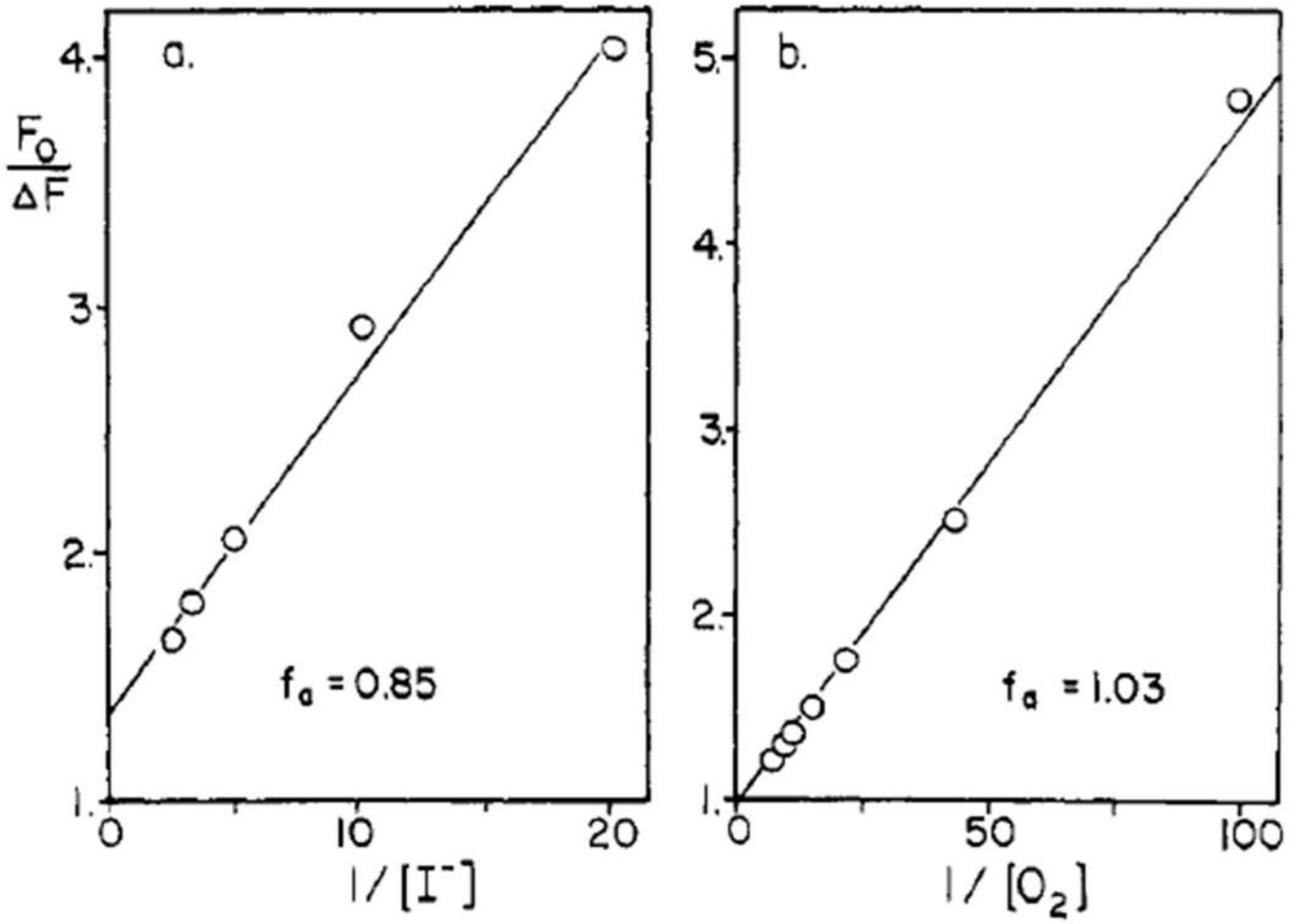

FIGURE 5:

Modified Stern–Volmer plots to determine the fraction of accessible tryptophans in pepsin by iodide (a) or oxygen (b) quenching. Both samples were excited at 290 nm, and the emission intensity recorded at 342 nm: I^- quench, 0.01 M HCl, [KCl] + [KI] = 0.5 M, *ca.* 10^{-4} M $Na_2S_2O_3$; O_2 quench, 0.01 M HC1.

TABLE I:

Effect of Oxygen Pressure on the Specific Activity of Enzymes.[a]

	Sp Act. (Units/mg)	
Enzyme	**Air**	**1500 psi of O_2**
Carboxypeptidase A	56.1	58.5
Carbonic anhydrase	1450	1500
Trypsin	204	205
α-Chymotrypsin	29.8	28.3
Lysozyme	555	560

[a]The special procedures required for these assays are described in detail in the previous paper.

TABLE II:

Oxygen Quenching Constants and Fluorescence Lifetimes for Proteins.[a]

Proteins	K (M^{-1})	τ_0[b] (nsec)	$K/\tau_0 \times 10^{-10}$ (M^{-1} sec^{-1})	Fluorescence Emission Max
α-Chymotrypsin[c]	4.1	2.1	0.20	332
Bovine serum albumin[d]	15.2	6.2	0.24	342
Human serum albumin[d]	14.7	6.0	0.25	342
Edestin[e]	8.0	3.2	0.25	328
Carbonic anhydrase[f]	11.4	4.4	0.26	341
Aldolase[c]	6.1	2.3	0.27	328
Azurin[d,i]	10	3.4	0.30	
Carboxypeptidase A[f]	6.4	1.7	0.38	330
Trypsinogen[c]	8.7	2.0	0.43	334
IgG[d]	10.1	2.2	0.46	332
Lysozyme[d]	7.4	1.5	0.48	340
Pepsin[g]	28.6	5.5	0.52	342
Trypsin[c]	10.2	1.9	0.54	335
Ribonuclease[h]	8.6	1.2	0.71	304

[a] 280 ± 2 nm excitation was used for all proteins. In determining the Stern–Volmer quenching constant (K), the fluorescence intensity was monitored at the maximum of emission (±3 nm).

[b] For lifetime measurements a Corning 7-54 filter was used to block the parasitic visible light which is particularly evident with the 0.25-m Jarrell-Ash monochromator used for excitation. The fluorescence emission was observed through a Corning 0-54 filter.

[c] 0.001 M HC1, pH 3.

[d] 0.1 M sodium phosphate, pH 7.0.

[e] 0.05 M sodium phosphate + 1 M NaCl, pH 7.0.

[f] 0.025 M Tris-HCl + 0.5 M NaCl, pH 7.5.

[g] 0.01 M HC1.

[h] 0.005 M sodium phosphate, pH 7.14.

[i] The fluorescence intensity of the tryptophan was followed at 355 nm, which does not correspond to the fluorescence emission maximum.

TABLE III:

Quenching Constants and Lifetimes for Proteins Using 305-nm[a] Excitation.

Protein[b]	K (M^{-1})	τ_0 (nsec)	$K/\tau_0 \times 10^{-10}$ (M^{-1} sec^{-1})	% Change[c]
Pepsin	22.9	4.7	0.48	−16
Lysozyme	6.7	1.7	0.39	−19
Carboxypeptidase A	4.5	1.7	0.25	−34
α-Chymotrypsin	4.1	1.8	0.23	+ 15

[a] 2-nm bandpass.

[b] Buffers are listed in Table II.

[c] Per cent change relative to $K/\tau()$ measured with 280-nm excitation.

TABLE IV:

Oxygen Stern–Volmer Quenching Constants as Observed by Fluorescence Lifetimes.

Protein	K (M^{-1}) (from τ_0/τ)	% Change[a]
α-Chymotrypsin	5.3	+29
Carbonic anhydrase	14	+22
Aldolase	6.1	0
Trypsinogen	8.7	0
Trypsin	10	−2
Pepsin	25	−13
Carboxypeptidase A	4.7	−27
Azurin	7.0	−30
Bovine serum albumin	9.0	−43
Human serum albumin	6.5	−53

[a]Per cent change from the Stern–Volmer constant obtained from measurement of fluorescence yield *vs.* oxygen concentration.

TABLE V:

Iodide Quenching Constants for Proteins.[a]

Protein	K (M^{-1})	$K/\tau_0 \times 10^{-10}$ (M^{-1} sec^{-1})
Trypsinogen	0.67	0.034
Carboxypeptidase A	0.54	0.032
Carbonic anhydrase	0.85	0.019
Pepsin	11.2–7.7[b]	0.21–0.14[b]

[a] 290 ± 2 nm excitation was used. The buffers described in Table II were used except for [KC1] + [KI] = 0.5 M and *ca.* 10^{-4} M $Na_2S_2O_3$.

[b] Downward curvature was seen in the Stern–Volmer plot.

TABLE VI:

Quenching Constants[a] and Lifetimes in 6 M Guanidine Hydrochloride.

Fluorophore or Protein	Emission Max	τ_0 (nsec)	K (M^{-1}) Oxygen	K (M^{-1})[c] Iodide
Indole[b]	350	3.5	22.9	15.8
Tryptophan[b]	355	2.5	14.7	4.82
N-Ac-Trp-NH_2[b]	355	2.7	19.8	5.79
Pepsin	347	4.6	19.5	8.27
Trypsinogen	352	2.7	16.5	3.32
Carboxypeptidase A	355	3.0	11.3	3.05
Carbonic anhydrase	355	2.8	11.8	3.0

[a] 290 ± 2 nm excitation was used for all solutions.

[b] 0.1 M sodium phosphate, pH 7, in addition to 6 M Gdn·HCl.

[c] For all the iodide quenching solutions [KC1] + [KI] = 0.5 M, *ca.* 10^{-4} M $Na_2S_2O_3$.

TABLE VII:

Oxygen and Iodide Bimolecular Quenching Constants for Fluorophores in the Presence and Absence of 6 M Guanidine Hydrochloride.

		$K/\tau_0 \times 10^{-10}$ (M^{-1} sec^{-1})	
Compound	**6 M Gdn·HCl**	**Oxygen**	**Iodide**
Indole	–	1.23	0.834
	+	0.65	0.450
Tryptophan	–	1.2	0.392
	+	0.59	0.193
N-Ac-Trp-NH_2	–	1.16	0.383
	+	0.73	0.214

TABLE VIII:

Normalized[a] Oxygen and Iodide Bimolecular Quenching Constants for Proteins.

		$K/\tau_0 \times 10^{-10}$ (M^{-1} sec^{-1})	
Protein	**6 M Gdn·HCl**	**Oxygen**	**Iodide**
Pepsin	–	0.57	0.21–0.14
	+	0.82	0.35
Trypsinogen	–	0.43	0.034
	+	1.17	0.24
Carboxypeptidase A	–	0.38	0.032
	+	0.73	0.21
Carbonic anhydrase	–	0.26	0.019
	+	0.81	0.20

[a]All values in 6 M Gdn·HCl were multiplied by 1.92 for reasons described in the text.