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On the use of self-quenching fluorophores in the study of membrane fusion kinetics

The effect of slow probe redistribution

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In glycoprotein-mediated pH-induced fusion of virus to animal cells, the mixing of materials between membranes or between cytoplasmic spaces occurs after the virus-cell complex has gone through a number of activation reactions. The monitoring of the fluorescence changes measured in a fusing system using self-quenching probes could reflect not only the kinetics of activation, but also the redistribution reaction of probes. For instance, time delay seen in the onset of fluorescence changes after triggering the fusion reaction (S.J. Morris, D.P. Sarkar, J.M. White and R. Blumenthal, J. Biol. Chem. (1989) 3972), could be due to rate-limiting probe redistribution kinetics. In this paper we examined in detail the effect of probe redistribution rates on fusion kinetics. Simulations were performed using a very simple model with two fusion-activation steps and an exponential probe redistribution kinetics. We conclude that if the rates of probe redistribution are faster than or equal to those of viral glycoprotein activation, the kinetics of the fusion reaction are not significantly affected.

1. Introduction

For enveloped viruses, the entry of their viral genome material into target cells is mediated by a process called 'membrane fusion' [1–3]. That is, virions and cells have to overcome the repulsive forces between them and integrate their membranes into a single continuous bilayer. Consequently, channels or pathways for the exchange of genome materials can be formed at the junctional area. It is known that virus-cell membrane fusion is a complex event involving many biochemical and biophysical interactions [4,5]. For example, fusion of influenza virus and animal cells requires a series of activation reactions after the binding of virus to the receptors on the target cells: protonation of hemagglutinin (HA) molecules, pH-induced conformational changes and aggregation of HA trimer-receptor complexes to form a fusion junction, etc. In general, the mixing of lipids, lipid-like probes, and other membrane components between the two fusing membranes occurs only after the virus-cell complex has been activated.

In the past, most biophysical and biochemical studies on virus-cell fusion had been centered on the elucidation of structures of the spike proteins and their conformational changes, etc. [6,7]. Little has been devoted to the kinetics or mechanisms of the activation reactions. Part of the reason is the lack of proper markers for labelling the intermediate states of the reactions. Recently, a series of studies on fusion-activation kinetics have been

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reported by a number of laboratories [8–13], based on fluorometric measurements using lipid-like dyes, R-18 (octadecyl rhodamine B chloride). The principle of the method relies upon the self-quenching properties of this dye: the intensity of total fluorescence decreases when the density of the dye increases and vice versa. Thus, upon fusing of R-18-containing membranes with membranes devoid of the probe, the surface density of the dye decreases because of dye redistribution, resulting in an increase in the total fluorescence.

In a typical virus-cell fusion system where R-18 molecules are initially loaded only to the virus membrane, the kinetics of the fusion event is usually studied in terms of the quantity, called the degree of dequenching, defined as [9,10]:

$$\sigma(t) = \frac{F(t) - F_0}{F_\infty - F_0} \tag{1}$$

where F_0 and $F(t)$ denote the total fluorescence of the system at times zero and t respectively, and F_∞ is the total fluorescence of the system when all the fluorophores are completely dequenched by disruption with detergent. This $\sigma(t)$ can be shown to be directly equal to the degree of activation (or fusion) if the following two assumptions are satisfied (ref. 9; see also below). First, the dyes are assumed to be completely dequenched after fusion. Second, the rate of redistribution of R-18 between the viral and cell membranes is assumed not to be rate-limiting. That is, the dyes can re-equilibrate instantaneously right after the membranes are fused and the equilibrium density of dyes on the fused membrane is extremely low so that self-quenching is absent. In this case, the degree of dequenching becomes a very useful quantity in the elucidation of mechanisms of fusion-activation reactions.

Since the cell membrane surface is very large compared to the virus membrane surface, the first assumption is rather reasonable. In general, if redistribution of R-18 is mediated by a free diffusion process, the rate of redistribution would be very fast. However, if there are restrictions to probe movement through the 'fusion junction', the redistribution process might be slowed down or retarded [14]. This prompts us to address the following two questions: (1) How fast does the probe-redistribution process have to be in order not to be rate-limiting? In other words, what is the lower limit of the redistribution rate constant if the $\sigma(t)$ in eq. 1 is still considered as approximately equal to the degree of activation?; (2) If the rate of redistribution is indeed slow, is it possible to extract the information on the kinetics of the activation reactions from the dequenching data? In order to obtain answers to the above, the basic relationship between the degree of dequenching and both the activation and redistribution reactions must be established. This is the goal of the present paper. Specifically, we show that an explicit expression for $\sigma(t)$ can be derived in terms of the rate constants of both reactions. As a result, the effect of slow redistribution on the shape of $\sigma(t)$ can be discussed and information regarding the kinetics of activation reactions can be obtained. Equations for other fusion-related quantities, such as the extent of lipid mixing and the degree of activation, will also be derived and discussed.

2. Elementary fusion reactions and probe redistribution

We first define the system and some of the terms to be used in this paper. Following the same experimental procedure and assumptions made previously [10], we consider a system of prebound complexes of cells and R-18-loaded viruses and study the change in fluorescence of these complexes after a pH change (the onset of activation). That it, we disregard the binding step in the kinetic study and consider only a system of bound virus-cell complexes. We also assume that the dissociation of these complexes during the entire process of measurement of the fusion kinetics is negligible and that the fusion of one virus will not interfere with that of another (no cooperative effect between viruses). In this case, each virus-cell complex can be considered as an independent object (unit) of the system and the total number of objects in the system is fixed. It should be borne in mind that usually more than one virus can bind to a cell. Thus, the word 'cell' in 'virus-cell com-

plex' does not necessarily mean the entire cell, but just the part of the cell that is associated with the virus. In other words, each object in our system contains a virus and the piece of the cell membrane to which the virus is attached.

A membrane fusion event is defined here as an irreversible process of merging two separate bilayers into an equilibrium structure that is continuous in both the bilayer membrane and the core space. Thus, as schematically illustrated in fig. 1, a pH-induced virus-cell fusion process involves at least the following three steps: (1) Binding of protons to the glycoproteins followed by conformational changes in the glycoprotein-receptor complex (from state [1] to [2]); (2) The coalescing of the two bilayers and formation of pores or channels at the junctional area (from [2] to [F1]); (3) Expansion of pores or channels until the final equilibrium state is achieved (from [F1] to [F2] to [F]). The transitions between the states in fig. 1 are called the 'elementary fusion reactions' of the fusion event. One should note that the elementary fusion reactions of a fusion event are properties of the virus and the cell involved and are not affected by the occurrence of redistribution of R-18 molecules in the system. In other words, upon pH-induced activation taking place, virus and cell membranes will fuse according to the elementary fusion reactions of the system, irrespective of the presence or absence of R-18 molecules in the system.

Fluorescence dequenching due to redistribution of R-18 molecules between the virus and cell membranes takes place when the complex is in one of the 'open' states for lipid redistribution ([F1], [F2], and [F] in fig. 1b). The elementary fusion reactions leading to the first lipid-mixing state [F1] are referred to as the 'activation reactions' of the fusion process. For simplicity, the loss of R-18 molecules from the virus surface (the probe redistribution reaction) will be assumed to follow an exponential function (eq. 10 below). In fact, the formalism presented below can be extended easily to other functional forms. The main purpose of this paper is to investigate the relation between the kinetics of fluorescence dequenching of R-18 and the kinetics of the activation and probe redistribution reactions.

3. The degree of dequenching

As experimentally observed [10,11], the measured degree of dequenching at infinite time in virus-cell fusion systems does not reach 100%. This means that some of the virus-cell complexes are not active. To eliminate the contribution from inactive complexes, we shall consider the normalized degree of dequenching, defined as

$$X(t) = \frac{\sigma(t)}{\sigma(\infty)} = \frac{F(t) - F_0}{F(\infty) - F_0}. \tag{2}$$

where $F(\infty)$ is the total fluorescence of the system at infinite time when all dequenching processes

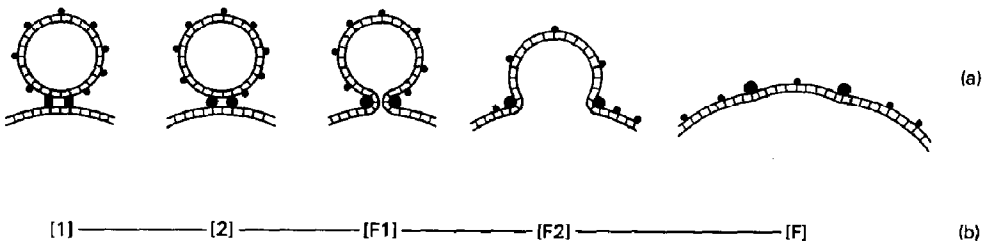

Fig. 1. The states of a hypothetical virus-cell fusion model. The small dots on the small vesicle represent the fluorescent R-18 molecules. The solid rectangular and circular blocks between the two fusing membranes represent the glycoprotein-receptor complexes before and after protonation, respectively. Redistribution of R-18 molecules occurs only after the two membranes have coalesced in states [F1], [F2], and the final fused, equilibrium state [F]. Thus, states [1] and [2] are referred to as the 'closed' states for lipid mixing and states [F1], [F2], and [F] as the 'open' states.

have stopped. Experimentally, $X(t)$ can be obtained without the Triton disruption step.

We consider a fusion system of M_t virus-cell complexes, of which M are active. Let the mean fluorescence intensity of an active complex be denoted as $\langle F(t)\rangle$ and that of an inactive one as F_I. Then,

$$F(t) = M\langle F(t)\rangle + (M_t - M)F_I \quad (3)$$

$$F(\infty) = M\langle F(\infty)\rangle + (M_t - M)F_I \quad (4)$$

$$F_0 = M\langle F(0)\rangle + (M_t - M)F_I. \quad (5)$$

Substituting eqs. 3–5 into eq. 2, we have

$$X(t) = \frac{\langle F(t)\rangle - \langle F(0)\rangle}{\langle F(\infty)\rangle - \langle F(0)\rangle} \quad (6)$$

This is the basic equation for deriving the relationship between the normalized degree of dequenching of R-18 molecules ($X(t)$) and the underlying fusion and R-18 redistribution reactions of the system.

For convenience, we shall use the kinetic diagram in fig. 2a as the basis of our derivation. However, the conclusions obtained should be applicable to any model.

We consider a system of M active virus-cell complexes undergoing the set of elementary fusion reactions shown in fig. 2a. Each virus of the complexes is loaded with a particular number of R-18 molecules and each complex is in state [1] before the onset of activation (pH change). Let N_0 be the number of R-18 molecules on the virus membrane of a complex at time zero, $N(t)$ being that at time t after the onset of activation. Then, the total fluorescence of the complex at t is equal to

$$F(t) = N(t)f_v(t) + [N_0 - N(t)]f_d \quad (7)$$

where $f_v(t)$ denotes the intrinsic fluorescence of a single R-18 on the virus membrane at time t and f_d that of a completely dequenched single R-18. The first term on the right-hand side of eq. 7 is the total fluorescence from the virus membrane, the second term corresponding to that from the cell membrane. It should be noted that the use of f_d in the second term implies a complete dequenching of R-18 on the cell surface. This is reasonable,

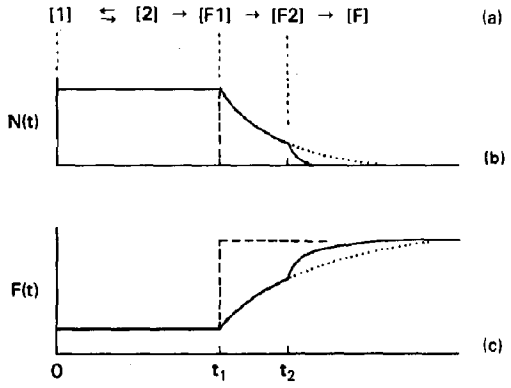

Fig. 2. The time course of N (number of R-18 molecules remaining on the virus surface) and F (total fluorescence intensity of a virus-cell complex). The value of N remains at N_0 (number of R-18 initially loaded onto the virus surface) until the complex reaches the first open state [F1]. Then, N decays exponentially according to eq. 10b.After reaching the second open state [F2], $N(t)$ follows another different exponential decay course, when the transport coefficient in state [F2] differs from that in state [F] (solid curve). Otherwise, it will follow the same exponential decay (dotted line). The dashed line represents the situation where the rate of redistribution of R-18 is infinitely fast. The corresponding fluorescence intensity of a single virus-cell complex is shown in panel c.

since the surface of a cell is much larger than that of a virus. The case in which R-18 molecule on the target cell surface are not completely dequenched, as in cell-cell fusion systems, is discussed in appendix A.

The value of $f_v(t)$ is always smaller than f_d due to the self-quenching of R-18. As shown by Hoekstra et al. [8], the degree of quenching is linearly proportional to the density of probes if the surface density is less than 7%:

$$f_v(t) = f_d\left[1 - \frac{kN(t)}{A_v}\right] \quad (8)$$

where k is a constant. It is obvious that $f_v(t)$ equals f_d only when $N(t)$ tends to zero (infinite dilution).

With eq. 8, eq. 7 becomes

$$F(t) = f_d N_0 - \frac{f_d k}{A_v} N(t)^2. \quad (9)$$

which represents the fluorescence intensity of a

single complex as a function of time. The average fluorescence of a complex in the system can be obtained by taking the ensemble average of $F(t)$ over the distribution of N_0 (R-18 is not loaded uniformly). To do this, we must express the $N(t)$ in eq. 9 in terms of N_0. As discussed before, $N(t)$ will remain at N_0 until the complex enters the first open state [F1]. Hereafter, $N(t)$ will decrease exponentially. Let t_1 be the time required for a complex to reach state [F1] from state [1] and assume that all open states have the same redistribution kinetics. Then, $N(t)$ can be expressed explicitly as:

$$N(t) = N_0,\ 0 < t < t_1 \tag{10a}$$

$$N(t) = N_0 e^{-\gamma(t-t_1)},\ t > t_1 \tag{10b}$$

where γ is a constant.

As can be seen from eqs. 9 and 10, $F(t)$ depends not only on N_0 but also on γ and t_1. The $N(t)$ and the corresponding $F(t)$ of a single complex are schematically shown in fig. 2b and c.

Due to the stochastic nature of chemical reactions, the value of t_1 will be different from one complex to the other. Thus, to obtain the average value of the fluorescence of a single complex, we must average $F(t)$ not only over the distribution of N_0, but also over that of t_1. Let $f(N_0)$ and $g(t_1)$ designate the respective probability distribution functions (pdf) of N_0 and t_1. Then

$$\begin{aligned}\langle F(t)\rangle &= \int_0^\infty \int_0^\infty F(t) f(N_0) g(t_1)\, \mathrm{d}N_0\, \mathrm{d}t_1 \\ &= f_d\langle N_0\rangle - \frac{k}{A_v} f_d \int_0^\infty \int_0^\infty N(t)^2 f(N_0) \\ &\quad \times g(t_1)\, \mathrm{d}N_0\, \mathrm{d}t_1 \end{aligned} \tag{11}$$

where

$$\langle N_0\rangle \equiv \int_0^\infty N_0 f(N_0)\, \mathrm{d}N_0$$

In general, $g(t_1)$ of an arbitrary biochemical diagram such as that of fig. 2a can be expressed in terms of exponentials as (see below, for example):

$$g(t_1) = \sum_i a_i e^{-\alpha_i t_1} \tag{12}$$

where a_i and α_i are functions of the rate constants of the activation reactions.

With $N(t)$ given in eqs. 10 and $g(t_1)$ in eq. 12, the integration in eq. 11 can be carried out easily. The final result is:

$$\begin{aligned}\langle F(t)\rangle = f_d\langle N_0\rangle - \frac{k}{A_v} f_d \langle N_0^2\rangle \cdot \Big\{ &\sum_i \frac{a_i}{\alpha_i} e^{-\alpha_i t} \\ &+ \sum_i \frac{a_i}{(\alpha_i - 2\gamma)} (e^{-2\gamma t} - e^{-\alpha_i t}) \Big\}\end{aligned} \tag{13}$$

with $\langle N_0^2\rangle$ defined as

$$\langle N_0^2\rangle \equiv \int_0^\infty N_0^2 f(N_0)\, \mathrm{d}N_0.$$

Thus, the average fluorescence intensities at time zero and infinite time can be shown to be

$$\langle F(0)\rangle = f_d\langle N_0\rangle - \frac{k}{A_v} f_d \langle N_0^2\rangle, \tag{14}$$

$$\langle F(\infty)\rangle = f_d\langle N_0\rangle. \tag{15}$$

From eqs. 13–15, the $X(t)$ in eq. 6 can be expressed as

$$\begin{aligned}X(t) = &\sum_i \frac{a_i}{\alpha_i} (1 - e^{-\alpha_i t}) \\ &- \sum_i \frac{a_i}{(\alpha_i - 2\gamma)} (e^{-2\gamma t} - e^{-\alpha_i t}).\end{aligned} \tag{16}$$

This equation presents the general relationship between the measured degree of dequenching $X(t)$ and the kinetic parameters of the processes of activation (a_i, α_i) and lipid redistribution (γ). A few interesting points can be readily obtained from inspection of eq. 16. First, the dequenching ratio $X(t)$ of the system does not depend on the distribution of N_0; i.e., the way in which the probes are loaded initially on to the viruses has no effect on the measured kinetic curve of $X(t)$. Second, as discussed in section 4, the first term in the above equation corresponds to the degree of activation (or the degree of fusion as usually referred to). Obviously, $X(t)$ is equal to the degree of activation only when the redistribution reaction is very fast ($\gamma \to \infty$). It seems that the second term is always negative (see below). Thus, $X(t)$ will always underestimate the true degree of activa-

tion. Third, when γ is very small ($\gamma \ll \alpha_i$), it is easy to show that the $X(t)$ in eq. 16 reduces to

$$X(t) = 1 - e^{-2\gamma t}. \tag{17}$$

This equation implies that single-exponential kinetics will be observed in the dequenching rate and that the value of the rate constant is equal to twice the transport coefficient of probe molecules.

4. Degree of activation and degree of mixing

By definition, the degree of activation (η_A) is the fraction of (active) complexes that are activated for lipid redistribution. That is, it is equal to the probability of a complex being in any one of the open states for lipid exchange ([F1], [F2] and [F] in fig. 1b or 2):

$$\eta_A(t) = p_{F1}(t) + p_{F2}(t) + p_F(t). \tag{18}$$

For the model in fig. 2a, this probability is exactly equal to the cumulative distribution function of $g(t_1)$. Thus,

$$\eta_A(t) = \int_0^t g(t_1)\, dt_1 = \sum_i \frac{a_i}{\alpha_i}(1 - e^{-\alpha_i t}). \tag{19}$$

The first sum of $X(t)$ in eq. 16 is exactly equal to the degree of activation. That is, $X(t)$ is equal to the degree of activation when γ is very large.

We wish to emphasize that $\eta_A(t)$ is a property of the elementary fusion reactions. It has nothing to do with the presence of R-18 or the redistribution of R-18. In general, η_A cannot be evaluated from R-18 dequenching data when the redistribution of R-18 is not extremely fast. The formal way of evaluation is to measure the concentration of complexes that are in the open states. However, since this is equal to the fraction of complexes that have been committed to lipid mixing, $\eta_A(t)$ may be evaluated from the dequenching data by a simple activation-deactivation experiment. If one can find a way to terminate the activation reactions without interfering with the redistribution reaction, then the final extent of dequenching after deactivation represents the fraction of activated complexes at the time of deactivation. That is, $\eta_A(t)$ can be estimated by terminating the activation reactions at different times after activation and measuring the final extent of dequenching as a function of the time between activation and deactivation.

The degree of lipid mixing (η_M) is defined as the fraction of the total number of R-18 molecules in the system that have diffused from the virus to the cell membrane. If $\langle N(t)\rangle$ denotes the mean number of R-18 molecules remaining on the virus membrane, then

$$\eta_M(t) = 1 - \frac{\langle N(t)\rangle}{\langle N_0\rangle}. \tag{20}$$

With eqs. 10 and 12, $\langle N(t)\rangle$ can be readily evaluated to yield:

$$\eta_M(t) = \sum_i \frac{a_i}{\alpha_i}(1 - e^{-\alpha_i t}) - \sum_i \frac{a_i}{(\alpha_i - \gamma)}(e^{-\gamma t} - e^{-\alpha_i t}). \tag{21}$$

Similar to $X(t)$, the first term in eq. 21 is exactly equal to the degree of activation. When γ is very large, $X(t)$ and $\eta(t)$ become identical to one other and both are equal to $\eta_A(t)$. On the other hand, when γ is very small, eq. 21 can be reduced to

$$\eta_M(t) = 1 - e^{-\gamma t}. \tag{22}$$

From eqs. 17 and 22, we have

$$\eta_M(t) = 1 - \sqrt{1 - X(t)}. \tag{23}$$

That is, $\eta_M(t)$ can be evaluated approximately from the measured $X(t)$ when the rate of redistribution of R-18 is either very fast or very slow. When the redistribution reaction is comparable to the fusion reactions, the kinetic data from dequenching fluorophores cannot be used directly to evaluate the degree of mixing.

On the other hand, $\eta_M(t)$ may be obtained from fluorescence measurements that do not rely on self-quenching. For example, if probes become fluorescent only when they are bound to some molecules on the cell membranes, then the total fluorescence of the system would be a direct measure of the degree of mixing.

5. Illustrative numerical calculations

We shall now deal with the case of the relationship between $X(t)$, $\eta_A(t)$, and $\eta_M(t)$ and demonstrate numerically with the simple fusion model in fig. 2a. The main step of the calculation involves evaluation of the pdf of the first passage time $g(t_1)$ for the activation reactions:

$$[1] \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} [2] \xrightarrow{k_f} [F^*], \tag{24}$$

Since the probability of being in state $[F^*]$ is equal to the cumulative probability of $g(t_1)$:

$$p_{F^*}(t) = \int_0^t g(t_1)\, dt_1, \tag{25}$$

we have

$$g(t_1) = \frac{dp_{F^*}(t)}{dt} = k_f p_2(t). \tag{26}$$

Thus, $g(t_1)$ can be obtained by solving the differential equations describing the kinetics of the reactions in eq. 24 with the following initial conditions:

$$p_1 = 1,\ p_2 = p_{F^*} = 0 \text{ at } t = 0. \tag{27}$$

For this simple kinetic scheme, $g(t_1)$ can be obtained analytically as:

$$g(t_1) = a_1 e^{-\alpha_1 t_1} + a_2 e^{-\alpha_2 t_1} \tag{28}$$

where

$$a_1 = \frac{\alpha_1 \alpha_2}{\alpha_2 - \alpha_1}$$

$$a_2 = \frac{\alpha_1 \alpha_2}{\alpha_1 - \alpha_2}$$

$$\alpha_{1,2} = \tfrac{1}{2}(k_{12} + k_{21} + k_f) \mp \tfrac{1}{2}\sqrt{(k_{12} + k_{21} + k_f)^2 - 4k_{12}k_f}$$

with α_1 and α_2 referring to the negative and positive signs, respectively. Thus, with the rate constants in eq. 24 being given, the values of $X(t)$, $\eta_A(t)$ and $\eta_M(t)$ can be evaluated as a function of γ values.

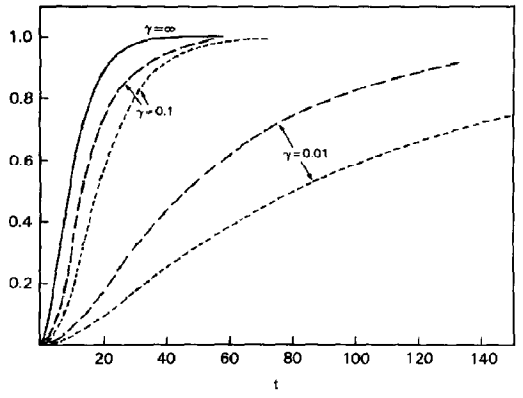

Fig. 3. Calculated $X(t)$ (- - - - -), $\eta_A(t)$ (———) and $\eta_M(t)$ (— — —) for the model in fig. 2a with the following rate constants: $k_{12} = 0.2$; $k_{21} = 0.02$; $k_f = 0.2$. The transport coefficient for R-18 redistribution is assumed to be the same for all open states.

For complicated activation reactions, the differential equations must be solved numerically. The procedures are standard and are easy to carry out using a computer.

Fig. 3 shows the calculated results for a number of γ values with the set of parameters for the model:

$$k_{12} = 0.2,\ k_{21} = 0.02,\ k_f = 0.2$$

The unbroken curve in fig. 3 refers to the degree of activation of the model. It depends only on the rate constants of the activation reactions and is independent of the γ value. At $\gamma = \infty$, both $X(t)$ and $\eta_M(t)$ are identical to $\eta_A(t)$. As γ decreases, the three quantities deviate from each other as expected.

6. Discussion

In this paper the quantitative relationship between the kinetics of dequenching of lipid-like R-18 molecules and the underlying biochemical reactions responsible for fluorescence changes in a virus-cell fusion system are examined. The purpose is to investigate the effect of the redistribution kinetics of R-18 molecules in fused membranes on the rate of fluorescence dequenching measured in a macroscopic system. Usually, the

rate of redistribution of R-18 in a fused membrane has been assumed to be very fast so that it makes no contribution to the kinetic behavior of dequenching. As a result, the kinetics of R-18 dequenching has been used directly to evaluate those of fusion activation. However, a recent single-object video-microscopy study on membrane fusion [14] indicated that redistribution of aqueous and lipid probes on fused membranes might be restricted. Motivated by this finding, we derive in this paper an equation expressing explicitly the degree of dequenching in terms of the rate constants of both activation and redistribution reactions. As shown in eq. 16 and in fig. 3, the rate of redistribution does have some effect on the kinetics of dequenching. In fact, the degree of dequenching always underestimates the true degree of activation (or fusion) at any time point, except when the system is in equilibrium at infinite time. This implies that the degree of dequenching defined in eq. 1 (or eq. 6) is not equivalent to the extent of fusion as usually assumed and that the kinetic parameters of virus-cell fusion systems obtained directly from the degree of dequenching without corrections for probe redistribution may contain errors, if the redistribution of R-18 is rate-limiting. However, as shown in fig. 3, the degree of activation does not deviate excessively from that of dequenching even when the rate of redistribution of probes (γ) is of the same order of magnitude as that of the activation reactions (k_{12}, etc.). Only when γ is at least 10-fold smaller than the k values do large differences between $X(t)$ and $\sigma(t)$ appear. In other words, although in principle the rate of redistribution of probes must be infinitely large in order that $X(t)$ and $\sigma(t)$ are identical, in practice the two are approximately equal to each other even when the rate of redistribution is about the same as that of the activation reactions. Of course, this conclusion may depend on the model used.

It is worthwhile pointing out that the elementary fusion reactions shown in fig. 1a can be interpreted by the 'allosteric gating' model of Blumenthal [12]. In this model, the flow of lipid-like R-18 molecules is controlled by the glycoprotein-receptor complexes at the junction of a virus-cell complex. That is, each complex serves both as the regulator and as the pathway for redistribution of nonequilibrium R-18 molecules. Each glycoprotein-receptor complex is an allosteric oligomer consisting of a number (three in the case of HA) of allosteric ligand-binding subunits. When all units are bound with protons (allosteric ligands), the complex adopts a different conformation suitable for aggregation. When three (or more) protonated glycoprotein-receptor complexes are aggregated, the membranes of the virus and the cell can coalesce and integrate to form a continuous bilayer. Enlargement of the pore at the center of the aggregated complexes, through which core materials can pass, is accomplished by aggregation of more protonated glycoprotein-receptor complexes.

It is important to point out that we have used a few assumptions in deriving eq. 16. First, as discussed by Hoekstra et al. [8], the degree of quenching of R-18 is assumed to be linearly proportional to the density of the probe. Second, the loss of R-18 molecules from the virus surface to the cell surface in an open state for lipid mixing is assumed to follow a simple exponential function. Third, the probe transfer rates for all open states are assumed to be the same. Fourth, as shown in fig. 2a, the 'membrane-coalescing' step and the channel-expansion steps in the elementary fusion reactions are assumed to be irreversible. These assumptions were used for the purpose of simplifying the mathematical analyses. As will be discussed in a separate report, the same qualitative conclusions can be obtained even without these assumptions.

The degree of activation (η_A), defined as the fraction of virus-cell complexes whose membranes have coalesced, is an important quantity for modelling the mechanism of a fusion process. In general, this quantity cannot be obtained directly from the cuvette dequenching data. However, as discussed in section 4, since it is equal to the fraction of complexes that will eventually become fused, η_A may be evaluated by terminating the activation reactions at different times after activation and measuring the extent of fusion at long times. In single-object video microscopy measurements, η_A may be estimated directly from the measured $g(t_1)$ (see. eq. 19).

The degree of mixing (η_M), defined as the

fraction of total probes that have diffused from the virus to the cell membranes, is the natural physical quantity related to the redistribution of probes in a fusion system. This can be readily obtained if the concentrations of probes on the virus and cell surfaces are measurable. For example, if probes become fluorescent only when bound to certain molecules in the cell membrane, then the total fluorescence can be used to estimate η_M. Like the degree of activation, η_M can be estimated readily from single-object video microscopy measurements. On the other hand, as discussed in section 4, η_M may be evaluated from dequenching data using self-quenching probes provided the rate of probe redistribution is either very small or very large.

In conclusion, we have derived an explicit expression for the degree of dequenching in terms of the rate constants of the fusion activation and probe redistribution reactions. From this equation, the relation between the degree of dequenching and real fusion-related quantities, such as the degrees of activation and lipid mixing, can be assessed. It is found that, if the rate of redistribution of self-quenching probes is rate-limiting, then the measured degree of dequenching underestimates the degree of activation (or fusion). However, the deviation of the two functions may not be too serious even when the rate of redistribution is comparable to that of activation. The formalism could be used in analyzing the kinetics of fusion activation with dequenching data even when the rate of redistribution of probes is rate-limiting. Finally, the analyses presented in this paper could be extended to fusion experiments with aqueous fluorophores [14].

Appendix A: Derivation of $X(t)$ in cell-cell fusion systems

Here, the expression for the degree of dequenching in terms of fusion and redistribution reaction rate constants will be derived for cell-cell fusion systems.The main difference between a virus-cell and a cell-cell fusion system is that the surface areas of the two fusion membranes in the latter case are not very different. As a result, probe dequenching may not be complete in cell-cell fusion systems. Let the surface areas of the two fusing membranes of a cell-cell complex be denoted A_1 and A_2, respectively, with N_0 and $N(t)$ representing the corresponding number of molecules of R-18 initially loaded onto membrane 1 and remaining there at time t. The intrinsic fluorescence of a single R-18 molecule on the two membranes at time t can then be expressed as:

$$f_1(t) = f_d\left[1 - \frac{kN(t)}{A_1}\right] \tag{A1}$$

$$f_2(t) = f_d\left[1 - \frac{k(N_0 - N(t))}{A_2}\right]. \tag{A2}$$

The total fluorescence of a complex at time t is then equal to

$$\begin{aligned} F(t) &= N(t)f_1(t) + [N_0 - N(t)]f_2(t) \\ &= f_d N_0 - \frac{f_d k N_0^2}{A_2} + \frac{2f_d k N_0}{A_2} N(t) \\ &\quad - f_d k\left[\frac{1}{A_1} + \frac{1}{A_2}\right] N(t)^2. \end{aligned} \tag{A3}$$

Substituting eq. A3 into the first integral in eq. 11 and using the $N(t)$ function in eq. 10, we obtain after the integration the mean fluorescence of a fusing complex:

$$\begin{aligned} \langle F(t)\rangle &= f_d\langle N_0\rangle - \frac{f_d k}{A_2}\langle N_0^2\rangle + \frac{2f_d k}{A_2}\langle N_0^2\rangle G_1 \\ &\quad - f_d k\left[\frac{1}{A_1} + \frac{1}{A_2}\right]\langle N_0^2\rangle G_2 \end{aligned} \tag{A4}$$

where

$$G_1 = \sum_i \left\{\frac{a_i}{\alpha_i} e^{-\alpha_i t} + \frac{a_i}{\alpha_i - \gamma}\left(e^{-\gamma t} - e^{-\alpha_i t}\right)\right\} \tag{A5}$$

$$G_2 = \sum_i \left\{\frac{a_i}{\alpha_i} e^{-\alpha_i t} + \frac{a_i}{\alpha_i - 2\gamma}\left(e^{-2\gamma t} - e^{-\alpha_i t}\right)\right\} \tag{A6}$$

Thus,

$$\langle F(0)\rangle = f_d\langle N_0\rangle - \frac{f_d k}{A_1}\langle N_0^2\rangle \tag{A7}$$

$$\langle F(\infty)\rangle = f_d\langle N_0\rangle - \frac{f_d k}{A_2}\langle N_0^2\rangle \tag{A8}$$

Substituting eqs. A4–A8 into eq. 6, we obtain

$$X(t) = 1 + [rG_1 - (1+r)G_2]/(1+r) \qquad \text{(A9)}$$

where $r \equiv A_1/A_2$. When $A_2 \gg A_1$, $r \to 0$ and eq. A9 is reduced to eq. 16.

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