A Double-Quenching Method for Studying Protein Dynamics: Separation of the Fluorescence Quenching Parameters Characteristic of Solvent-Exposed and Solvent-Masked Fluorophors†

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ABSTRACT: A novel experimental method, suitable for separate analysis of the quenching parameters characteristic of solvent-exposed and solvent-masked fluorophors of macromolecules, is described. The method is based on the modified Stern–Volmer analysis and requires simultaneous application of two kinds of quencher: one that can selectively quench the emission of exposed fluorophors (e.g., ionic quenchers) and another that is nonselective (e.g., oxygen or, in many cases, acrylamide), capable of quenching the fluorescence of both exposed and masked groups. In order to examine the accuracy of the model, a computer simulation was performed. The results showed that the errors are comparable to those arising from the conventional quenching experiments. The method is applicable to phosphorescence quenching as well and is extendable to time-resolved measurements (by replacing fluorescence intensities with lifetimes). The method was applied to resolve the quenching parameters of lysozyme fluorescence by the use of iodide as selective and acrylamide as nonselective quenchers. The determination of the acrylamide quenching constant associated with the internal fluorophor, Trp-108 (K_q = 3.5 M^{-1}), permits specific studies on the dynamics of internal regions of the protein. The quenching constant determined for the more exposed residue Trp-62 (K_q = 1.6 M^{-1}) provides local information about the surface independent of the electrostatic effects observed when an ionic quencher is used.

Fluctuational behavior is a characteristic property of the collection of atomic particles composing macromolecules such as proteins. The possible role of the internal dynamics of enzyme molecules in their catalytic and regulatory functions has been given increasing attention in the last 10 years or so (Somogyi & Damjanovich, 1971, 1975; Damjanovich & Somogyi, 1973; Gurd & Rothgeb, 1979; Karplus & McCammon, 1981; Somogyi et al., 1984a). A number of physical methods have been developed for studying protein dynamics, both in the crystalline state and in solution. One such method, the quenching of tryptophan fluorescence, has become a powerful tool for studying nanosecond structural fluctuations of protein molecules in solution (Lakowicz & Weber, 1973; Eftink & Ghiron, 1975, 1977; Barboy & Feitelson, 1978; Matko et al., 1980).

The fluorescence behavior of tryptophan residues in a native protein is not uniform, i.e., each residue is characterized by unique microenvironmental properties. Depending on their location in the protein matrix, they may be fully or partially exposed to the solvent (Lee & Richards, 1971; Richards, 1977). Ionic quenchers (I^-, NO_3^-, Br^-, Cs^+, etc.), due to their charged and strongly hydrated character, can extinguish only fluorescence emitted by fluorophors located at, or near to, the surface of the protein (Lehrer, 1971; Burnstein et al., 1973). Oxygen (a nonionic quencher), in contrast, can penetrate the protein matrix and quench all the tryptophan residues, buried and exposed (Calhoun et al, 1983a,b; Hagaman & Eftink, 1984; Gratton et al., 1984; Jameson et al., 1984; Lakowicz & Weber, 1973). In the case of another nonionic quencher, acrylamide, there is no clear evidence as to whether it penetrates the protein matrix (thereby reporting on the internal fluctuation of the macromolecule) or whether it quenches the fluorescence of the buried fluorophors only when they are transiently exposed to the solvent (Calhoun et al., 1983a,b; Englander & Kallenbach, 1983; Hagaman & Eftink, 1984; Eftink & Ghiron, 1984). In this latter case, the acrylamide quenching data also contain information about protein dynamics; but the interpretation will be different from what is used in the case of penetration. Similarly, the oxygen quenching data of proteins presenting both solvent-exposed and buried fluorophors contain mixed information.

Such considerations indicate that the separation of quenching parameters associated with buried and exposed fluorophors is of special importance for the further understanding of protein fluctuations studied by fluorescence, or phosphorescence, quenching. In a recent attempt to determine the acrylamide quenching constant for the buried tryptophan (Trp-314) of horse liver dehydrogenase (Eftink & Selvidge, 1982), the authors used iodide, in relatively high concentration (0.45 M), to quench about 95% of the fluorescence of the exposed tryptophan. Subsequently, acrylamide was added to the mixture; the decrease of fluorescence then characterized the acrylamide quenching of the buried tryptophan. A similar approach was used by Hagaman & Eftink (1984) to study the fluorescence quenching of the same tryptophan residue by oxygen. In this case, 1 M acrylamide was used to quench the surface tryptophan residue (Trp-15). Although this approach seems attractive and simple, there are several problems asso-

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ciated with it. For example, it is impossible to achieve 100% quenching of the exposed fluorophor. The remaining fluorescence then might generate a considerable error. Further, it is difficult to generalize the method owing to the uncertainty of the quencher concentration necessary to quench "totally" the fluorescence of the exposed fluorophors. Another difficulty might also arise from the potentially high concentration of the "selective" quencher since it could result in gross conformational transitions, or even denaturation, of the protein (Eftink & Selvidge, 1982).

Thus, it is important to find a suitable method using "mild" conditions to separate the quenching parameters related to the "internal" and "external" fluorophors. Our principal aim was to develop such a method, with general applicability ensured by use of low (or moderate) quencher concentrations.

THEORY

Let us consider a hypothetical protein having three different classes of intrinsic fluorophors: (a) exposed (external), (b) buried (internal), and (c) nonquenchable. The emission intensities for these classes, F_{0e} ($=\alpha F_0$), F_{0i} ($=\beta F_0$), and F_{0n} ($=\gamma F_0$) respectively, denote the appropriate fractions of the total fluorescence intensity, F_0. Therefore

$$\alpha + \beta + \gamma = 1 \tag{1}$$

According to Eftink & Ghiron (1976), in the presence of nonionic quencher, Q_1, which can quench both external and internal fluorophors with quenching constants K_{1e} and K_{1i}, respectively, we have

$$\frac{F_1}{F_0} = \frac{\alpha}{1 + K_{1e}[Q_1]} + \frac{\beta}{1 + K_{1i}[Q_1]} + \gamma \tag{2}$$

where F_1 is the fluorescence intensity at the quencher concentration $[Q_1]$. It is assumed that the quenching constants of the applied quenchers for the γF_0 fraction of the total fluorescence are equal to 0. The subscripts i and e refer to the fluorescence of internal (buried) and external (exposed) fluorophors, respectively. It is further assumed that all the external and internal fluorophors are homogeneous (or quasi-homogeneous) populations with respect to quenching. Therefore, two constants, K_{1i} and K_{1e}, will describe the collisional quenching over the whole concentration range of the quencher concentration.

Adding an ionic (selective) quencher, Q_2, to the mixture containing the protein and the nonionic (nonselective) quencher, one obtains

$$\frac{F_{1,2}}{F_0} = \frac{\alpha}{1 + K_{1e}[Q_1] + K_2[Q_2]} + \frac{\beta}{1 + K_{1i}[Q_1]} + \gamma \tag{3}$$

where $F_{1,2}$ is the fluorescence intensity when both types of the quencher are present. Here it is assumed that the static quenching by both quenchers can be neglected.

Combining eq 2 and 3, we obtain

$$\frac{\Delta F}{F_0} = \frac{F_1 - F_{1,2}}{F_0} = \frac{\alpha}{1 + K_{1e}[Q_1]} - \frac{\alpha}{1 + K_{1e}[Q_1] + K_2[Q_2]} \tag{4}$$

Rearrangement of eq 4 leads to

$$\frac{F_0}{\Delta F} = \frac{1 + K_{1e}[Q_1]}{\alpha} + \frac{(1 + K_{1e}[Q_1])^2}{\alpha K_2}\frac{1}{[Q_2]} \tag{5}$$

Equation 5 shows that, by maintaining $[Q_1]$ constant and changing the value of $[Q_2]$, the plot of $F_0/\Delta F$ against $1/[Q_2]$ results in a straight line with the slope

$$\xi = \frac{(1 + K_{1e}[Q_1])^2}{\alpha K_2} \tag{6}$$

and with the intercept

$$\omega = \frac{1 + K_{1e}[Q_1]}{\alpha} \tag{7}$$

The above equations are not unique. If one considers quenching as the equivalent of the competitive inhibition of tryptophan-"catalyzed" conversion of absorbed quanta to emitted quanta and if we further assume that the affinity for light is high ($K_m > S$), then eq 2 becomes another version of the Dixon plot of enzymology. The further algebraic manipulations and plots are consequently similar to those used with steady-state enzyme kinetics.

Equation 5 contains three unknowns, K_{1e}, K_2, and α; thus, in order for eq 5 to be useful, we have to obtain independent estimates for two of the unknowns. In the presence of only the ionic quencher, $[Q_1] = 0$, eq 5 reduces to the modified Stern–Volmer equation (Lehrer, 1971). The variables ξ and ω, denoted for such a case with subscript o, reduce to

$$\omega_o = \alpha^{-1} \tag{8}$$

$$\omega_o/\xi_o = K_2 \tag{9}$$

A separate experiment then allows us to determine K_2 and α. With these values inserted into eq 5, we can estimate K_{1e}. Here we have to note that eq 5 contains information about the value of K_{1e} both in its intercept and slope. Owing to the low level of the quencher concentration, the error of the intercept is usually higher than that of the slope. Therefore, it seems more appropriate to use the slope of eq 5 to determine the value of K_{1e}. Taking the square root of eq 6, we arrive at

$$\xi^{1/2} = \frac{1}{(\alpha K_2)^{1/2}} + \frac{K_{1e}}{(\alpha K_2)^{1/2}}[Q_1] \tag{10}$$

The value of K_{1e} can be obtained by dividing the slope of the $\xi^{1/2}$ vs. $[Q_1]$ plot by its intercept.

Having now one more parameter (K_{1e}) calculated, we move on to determine the values of K_{1i}, γ, and β. For this, let us rearrange eq 2 into the form:

$$X = \left(\frac{F_1}{F_0} - \frac{\alpha}{1 + K_{1e}[Q_1]}\right) = \frac{\beta}{1 + K_{1i}[Q_1]} + \gamma \tag{11}$$

The expression on the left-hand side of eq 11, X, can be evaluated by using the known values of α and K_{1e} and the measured values of F_1 and F_0. Denoting the value of X at $[Q_1] = 0$ by X_0, we write

$$\frac{1}{X_0 - X} = \frac{1}{\beta} + \frac{1}{K_{1i}\beta}\frac{1}{[Q_1]} \tag{12}$$

Plotting $(X_0 - X)^{-1}$ vs. $[Q_1]^{-1}$ results in a straight line with intercept β^{-1} and slope $(\beta K_{1i})^{-1}$. Dividing the intercept by the slope yields K_{1i} while the reciprocal value of the intercept gives β. By now using eq 1, the value of γ is readily available.

To assess the propagation of error through the series of plots represented by eq 5–12, we have carried out a series of computer-simulated experiments. We start by calculating quenching curves according to eq 5 (Figure 1), using a set of arbitrary, but fixed, parameters described in the legend to Figure 1. We then simulate a series of fictitious data points obtained from a system described by the same parameters. However, we added a random error to each of the experimental steps necessary for producing the fluorometric and concentration values appearing in eq 5. The difference between the

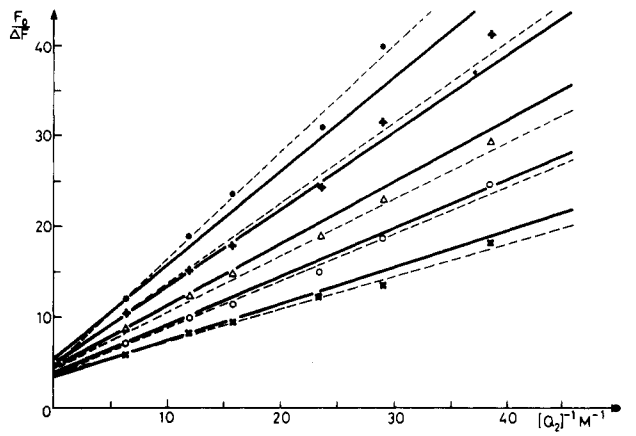

FIGURE 1: Solid lines represent $F_0/\Delta F$ as a function of $1/[Q_2]$ calculated according to eq 5 with $\alpha = 0.3$, $K_2 = 8.2\ M^{-1}$, $K_{1e} = 1.5\ M^{-1}$, and the following values of $[Q_1]$: 0 (×), 0.1 (○), 0.2 (△), 0.3 (+), and 0.4 M (●). The points represent simulated experimental values that were calculated with the same set of parameters but amended by adding to each step used in experimental calculation of ΔF, F_0, and $[Q_2]$ the random error, 0.5% for spectrofluorometry, and the appropriate dilution error. The dashed lines are fitted to the points by a least-squares procedure. The difference between the two sets of lines represents an estimate of the error produced by random error in the series of measurements carried out. (A whole family of simulated curves, 15, was then used to estimate the expected standard deviations, Figures 2 and 3).

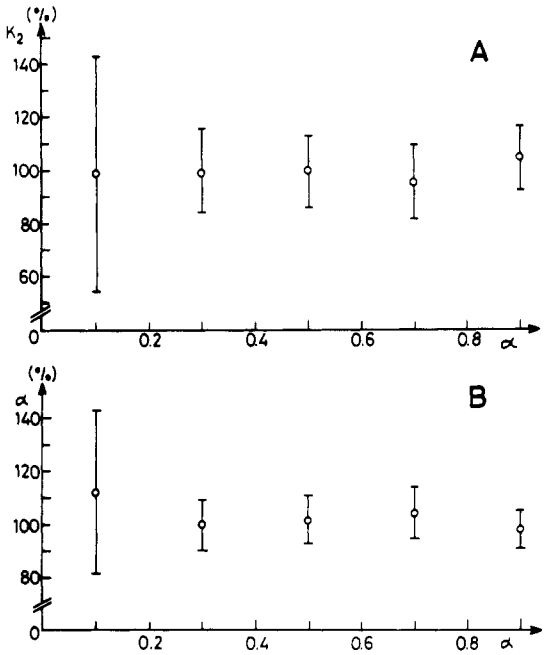

FIGURE 2: Mean percental values and standard deviations for estimates of K_2 (A) and α (B), obtained by averaging and least-squares fitting of 15 simulated runs produced as described in the legend to Figure 1, are plotted as a function of α. The initial values for constants K_2 and K_{1e} used in the calculations were the same as given in Figure 1.

calculated curves and the curves fitted to simulated points represents an example of the influence of random error in measurements.

The simulation is repeated 15 times, and the data are averaged. These data and further simulated data were used for similar comparisons of the curves described by eq 10 and 12. In addition to the values given in the legend to Figure 1, 0.6 and 4.5 M^{-1} were used for β and K_{1i}. The averaging of 15 simulated data sets allows us finally to estimate the error in constants K_2, α, β, K_{1i}, and K_{1e} obtained by use of the double-quenching method. The expected mean percental values

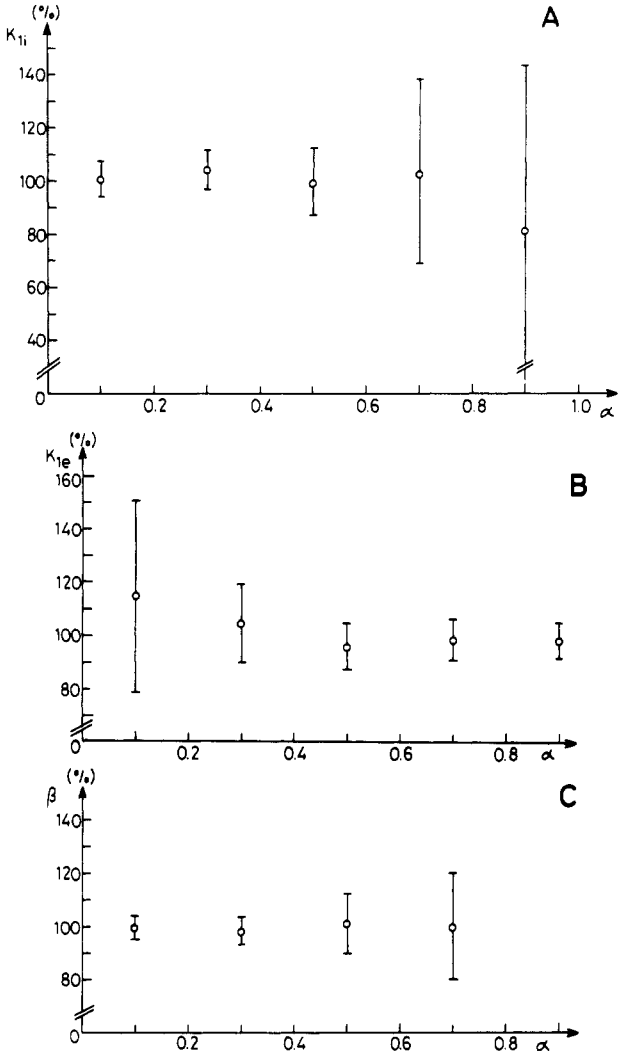

FIGURE 3: Mean percental values and standard deviations of K_{1i} (A), K_{1e} (B), and β (C) plotted as a function of α. The initial values for constants K_2 and K_{1e} were the same as before. For the other constants, $\beta = 0.6$, $\gamma = 0.1$, and $K_{1i} = 4.5\ M^{-1}$ values were used with the exception of γ, which was set to 0.05 when $\alpha = 0.9$. The data are from the averages of the 15 simulated runs.

and standard deviations for the experimentally determined K_2 and α are given in Figure 2 as a function of α. Figure 3 shows the mean percental values and standared deviations for K_{1i}, K_{1e}, and β again as a function of α. According to these data, the errors of β, K_{1e}, and K_{1i} are comparable with those of α and K_2. Note that α and K_2 are independently determined from the usual modified Stern–Volmer plot (see eq 5 when $[Q_1] = 0$); therefore, the comparison of their error with those of the other parameters represents a comparison of the two (the conventional and the presented) methods from the viewpoint of accuracy. As is shown by Figure 3, higher values of error for K_{1e} and K_{1i} occur when α is exceptionally small or large. This is due to the small values of ΔF (in eq 4 and 5) and X (in eq 11).

Materials and Methods

Lysozyme (egg white) was obtained from Worthington Biochemical Corp. KI (ultra pure) was a Merck product. Acrylamide (Serva) was recrystallized from acetone (Merck, spectroscopic grade) before use. All other chemicals (of analytical grade) were obtained from Reanal Chemical Corp. (Hungary). Fluorescence measurements were done on a Hitachi Perkin-Elmer MPF-4 spectrophotofluorometer operating in ratio mode and equipped with a thermostated cell

Table I: Collisional Quenching Constants for the Quenching of Lysozyme Fluorescence Evaluated by Double-Quenching Technique

quencher	collisional quenching constants (M^{-1})	fraction of accessible fluorescence to iodide, α	fraction of fluorescence accessible only to acrylamide, β	fraction of inaccessible fluorescence to both iodide and acrylamide, γ
iodide	$K_2 = 8.13$	0.33	0.63	
acrylamide	$K_{1e} = 1.62$			0.04
	$K_{1i} = 3.57$			
	$\bar{K}_{measd} = 3.02$			
	$\bar{K}_{calcd} = 3.03$			

holder. The analog output signal was monitored by a sampling unit and printed by a line printer. The collected data were computer-averaged. During the experiments, the temperature was held constant within 0.1 K. The fluorescence quenching experiments were performed in 1 × 1 cm quartz cells. Tryptophan residues were excited selectively at 297 nm with a 5-nm bandwidth for excitation.

The protein solutions containing acrylamide were prepared immediately prior to titration with the ionic quencher (KI). Protein fluorescence, monitored at 337 nm, was quenched by repetitive addition of different amounts of 2.2 M KI stock solutions, always containing acrylamide in the same concentration (0–0.4 M) as did the protein solution.

Factors were applied to correct the effects due to dilution and to optical density of acrylamide at the excitation wavelength. The purified acrylamide used in these experiments had a molar extinction coefficient of 0.24 M^{-1} cm^{-1} at 297 nm, measured with a Beckman ACTA CV spectrophotometer. Both experimental and calculated data were routinely fitted by standard linear regression methods.

Results

The method described under Theory was applied to the quenching of lysozyme fluorescence by iodide and acrylamide. The presence of the two quenchers together caused no change in the shape of the fluorescence emission spectra, but at the highest concentration of iodide (180 mM) the peak of the fluorescence emission spectrum shifts (by about 2–3 nm) to lower wavelength. This observation is in agreement with earlier data (Lehrer, 1971) showing spectral changes upon addition of I^-. Figure 4 shows the iodide quenching curves measured with different concentrations of acrylamide (0–0.4 M) and plotted according to eq 5. The quenching curve at zero concentration of the quencher Q_1 (acrylamide) is identical with the modified Stern–Volmer plot introduced by Lehrer (1971). All of the quenching curves were linear at the applied concentrations of the two quenchers (see legend to Figure 4). The quenching constant (K_2) characterizing the iodide quenching of the tryptophans and the fraction of accessible fluorescence (α) were determined with data from Figure 4 and are collected in Table I.

In order to obtain the acrylamide quenching constant (K_{1e}) characteristic of the external fluorophor, the square roots of the slopes of the curves shown in Figure 4 ($\xi^{1/2}$) were calculated and replotted against the acrylamide concentration. This plot gave a good fit to a linear function, allowing estimation of K_{1e} (see Table I).

In order to calculate the acrylamide quenching constant (K_{1i}) characterizing the buried tryptophans (i.e., those inaccessible to iodide), $(X_0 - X)^{-1}$ values (see eq 12) were plotted against the inverse of the acrylamide concentration (data not shown). The values of K_{1i} and β (see Table I) were directly obtained from this plot, which showed no deviation from the linear curve expected. The value of γ was calculated according to eq 1.

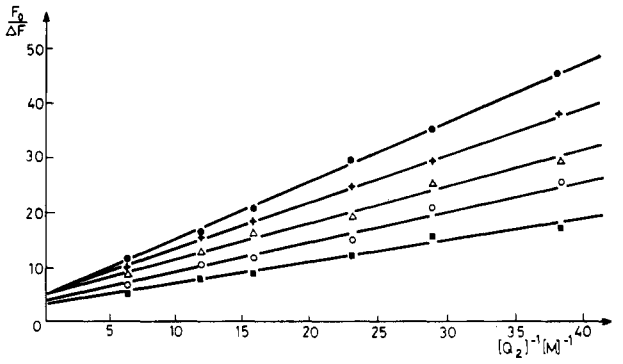

FIGURE 4: Experimentally measured quenching of lysozyme by iodide (Q_2) in the presence of varying amounts of acrylamide (Q_1) plotted according to eq 5. The acrylamide concentrations were as follows: 0 (×), 0.1 (○), 0.2 (△), 0.3 (+), and 0.4 M (●). The concentration of iodide was varied from 0 to 0.18 M. The buffer was on all occasions 50 mM phosphate, pH 7.0, containing 0.1 mM $Na_2S_2O_3$ (to prevent I_3^- formation). The excitation wavelength was 297 nm, and the emission was monitored at 337 nm. A bandwidth of 5 nm was used in all measurements. The temperature was kept constant at 303 ± 0.1 K.

The fluorescence of lysozyme was also quenched by acrylamide alone in a separate experiment. The modified Stern–Volmer plot was found to be linear. To compare this independent determination directly with the results of double-quenching experiments, we have also plotted a calculated Stern–Volmer plot with the values for α, β, K_{1e}, and K_{1i} obtained from the double-quenching experiment reported. The average quenching constant $\bar{K}$ is calculated from both the experimental and the calculated curve. The values 3.02 and 3.03 M^{-1} show a gratifying agreement.

Discussion

In biological macromolecules many different types of fluctuation (e.g., in energy, volume, and conformation) occur (Lumry & Gregory, 1985). Since protein molecules interact dynamically with the solvent medium, the system also exhibits various kinds of fluctuation at the interface (Careri et al., 1979). While some methods (e.g., NMR and isotope exchange) have been used widely to study structural fluctuations at the "slower" end of the time scale (Woodward et al., 1982; Gregory & Rosenberg, 1985), fluorescence spectroscopy yields insight into the nanosecond dynamics of protein molecules (Hevessy et al., 1981; Haas & Steinberg, 1984; Matko et al., 1984; Somogyi et al., 1984b). The fluorescence quenching method was first introduced, as a probe for protein dynamics, by Lakowicz & Weber (1973). Since then, a number of studies have demonstrated the usefulness of this method as an indicator of the structural fluctuations within protein matrices (Eftink & Ghiron, 1975, 1981; Barboy & Feitelson, 1978; Matko et al., 1980; Gratton et al., 1984; Jameson et al., 1984).

The interpretation of quenching data for multitryptophan proteins having both exposed and buried residues is, however,

not necessarily straightforward. Experiments using acrylamide as a quencher are especially troublesome, because both buried and exposed residues can be quenched, but to a differing degree. The average quenching constant (a weighted average of the individual constants belonging to different classes of emitters) used for describing such systems can at best provide only a first approximation of the pattern of quenching reactions involved.

The method presented here provides a separation of the quenching parameters belonging to the buried and exposed subpopulations of the fluorophors. Since the quenching of fluorescence from these two subgroups of emitters probably occurs by different mechanisms (reporting on the internal and surface dynamics of the macromolecule, respectively), this separation makes it possible to study selectively two different aspects of protein dynamics.

The method described here is based on the well-known assumption of steady state and on a new variation of the classic Stern–Volmer type of experimental approach. We simultaneously use two quenchers, one selectively quenching only the emission from solvent-exposed residues and another, nonselective quencher able to quench emission from both exposed and buried fluorophors.

The plot shown in Figure 4 is a set of data from a quenching experiment carried out by using the selective quencher (I^- in the present case) to titrate the sample at different concentrations (but constant during each titration) of the nonselective quencher (acrylamide). When the sample does not contain nonselective quencher, the titration data submit to the modified Stern–Volmer plot (the lowest curve on Figure 4) and may be used to determine the quenching constant, K_2, and the accessibility, α, for the selective quencher.

Following eq 10, the slopes of the curves in Figure 4 are further processed and used to get a secondary plot for the evaluation of K_{1e}, the quenching constant of the nonselective quencher for the exposed fluorophors. The primary data (after correcting for dilution and inner filter effects) are then used, along with the values of α and K_{1e} (already determined from the primary and secondary plots), to calculate the fraction of fluorescence from the buried emitters in the presence of quenchers, X (see eq 11). Further, by a tertiary plot, one can determine the quenching constant, K_{1i}, of the nonselective quencher (acrylamide) for the buried emitters and the fraction of fluorescence quenchable only by the nonselective quencher, β (see eq 12). Finally, to get the fraction γ (the fraction of fluorescence that is nonquenchable by the applied quenchers) one has only to subtract the values of α and β from 1 (see eq 1).

To examine the accuracy of the method, computer simulations were carried out. Although this simulation cannot be regarded as a complete error analysis, it shows that the parameters α and K_2 (which are evaluated in exactly the same way as for a regular analysis using the modified Stern–Volmer plot) have similar errors as β, K_{1i}, and K_{1e} obtained from our analysis. The accuracy of our mehod then is comparable to that in the usual treatment of quenching data.

The method is equally applicable for the quenching analysis of both fluorescence and phosphorescence. For phosphorescence quenching, one usually neglects the presence of static quenching, since the excited-state lifetime is long enough (up to seconds) to ensure the dominance of dynamic quenching (i.e., significant extent of dynamic quenching occurs in the concentration range of quencher where the static component is practically absent). The presence of a static component, however, has to be considered when the method is applied to fluorescence. The static component for globular proteins is usually about one-tenth, or so, of the dynamic one and can often be neglected (Eftink & Ghiron, 1976). When static quenching is significant, it usually manifests itself as an upward curvature of the F_0/F plot. In our case, its presence should result in curvature of the primary, secondary, or tertiary plots, depending on which class of the emitters experience static quenching (and which of the quenchers is responsible for it). The lack of any curvature, along with other observations (Lehrer, 1971; Eftink & Ghiron, 1976), indicates that neither the iodide nor the acrylamide produces significant static quenching.

In general, the presence of static quenching introduces an extra complication. For a simple Stern–Volmer plot, the static contribution can be estimated from the magnitude of nonlinearity (Eftink & Ghiron, 1976). In our case, the presence of three different static contributions usually does not allow such empirical corrections. However, it should be pointed out that although we have used steady-state fluorescence intensities in our equations, a set of similar equations can be written by replacing intensities with the appropriate lifetimes. Studies of time-resolved fluorescence are not influenced by static quenching, and the use of similar sequences of plots as described here would provide estimates of the same set of quenching parameters.

A number of possible sources for systematic errors in such measurements have to be considered. Considering quencher effects on the conformation of lysozyme, acrylamide up to 0.4 M, did not cause denaturation (Eftink & Ghiron, 1976). Acrylamide has been reported to react with SH groups in proteins, which might affect either of the quenching constants determined. The effect, however, is unlikely in view of the observed reversibility of acrylamide quenching reactions (Matko et al., 1980). In titration with potassium iodide, it is usual to substitute part of KI for KCl to avoid the change of ionic strength. In our particular case, however, it was not necessary since it is shown that the change of ionic strength alone, up to 2 M KCl has no significant effect on the fluorescence of lysozyme (Altekar, 1977a,b). The simultaneous presence of two quenchers, if at sufficiently high concentration levels, could introduce a further complication. The activity coefficients of each of them could be influenced by the presence of the other. The relatively low concentrations of the quenchers and the observed linearity of the plots, however, argue against such an effect.

The possible effect of the existence of a charge-transfer complex between I^- and acrylamide (Eftink & Selvidge, 1982) has also been considered. On the basis of differential-absorption measurements (around 274 nm where the difference absorption maximum of the complex can be observed), we can conclude that the concentration of the complex did not exceed 1–2% of the total quencher concentration. Thus, its effect can be neglected here.

In order to convert the quenching constants into bimolecular rate constants, we started with the observations of Imoto et al. (1971) showing that about 90% of the total fluorescence intensity of lysozyme can be attributed to Trp-108 and Trp-62 residues. According to Lee–Richards criterion (Lee & Richards, 1971; Richards, 1977), Trp-62 is highly exposed to the solvent while Trp-108 is largely buried (Shrake & Rupley, 1973; Ichiye & Karplus, 1983). Then, by assigning the lifetimes $\tau_e = 2.04$ ns and $\tau_i = 2.26$ ns (Formoso & Forster, 1975) to the exposed and buried emitters, respectively, we can calculate the bimolecular rate constants. The rate constant for iodide was found to be 3.6×10^9 M^{-1} s^{-1} in agreement with

earlier data (Lehrer, 1971; Formoso & Forster, 1975). For acrylamide quenching, the bimolecular rate constants k_{qe} = 7.2×10^8 M^{-1} s^{-1} and k_{qi} = 1.7×10^9 M^{-1} s^{-1} were obtained. As expected, our constants are attenuated when compared to the bimolecular quenching constants for small indole compounds, 7×10^9 M^{-1} s^{-1}, and the value assigned to fully exposed residues in small peptides, 4×10^9 M^{-1} s^{-1} (Eftink and Ghiron 1976, 1977). The k_{qi} of 1.7×10^9 M^{-1} s^{-1} for Trp-108 lies between the values observed for human serum albumin and monellin, which in turn, lie by a factor of 5 below that obtained for RNase T_1, the most often used model for completely buried tryptophans. What is more interesting is that the more exposed residue (Trp-62), instead of being more easily quenched, is somewhat less accessible to acrylamide quenching. We have to remember, however, that, by considering rate differences described by a factor of 2, we are talking of activation energy differences below 1 kcal. Such differences can be accounted for by steric hinderance. Bimolecular rate constants for reactions between aromatic residues in proteins and organic reactants are notoriously sensitive to the nature of the reactant and the positioning of side chains around the reactive residue (Hallaway et al., 1980). Faster reaction rates for buried residues have been reported on many occasions (Skov et al., 1964; Beaven & Gratzer, 1968). Before attempting to interpret these differences in terms of structural parameters, we would have to ascertain that the chemical modifications used in lifetime experiments did not result in changes of lysozyme structure, a task outside the scope of this investigation.

In summary, it can be stated that, in spite of the experimental and theoretical limitations discussed, the method seems to be suitable for selective study of the dynamic exposure of Trp residues located inside and at the surface of the proteins, by the technique of double quenching of the protein fluorescence or phosphorescence.

Registry No. I^-, 20461-54-5; lysozyme, 9001-63-2; acrylamide, 79-06-1; L-tryptophan, 73-22-3.

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