GATED QUENCHING OF INTRINSIC FLUORESCENCE AND PHOSPHORESCENCE OF GLOBULAR PROTEINS

An Extended Model

BÉLA SOMOGYI, JOHN A. NORMAN, AND ANDREAS ROSENBERG
Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT We present a theoretical model to account for the quenching data of macromolecular fluorescence and phosphorescence when the accessibility to the quencher is gated by a dynamic mechanism coupled to the fluctuation of the macromolecular matrix. We show that the model currently in use to interpret gated quenching processes gives only approximate results in both qualitative and quantitative terms, and it can be regarded as a specific case of the presented model. We show that the gating dynamics affect both the apparent accessibility (α_{obs}) and K_{sv} values obtained by the modified Stern-Volmer plot. The effect of gating on α_{obs} and K_{sv} depends upon the relative rate of gating compared to the excited state lifetime. The model allows us to predict the effect of viscosity on quenching if it takes place by a gated mechanism. The prediction can and is, in this case, compared to the existing data on glycerol effects on acrylamide quenching of the tryptophan fluorescence in RNAse T_1. The result shows that a simple gated model is not compatible with the observed quenching behavior.

INTRODUCTION

The recognition that protein dynamics plays an important role in the biological functions of proteins is now shared by many investigators (McCammon and Karplus, 1980; Somogyi et al., 1984*a*). This is indicated by the ever-increasing number of articles dealing with the possible biological role of fluctuations in macromolecules. The search for methods able to give information about details of the dynamics of macromolecules is of high priority (Austin et al., 1974; Hevessy et al., 1981; Karplus and McCammon, 1981; Xu and Weber, 1982; Parak and Knapp, 1984; Haas and Steinberg, 1984; Somogyi et al., 1984b, 1985). One of such methods, the study of the quenching of built-in fluorophores like tryptophan in proteins by agents in solution was initiated by Lehrer (1967, 1971) and Lakowicz and Weber (1973*a*, *b*). The use of iodide and oxygen in these investigations was followed by use of acrylamide and other organic molecules (Eftink and Ghiron, 1975, 1984).

Such studies, besides determining the quenchable nature of specific residues, yield information about the structural dynamics of the protein matrix. The precise nature of the dynamic parameters reflected in the quenching parameters is, however, still subject to uncertainty due to the ambiguous nature of the kinetic models used to describe the process of quenching. The interpretation of fluorescence data, as well as of phosphorescence data, is extensively based on a simple steady state model borrowed from the hydrogen exchange literature (Barksdale and Rosenberg, 1982; Calhoun et al., 1983 *a*, *b*)

$$A_{cl}^{*} \underset{k_{cl}}{\overset{k_o}{\rightleftharpoons}} A_o^{*} + Q \xrightarrow{k_+[Q]} A_0. \qquad (1)$$

Q is the quencher molecule in solution; A and A^* are the ground and excited states of the fluorophore; subscripts cl and o stand for the nonaccessible (closed) and accessible (open) states. The constant k_+ is the biomolecular quenching constant characteristic of the rate of the encounter complex formed between the exposed fluorophore and the quencher molecule. Detailed analysis of the rate equation derived from the above reaction scheme has led to the development of a model able to account for the oxygen quenching of porphyrin fluorescence in Mb^{desFe} and Hb^{desFe} (Gratton et al., 1984; Jameson et al., 1984). The corresponding molecular mechanism was one where oxygen molecules penetrate or diffuse through the protein matrix which remains in the native conformation state. Such a mechanism is quite similar to the so-called solvent penetration mechanism proposed for hydrogen exchange under certain conditions (Barksdale and Rosenberg, 1982; Woodward et al., 1982). The penetration mechanism, while now generally accepted as valid for oxygen quenching (Calhoun et al., 1983*b*; Hagman and Eftink, 1984;

Dr. Somogyi's permanent address is Department of Biophysics, University of Medical School, Debrecen, H-4012 Debrecen, Hungary

Jameson et al., 1984), remains to be proven for other nonionic quenchers such as acrylamide (Eftink and Ghiron, 1984; Englander and Kallenbach, 1984). However, when considering ionic quenchers, such as iodide, the penetration scheme seems quite unlikely.

For quencher molecules which cannot penetrate the protein matrix, quenching of an internal fluorophore of a protein requires the fluorophore to become occasionally exposed to the solvent (and therefore to the quencher molecules as well). In other words, the quenching in this case is "gated" by the dynamic fluctuation of the protein matrix (bringing the fluorophore to the exposed state and back again to the buried one).

The present work will explore the consequences in terms of kinetic equations and parameters of such a model based on a more detailed scheme than the one presented in Eq. 1. We also discuss proposed experimental variables to help determine how this mechanism, with the resulting kinetic model, allows interpretation of quenching data to identify gated quenching phenomena.

THE MODEL OF GATED QUENCHING

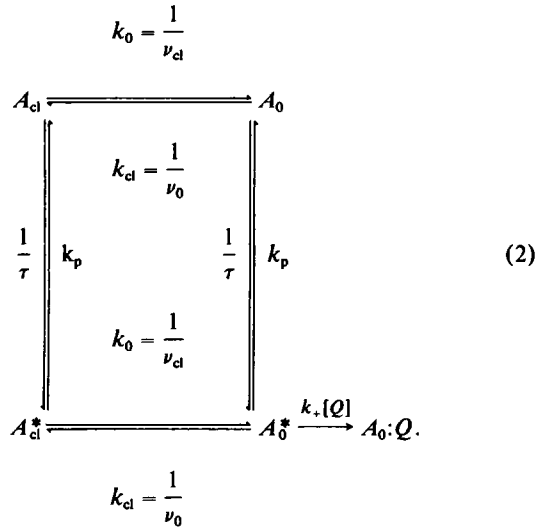
Regarding the molecular-level details of gated quenching, one first should consider the possible mechanism for the transient exposure of the otherwise buried (not exposed to the solvent) fluorophore. Basically, there are two processes able to account for transient exposure:

(*a*) Due to the relatively high level of fluctuations in the thermodynamic parameters such as volume, enthalpy, entropy, etc. of proteins (Cooper, 1976; Lumry and Gregory, 1986), there is a considerable probability that transient channels, or holes, open in the protein body forming direct connections between some, otherwise buried, protein groups and the solvent. These transient holes (which can be regarded as a kind of mobile-defect described by Lumry and Rosenberg, 1975) then guide the quencher molecules to the fluorophore. This view of gating is similar to the penetration model, since it is hard to assume that in each case these transiently formed holes close from the inside (to ensure that no water (or solvent) molecules are trapped within the protein body when closing is complete).

(*b*) A slightly different approach to transient exposure is offered by the transition state kinetics of the overall conformational fluctuations of proteins. The protein molecule, fluctuating around the free energy minima characterizing the native state, samples a number of high energy states along the side of the potential well towards the transition state. With certain probability it also samples the transition state, however, in some instances, it falls back to the native state instead of crossing the saddle point towards the unfolded state. Such substates of high energy are often described as being in partially or locally unfolded states (Englander and Kallenbach, 1984), i.e. where the nucleus of unfolded chain has not reached the critical size necessary for the major unfolding to occur.

We emphasize that in both cases, many peptide groups are usually involved in the process by which the buried fluorophore becomes exposed and then relaxes back again to the non-accessible state. Therefore, instead of using a well-defined potential shape to describe the buried-exposed transition (Lee, 1983), we treat it as a result of the movement of many independent groups, i.e. as the superposition of many independent local fluctuations. This means that we regard the two states as being separated by a potential barrier which is surmounted with a relatively small probability. As a consequence, both states can be characterized by some average lifetime (ν_{op} and ν_{cl}) and exponential depopulation in time (which assumes that the system has no "memory," i.e. the transition probabilities are independent of time) (McCammon and Karplus, 1981).

In formulating a reaction scheme, any sophisticated model of gated quenching has to take into account the $A^* \rightarrow A$ transitions as well as transitions which can take place equally well in both exposed and buried (i.e. open and closed) states. Accordingly, instead of the sequential reaction scheme of Eq. 1, the following, extended scheme should be used:



(2)

In the analysis we assume that $k_p \ll \tau^{-1}$, i.e. $[A^*] \ll [A] \simeq [A_0]$ with $[A_0]$ the total fluorophore concentration. This is the assumption used to derive the Stern-Volmer equation and is valid for the usual illumination conditions utilized in quenching experiments. Further, it is also assumed that both the $A_{cl} \rightarrow A_{cl}^*$ and $A_0 \rightarrow A_0^*$ excitation steps occur with the same k_p pumping rate.

The mathematical description of the gated quenching characterized by the above scheme might use several approaches (see e.g., McCammon and Northrup, 1981; Szabo et al., 1982). What we have found to be convenient is a probabilistic approach concentrating on the characterization of the quenching process itself. Of course, it is also possible to solve the appropriate differential equation system at steady-state conditions. By doing this, we obtain

exactly the same result as by using the presented approach but with considerably more algebra.

Let α be the quenchable fraction of the fluorescence (or phosphorescence) intensity, F_0 (i.e., the fraction of fluorescence emitted by those fluorophores involved in gating). Then, β $(= 1 - \alpha)$ is the fraction which is not affected by the quencher at all. Then, the total intensity can be written as

$$F_0 = F_\alpha + F_\beta = F_0\alpha + F_0\beta. \tag{3}$$

Since the α fraction of F_0 is, by definition, involved in gated quenching, the fluorescence intensity, $F_{\alpha Q}$, coming from that fraction in the presence of quencher molecules can be expressed as

$$F_{\alpha Q} = WF_\alpha = WF_0\alpha, \tag{4}$$

where W is the probability that the fluorescence constituting the fraction α of F_0 will not be quenched.

We then define the different independent events that can occur in the closed and opened states of the fluorophore when it is in the excited state. Considering first the closed state, the probability ω_{dc} of the excited fluorophore decaying (by either emitting a photon or by transferring energy to neighboring groups) before it enters the open state is

$$\omega_{dc} = \int_0^\infty \left[1 - \int_0^{t'} f_0(t)\mathrm{d}t\right] f_{dc}(t']\mathrm{d}t', \tag{5}$$

where $f_{dc}(t)$ and $f_0(t)$ are the density functions describing the decomposition of the excited fluorophore and the closed to open transition, respectively. As stated earlier, the closed to open transition follows an exponential distribution in time, with ν_{cl} being the average lifetime of the closed state. Therefore, the form of $f_0(t)$ is

$$f_0(t)\mathrm{d}t = \frac{1}{\nu_{cl}} \exp\left[-\frac{t}{\nu_{cl}}\right]\mathrm{d}t. \tag{6}$$

The decay of the excited state to the ground state also follows an exponential distribution, with an average lifetime of τ. Therefore,

$$f_{dc}(t)\mathrm{d}t = \frac{1}{\tau} \exp\left[-\frac{t}{\tau}\right]\mathrm{d}t. \tag{7}$$

Following the application of Eqs. 6 and 7, ω_{dc} becomes simply

$$\omega_{dc} = \frac{\tau^{-1}}{\tau^{-1} + \nu_{cl}^{-1}} = \frac{\nu_{cl}}{\nu_{cl} + \tau}. \tag{8}$$

Similarly, the probability ω_0 that the excited fluorophore becomes exposed to the solvent (the system enters the open state) before its decay to the ground state is

$$\omega_0 = \int_0^\infty \left[1 - \int_0^{t'} f_{dc}(t)\mathrm{d}t\right] f_0(t')\mathrm{d}t' = \frac{\tau}{\nu_{cl} + \tau}. \tag{9}$$

Considering next the open state, let $f_{cl}(t)$, $f_q(t)$ and $f_{do}(t)$ be the density functions characterizing the open to closed transition, the quenching process and the excited state to ground state transition (all possible decay pathways excluding quenching), respectively. Accordingly, the probability ω_{cl} that the open to closed transition takes place before quenching or other decay of the excited state is

$$\omega_{cl} = \int_0^\infty \left[1 - \int_0^{t'} f_q(t)\mathrm{d}t\right]\left[1 - \int_0^{t'} f_{do}(t)\mathrm{d}t\right] f_{cl}(t')\mathrm{d}t'. \tag{10}$$

Similarly, the probability ω_{do} that deactivation occurs in the open state prior to quenching is

$$\omega_{do} = \int_0^\infty \left[1 - \int_0^{t'} f_q(t)\mathrm{d}t\right]\left[1 - \int_0^{t'} f_{cl}(t)\mathrm{d}t\right] f_{do}(t')\mathrm{d}t'. \tag{11}$$

Writing similar equations for $f_q(t)$, $f_{do}(t)$ and $f_{cl}(t)$ as in Eq. 6 (with the characteristic lifetime of the appropriate processes being $(k_+[Q])^{-1}$, τ and ν_0, respectively) and integrating, we obtain

$$\omega_{cl} = \frac{\tau}{\tau + \nu_0 + k_+[Q]\tau\nu_0} \tag{12}$$

$$\omega_{do} = \frac{\nu_0}{\tau + \nu_0 + k_+[Q]\tau\nu_0}. \tag{13}$$

Here $[Q]$ is the quencher concentration and k_+ is the bimolecular rate constant of the encounter pair formed by the exposed fluorophore and the quencher molecule. k_+ can be expressed (see, e.g., Eftink and Ghiron, 1981) in the classical Smoluchowski formulation

$$k_+ = p4\pi NDr. \tag{14}$$

Here r and D are the sum of the radii and the sum of the diffusion constants of the molecules forming the encounter pair, respectively, N is Avogadro's number per millimole, and p is a probability factor accounting for the steric relations of the "collision." Of course, the probability ω_q of quenching during the time while the fluorophore stays in the open state could be calculated in a similar way, but can simply be stated as

$$\omega_q = 1 - \omega_{cl} - \omega_{do}. \tag{15}$$

Having defined all of the probabilities characterizing the different pathways of the excited fluorophores, we define the probability W by taking into account every possible event resulting in deactivation excluding quenching. This results in the expression

$$W = P_{cl}\left[\omega_{dc} + \omega_0\left[\omega_{do} + \omega_{cl}\left[\omega_{dc} + \omega_0\left[\omega_{do} + \ldots\right]\right]\right]\right] + P_0\left[\omega_{do} + \omega_{cl}\left[\omega_{dc} + \omega_0\left[\omega_{do} + \omega_{cl}\left[\omega_{dc} + \ldots\right]\right]\right]\right], \tag{16}$$

where P_{cl} and P_0 $(= 1 - P_{cl})$ are the probabilities that the excited fluorophore can be found in the closed and open states, respectively. The two infinite series composing the

r.h.s. of Eq. 16 can be rearranged to

$$W = [P_{cl}(\omega_{dc} + \omega_0\omega_{do}) + P_0(\omega_{do} + \omega_{cl}\omega_{dc})] \sum_{i=0}^{\infty} \omega_o^i\omega_{cl}^i. \quad (17)$$

Using the relationship

$$\sum_{i=0}^{\infty} q^i = \frac{1}{1-q}, \quad 0 < q < 1, \quad (18)$$

the closed form of W reads

$$W = \frac{P_{cl}(\omega_{dc} + \omega_o\omega_{do}) + P_o(\omega_{do} + \omega_{cl}\omega_{dc})}{1 - \omega_o\omega_{cl}}. \quad (19)$$

To obtain expressions for P_o and P_{cl}, we consider a single fluorophore for a time period, t ($t \gg \nu_o, \nu_{cl}$), in which case the time the system spends in the closed state is $P_{cl}t$, while the time spent in open state is $(1 - P_{cl})t$. Since ν_o and ν_{cl} are the average lifetimes of the appropriate states

$$\frac{P_{cl}t}{\nu_{cl}} = \frac{(1 - P_{cl})t}{\nu_o} \text{ if } t \gg \nu_o, \nu_{cl}. \quad (20)$$

By rearranging Eq. 20

$$P_{cl} = \frac{\nu_{cl}}{\nu_{cl} + \nu_o}, \quad (21)$$

and consequently

$$P_o = \frac{\nu_o}{\nu_{cl} + \nu_o}. \quad (22)$$

By inserting the appropriate expressions for the different probabilities into Eq. 19 and using Eq. 4, we arrive at

$$\frac{F_{\alpha Q}}{F_\alpha} = W = \frac{1 + P_{cl}\dfrac{k_+[Q]\tau}{1 + \dfrac{\tau}{\nu_{cl}} + \dfrac{\tau}{\nu_0}}}{1 + \dfrac{\tau + \nu_{cl}}{\nu_{cl}}\dfrac{k_+[Q]\tau}{1 + \dfrac{\tau}{\nu_{cl}} + \dfrac{\tau}{\nu_0}}}. \quad (23)$$

In the case defined by Eq. 3, i.e. when there is a fraction of fluorescence that cannot be quenched, the most suitable way to analyze the quenching data is to use the modified form of the Stern-Volmer equation (Lehrer, 1971)

$$\frac{F_o}{F_o - F} = \frac{F_o}{\Delta F} = \frac{1}{\alpha_{obs}} + \frac{1}{\alpha_{obs}}\frac{1}{K_{sv}}\frac{1}{[Q]}. \quad (24)$$

Here F_0 and F are the fluorescence intensities in the absence and the presence of quencher molecules, $[Q]$ is the quencher concentration, and α_{obs} is the fraction of fluorescence accessible to quenching by observation. K_{sv} is the Stern-Volmer constant, which is usually given in the form of

$$K_{sv} = k_+\tau, \quad (25)$$

where τ is the lifetime of the excited fluoropore in the absence of the quencher and k_+ is the bimolecular rate constant given by Eq. 14. (In the case of "weak" quenchers, when not every collision results in quenching, Eq. 25 should contain a proportionality factor, γ, accounting for this property (Eftink and Ghiron, 1984). To write the modified Stern-Volner equation for gated quenching, one can use Eqs. 3 and 4 to obtain F_0 and F. Since F is the fluorescence intensity in the presence of quencher, it is the sum of the nonquenchable fraction, βF_0, and the remaining part of the quenchable fluorescence, $F_{\alpha Q}$. Therefore, the ratio $(F_0 - F)/F_0$ is given by $\alpha(1 - W)$. This, in combination with Eq. 23, can be rearranged to yield

$$\frac{F_0}{\Delta F} = \frac{1}{\alpha}\frac{\nu_0 + \nu_{cl}}{\nu_0 + \nu_{cl}\dfrac{\tau}{\tau + \nu_{cl}}} + \frac{1}{[Q]}\frac{1}{\alpha P_0 k_+ \tau}. \quad (26)$$

Converting this to the form of Eq. 24, the parameters α_{obs} and K_{sv} appear as

$$\alpha_{obs} = \alpha\alpha_g \quad (27$$

and

$$K_{sv} = \frac{P_0 k_+ \tau}{\alpha_g}, \quad (28)$$

where α_g is the "gated" accessibility, i.e. the fraction of the total accessibility, α_{obs}, accounted for by the dynamics of the gating itself

$$\alpha_g = P_0\left(1 + \frac{P_{cl}}{P_0}\frac{\tau}{\tau + \nu_{cl}}\right). \quad (29)$$

DISCUSSION

Our model, by taking into account the finite lifetime of the excited state, allows the interpretation of a wide range of structural lifetimes in terms of quenching parameters. And, although our model was derived for fluorescence, it is readily applicable to quenching of phosphorescence as well. The complete model of gated quenching we present shows that, provided a gating mechanism operates (and static quenching is negligible), the quenching should be first order over the whole range of quencher concentration.[1]

The last corollary sets our equations apart from those most frequently used to interpret quenching data (Calhoun et al., 1983). These authors and others express the apparent quenching constant K_{sv} as

$$\frac{F_0}{F} - 1 = K_{sv}[Q] = \frac{k_0 k_+ [Q]}{k_{cl} + k_+[Q]}\tau. \quad (30)$$

Eq. 30 contains rate constants for the opening and closing

[1]The derived expressions are only valid (as they are) for uncharged quencher molecules. In the case of charged quenchers further complications appear as effects on activity coefficients and on charge repulsion terms.

TABLE I
THE QUENCHING PARAMETERS OF GATED QUENCHING IN THE LIMITED CASES

	Dynamic ($\nu_0, \nu_{cl} \ll \tau$)	Static ($\nu_0, \nu_{cl} \gg \tau$)
α_{obs}	α	$P_0\alpha$
K_{sv}	$P_0k_+\tau$	$k_+\tau$

of the gate k_0, k_{cl} and is derived from the scheme presented in Eq. 1 by assuming $k_{cl} \gg k_0$ (see e.g. McDaniel and Smoot, 1956).

Due to the inherent nonlinearity of Eq. 30 experimental data are as a rule fitted to the simplified expression valid for the condition $k_+ [Q] \gg k_{cl}$ (Calhoun et al., 1983)

$$K_{sv} = \frac{k_0}{k_{cl}} k_+\tau = K_{eq}k_+ \tau. \quad (31)$$

The derivation of Eq. 30 from scheme I, however, contains the tacit assumption that the accessibility is 1. Accordingly, Eq. 30 is correct even when k_{cl} is comparable to, or less than, $k_+[Q]$ (but $k_{cl} \gg k_0$) and the nonlinearity simply reflects the decreased accessibility. By comparing Eq. 30 with our result we can conclude that it represents a special case of our model, i.e. by equating $\alpha = 1$ and assuming $k_{cl} \gg k_0$ our Eq. 28 reduces to Eq. 30.

According to our model, the apparent accessibility (α_{obs}) and the Stern-Volmer constant (K_{sv}) obtained by the modified Stern-Volmer plot are shown by Eq. 26 to be complex functions of the various lifetimes. The model allows division of the gating into three time ranges: dynamic ($\nu_0, \nu_{cl} \ll \tau$), intermediate ($\nu, \nu_{cl} \simeq \tau$) and static ($\nu_0, \nu_{cl} \gg \tau$). In the two time extremes, the dynamic and the static, the quenching parameters can be simplified. The resulting expressions are shown in Table I. In the intermediate time range, the effect of gating on these parameters is described by Eqs. 27–29. (Note that the expressions "dynamic" and "static" describe the gating and must not be mistaken for the similar expressions characteristic of the quenching itself.)

The information obtainable from a linear function such as the Stern-Volmer plot we use, is limited to two parameters, the slope and the intercept. Obviously in order to extract information about the gating process, characterized by P_0 and ν_{cl}, we have to introduce new independent experimental variables in addition to quencher concentration. The influence of the variables on the terms appearing in the expressions for α_{obs} and K_{sv} can be predicted from studies of the quenching of model compounds and from studies of protein conformation by methods other than fluorescence. Natural choices for the new variables are temperature and viscosity.

Before application of our model and equations, one should consider criteria for the validity for our basic assumption, namely the gating mechanism itself. Alternate mechanisms of quenching (by neutral quenchers), notably by solvent and quencher penetration of the protein matrix

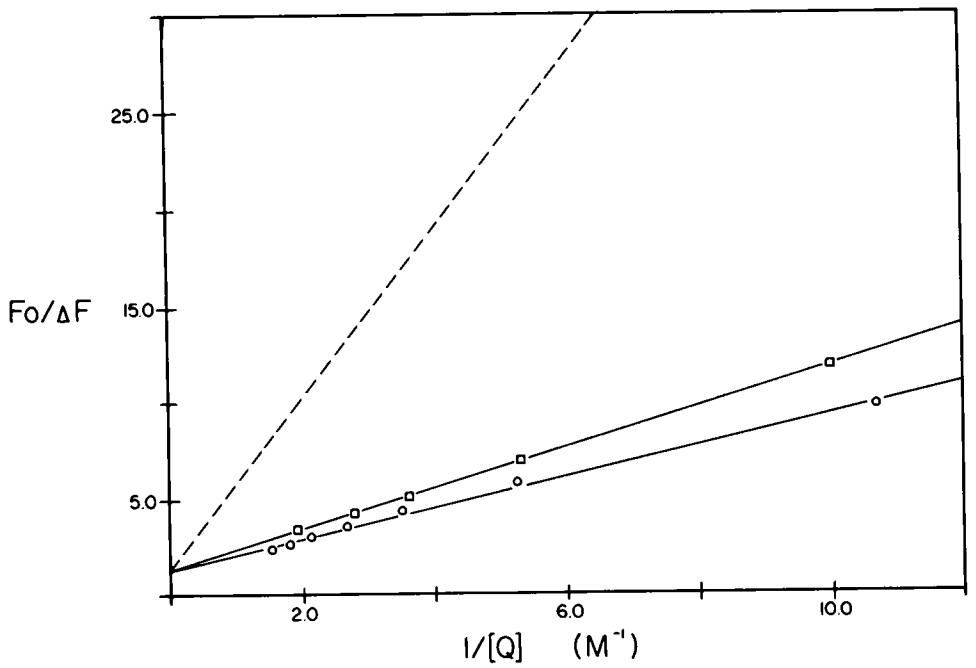


FIGURE 1 Effect of viscosity. The modified Stern-Volmer plot of acrylamide quenching of the single tryptophan residue in ribonuclease T_1 in the absence (O—O) and in the presence (□—□) of 50% glycerol in Tris buffer, pH 7.0, at room temperature. Data points are taken and transformed from the paper by Eftink and Ghiron, 1975. Straight lines were fitted by linear regression. The dashed line shows a curve having the slope predicted for the gated quenching mechanism in the presence of 50% glycerol (without correction for any other effect of glycerol but that of viscosity).

TABLE II
THE PARAMETERS OF THE MODIFIED STERN-VOLMER PLOT FOR THE ACRYLAMIDE QUENCHING OF THE TRYPTOPHAN RESIDUE IN RIBONUCLEASE T_1

	Buffer	50% Glycerol
slope (M)	0.80	1.05
K_{sv} (M^{-1})	1.63 (1.1*)	1.26 (0.9*)
α	0.76	0.75

*The K_{sv} values are those obtained by Eftink and Ghiron (1975) using the Stern-Volmer representation.

by a multistep random walk type of mechanism (Woodward et al., 1982; Gregory and Rosenberg, 1985), will not lead to the equations we present for gating. We have *a priori* no reason to believe that such mechanisms do not exist, especially in the case of very inaccessible residues.

In answer to that question, a useful relationship emerges from Eq. 26. The inverse of the slope, a product of α_{obs} and K_{sv}, is independent of α_q (the fraction of total accessibility accounted for by the dynamics of the gating process) over the whole range of τ/ν_{cl} ratios. Consequently it should, when studied as a function of viscosity, show linear dependence on viscosity, resulting from the well defined viscosity effect on k_+. This feature of our model can be applied as a diagnostic test. To determine whether the quenching is taking place via a gating mechanism one should study the viscosity dependence of the slope of the modified Stern-Volmer plot. Such a study, although as yet based on only two viscosity values, has been carried out for the quenching of the deeply buried tryptophan residue in ribonuclease T_1 by acrylamide (Eftink and Ghiron, 1975). To process their data, the authors used the Stern-Volmer representation which assumes the accessibility to be unity. In the case of gated quenching, however, the apparent accessibility can deviate from one (see above). This results in the necessity to use the modified Stern-Volmer representation which can easily be obtained by a transformation of the data points of the Stern-Volmer plot. Accordingly, the data points obtained by Eftink and Ghiron (1975) have been reprocessed (see Fig. 1) to see whether the acrylamide quenching of the tryptophan residue in ribonuclease T_1 fits the gating model. The re-plot resulted in a parameter set (see Table II) showing slight deviation (due to $\alpha < 1$) in K_{sv} values from the ones obtained by Eftink and Ghiron (1975). In contrast to our model, which predicts a 5.5-fold increase in the slope of the modified Stern-Volmer plot upon the 5.5-fold increase in viscosity (produced by 50% glycerol at room temperature), the ratio of the slopes, with and without the presence of 50% glycerol, is 1.31. This finding supports the conclusion by Eftink and Ghiron (1975) who suggested that quenching is taking place via an alternate mechanism such as penetration rather than gating, unless P_0 for the possible gating changes in a compensatory manner with viscosity. This is unlikely because both ν_{cl} and ν_0 are influenced in the same manner by viscosity and the specific effect of glycerol on the stability of open conformations (Gekko and Timasheff, 1981) is of direction that should enhance the apparent viscosity dependence. Another effect to consider is the effect of glycerol on the activity coefficient of acrylamide. According to solubility measurements, the appropriate activity coefficient increases to ~1.3 in the presence of 50% glycerol (Rosenberg, unpublished data) which, again, cannot be responsible for the fourfold difference in the slopes of the expected and measured cuves of Fig. 1. We should bear in mind that the quenching data, as kinetics in general, does not prove the existence of a definite alternate mechanism. The conclusion is limited to the observation that the gating models represented by Eqs. 1 and 2 are not sufficient. More detailed data are undoubtedly needed to validate alternate, more complex formulations for the quenching mechanism.

In the specific cases of quenching where the gating model appears to be applicable, relaxations appearing in the intermediate time range offer the most possibilities for study by additional experimental variables. The reported lifetimes for structural rearrangements related to thermal fluctuations are in the microsecond and millisecond range (Tsong, 1982). The quenching parameters in this case would definitely fall into the static gating range. Small amplitude thermal motions such as those visualized by molecular dynamics calculations presents lifetimes in the nano- and picosecond range falling thus most probably into the intermediate gating range. For study of the dynamic range, phosphorescence quenching with its long lifetimes provides a favorable experimental alternative.

For the residues where gating, rapid one step opening and closing of structural segments, results in accessible states, the quenching can be described by the equations we present. This allows explicit expression of the relationship between structural motion and quenching over the whole range of ratios for the structural and excited state lifetimes.

The authors wish to thank Attila Szabo and Maurice R. Eftink for their helpful discussions.

This investigation was supported by National Science Foundation grant PCM 8303021.

Received for publication 5 September 1985 and in final form 17 December 1985.

REFERENCES

Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and L. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* 14:5355–5373.

Barksdale, A. D., and A. Rosenberg. 1982. Acquisition and interpretation of hydrogen exchange data. Method of Biochemical Analysis. D. Glick, editor. Vol 28. John Wiley & Sons, N.Y. 1–113.

Calhoun, D. B., J. M. Vanderkooi, and S. W. Englander. 1983*a*. Penetration of small molecules into proteins studied by quenching of phosphorescence and fluorescence. *Biochemistry.* 22:1533–1539.

Calhoun, D. B., J. M. Vanderkooi, G. V. Woodrow, III, and S. W.

Englander. 1983*b*. Penetration of dioxygen into proteins studied by quenching of phosphorescence and fluorescence. *Biochemistry*. 22:1526–1532.

Cooper, A. 1976. Thermodynamic fluctuation in protein molecules. *Proc. Natl. Acad. Sci. USA*. 73:2740–2741.

Eftink, M. R., and C. A. Ghiron. 1975. Dynamics of a protein matrix revealed by fluorescence quenching. *Proc. Natl. Acad. Sci. USA*. 72:3290–3294.

Eftink, M. R., and C. A. Ghiron. 1981. On the analysis of the temperature and viscosity dependence of fluorescence-quenching reactions with proteins. *Arch. Biochem. Biophys*. 209:706–709.

Eftink, M. R., and C. A. Ghiron. 1984. Indole fluorescence quenching studies on proteins and model systems: use of the inefficient quencher succinimide. *Biochemistry*. 23:3891–3899.

Englander, S. W., and N. R. Kallenbach. 1984. Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys*. 16:521–655.

Gekko, K., and S. N. Timasheff. 1981. Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry*. 20:4677–4686.

Gratton, E., D. M. Jameson, and G. Weber, B. Alpert. 1984. A model of dynamic quenching of fluorescence in globular proteins. *Biophys. J*. 45:789–794.

Gregory, R. B., and A. Rosenberg. 1986. Protein associations by partitioning in two phases. *Methods Enzymol*.

Haas, E., and I. Z. Steinberg. 1984. Intramolecular dynamics of chain molecules monitored by fluctuations in efficiency of excitation energy transfer. A theoretical study. *Biophys. J*. 46:429–437.

Hagman, K. A., and M. R. Eftink. 1984. Fluorescence quenching of trp-314 of liver alcohol dehydrogenase by oxygen. *Biophys. Chem*. 20:201–207.

Hevessy, J., B. Somogyi, G. R. Welch, S. Papp, J. Matkó, S. Damjanovich. 1981. A fluorescence parameter reporting on the change of intramolecular fluctuations. *Int. J. Luminescence*. 24/25:811–814.

Jameson, D. M., E. Gratton, G. Weber, and B. Alpert. 1984. Oxygen distribution and migration with $MB^{DES\ Fe}$ and $HB^{DES\ Fe}$. *Biophys. J*. 45:795–803.

Karplus, M., and J. A. McCammon. 1981. The internal dynamics of globular proteins. *CRC Crit. Rev. Biochem*. 9:293–349.

Lakowicz, J. R., and G. Weber. 1973*a*. Quenching of fluorescence by oxygen. A probe for structural fluctuations in macromolecules. *Biochemistry*. 12:4161–4170.

Lakowicz, J. R., and G. Weber. 1973*b*. Quenching of protein fluorescence by oxygen. Detection of structural fluctuations in proteins on the nanosecond time scale. *Biochemistry*. 12:4171–4179.

Lee, B. 1983. Calculation of volume fluctuation for globular protein models. *Proc. Natl. Acad. Sci. USA*. 80:622–626.

Lehrer, S. S. 1967. The selective quenching of tryptophan fluorescence in proteins by iodide ion: lysozyme in the presence and absence of substrate. *Biochem. Biophys. Res. Commun*. 29:767–772.

Lehrer, S. S. 1971. Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry*. 17:3254–3263.

Lumry, R., and A. Rosenberg. 1975. Mobile defect hypothesis of protein function. *Colloques Internationaux du C.N.R.S. L'Eau et Les Systemes Biologiques*. 246:53–62.

Lumry, R., and R. B. Gregory. 1986. Free-Energy Management in Protein Reactions: Concepts, Complications and Compensation. *In* The Fluctuating Enzyme. G. R. Welch, editor. Wiley Interscience Press, New York. In press.

McCammon, J. A., and M. Karplus. 1980. Simulation of protein dynamics. *Annu. Rev. Phys. Chem*. 31:29–45.

McCammon, J. A., and S. H. Northrup. 1981. Gated binding of ligands to proteins. *Nature* (*Lond.*). 293:316–317.

McDaniel, D. H., and C. R. Smoot. 1956. Approximations in the kinetics of consecutive reactions. *J. Phys. Chem*. 60:966–969.

Parak, F., and E. W. Knapp. 1984. A consistent picture of protein dynamics. *Proc. Natl. Acad. Sci. USA*. 81:7088–7092.

Somogyi, B., G. R. Welch, and S. Damjanovich. 1984*a*. The dynamic basis of energy transduction in enzymes. *Biochim. Biophys. Acta*. 768:81–112.

Somogyi, B., J. Matkó, S. Papp, J. Hevessy, G. R. Welch, and S. Damjanovich. 1984*b*. Foerster-type energy transfer as a probe for changes in local fluctuations of protein matrix. *Biochemistry*. 23:3403–3411.

Somogyi, B., S. Papp, A. Rosenberg, I. Seres, J. Matkó, G. R. Welch, and P. Nagy. 1985. A double-quenching method for studying protein dynamics: separation of the fluorescence quenching parameters characteristic of solvent exposed and masked fluorophores. *Biochemistry*. 24:6674–6679.

Szabo, A., D. Shoup, S. H. Northrup, and J. A. McCammon. 1982. Stochastically gated diffusion-influenced reactions. *J. Chem. Phys*. 77:4484–4493.

Tsong, T. Y. 1982. Viscosity-dependent conformational relaxation of ribonuclease A in the thermal unfolding zone. *Biochemistry*. 21:1493–1498.

Woodward, C., I. Simon, and E. Tüchsen. 1982. Hydrogen exchange and the dynamic structure of proteins. *Mol. Cell. Biochem*. 48:135–160.

Xu, G., and G. Weber. 1982. Dynamics and time-averaged chemical potential of proteins. *Proc. Natl. Acad. Sci. USA*. 79:5268–5271.