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Leakage of Membrane Vesicle Contents: Determination of Mechanism Using Fluorescence Requenching

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ABSTRACT Agents such as antimicrobial peptides and toxins can permeabilize membrane vesicles to cause leakage of entrapped contents in either a graded or an all-or-none fashion. Determination of which mode of leakage is induced is an important step in understanding the molecular mechanism of membrane permeabilization. Wimley et al. (1994, *Protein Sci.* 3:1362–1378) have developed a fluorescence method for distinguishing the two modes that makes use of the dye/quencher pair 8-aminonapthalene-1,3,6 trisulfonic acid (ANTS)/*p*-xylene-bis-pyridinium bromide (DPX) without the usual need for the physical separation of vesicles from released contents. Their "requenching" method establishes the mode of release through the fluorescence changes that occur when DPX is added externally to a solution of vesicles that have released some fraction of their contents. However, the requenching method as originally stated ignored the possibility of preferential release of dye or quencher. Here we extend the theory of the method to take into account preferential release and the effects of graded leakage. The ratio of the rates of release of the cationic quencher DPX and anionic dye 8-aminonapthalene-1,3,6 trisulfonic acid can be estimated by means of the theory. For graded leakage, we show that the release of the markers does not coincide with the fluorescence changes observed in the standard leakage assay. This is true for self-quenching dyes as well and means that 1) the amount of released material will be overestimated and 2) the kinetics will be nonexponential and have artificially high apparent rates. We show how the extended requenching analysis allows the results of leakage experiments to be corrected for artifacts that result from graded and preferential leakage. Experimental evidence is presented for the existence of peptide-induced preferential graded leakage of DPX from both neutral and anionic vesicles.

INTRODUCTION

Membrane permeabilization is an important aspect of the interactions of proteins and other molecules with lipid bilayers. Induced leakage of a cell's contents is one of the most common killing mechanisms of peptides with antimicrobial, cytotoxic, or host defense functions (Habermann, 1972; Dempsey, 1990; Lehrer et al., 1991; Sansom, 1991). In addition, the nature of spontaneous leakage of molecules from lipid vesicles is important for drug encapsulation and delivery (Weinstein et al., 1977). A commonly used experimental approach to membrane leakage and/or membrane fusion utilizes vesicles that are loaded with fluorescent markers that change their properties upon release. The most frequently used markers are ANTS/DPX (an anion/cation fluorophore/quencher pair) (Ellens et al., 1984, 1986; Duzgunes et al., 1985; Parente et al., 1990; Wimley et al., 1994) and the self-quenching weak anionic dyes carboxyfluorescein (Barbet et al., 1984; Schwarz and Robert, 1990; Schwarz et al., 1992; Nagawa and Regen, 1992; Driessen et al., 1995) and calcein (Subbarao and MacDonald, 1994). One of the principal problems of leakage studies is the determination of the mechanism by which release occurs. Generally, leakage can be a graded process in which all of the vesicles release portions of their contents, or it can be all-or-none, in which some fraction of vesicles lose either all of their contents or none (Fig. 1).

Recently Wimley et al. (1994) showed that the exact leakage mechanism can be determined experimentally in some cases without resorting to the physical separation of vesicles from released material (Weinstein et al., 1981; Parente et al., 1990). Their fluorescence "requenching" procedure is based on the idea that those ANTS molecules that are released and those that are not will show different susceptibilities to quenching with externally added DPX. The technique in its original form makes the simplifying assumption that the leakage of ANTS is equal to the leakage of DPX. This is a reasonable assumption for leakage from vesicles treated with human defensins because of the formation of large pores (Wimley et al., 1994). In general, however, leakage rates must be sensitive to the physical properties of the markers and the leakage pathway. The antimicrobial peptide nisin, for example, induces leakage that strongly depends on the polarity of the charge of the different carboxyfluorescein derivatives used to examine leakage (Driessen et al., 1995). Because such phenomena operating in the ANTS/DPX system must be accounted for in order for the requenching method to be broadly useful, we undertook the investigations reported in this paper.

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Abbreviations used: POPC, palmitoyloleoylphosphatidylcholine; ANTS, 8-aminonapthalene-1,3,6 trisulfonic acid; DPX, *p*-xylene-bis-pyridinium bromide; pentapeptide, hydrophobic pentapeptide AcWLWLL; HNP-2, human neutrophil peptide (or defensin); rHNP-2, reduced and denatured form of HNP-2.

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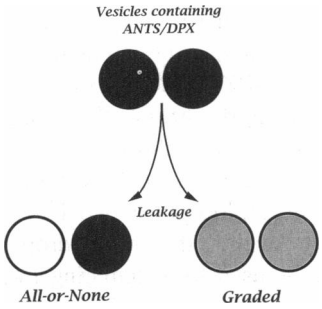

FIGURE 1 Schematic representation of the all-or-none and graded release of vesicle contents. Black vesicles are fully loaded with fluorescent marker and white vesicles are empty. Gray vesicles have leaked a fraction of their contents.

We first sought experimental evidence of preferential release from POPC vesicles loaded with ANTS/DPX by comparing release induced by a model hydrophobic pentapeptide with release induced by melittin. Preferential release of DPX over ANTS was observed to occur. We next considered the possibility of using fluorescence lifetime measurements to distinguish graded from all-or-none release under the assumption that the two cases shown in Fig. 1 would result in the same intensity but different lifetime distributions if quenching occurred by a dynamic mechanism. We found, however, that the primary quenching mechanism is a static one that may be due to the formation of a nonfluorescent complex between ANTS and DPX. This makes lifetime measurements impractical as a method for establishing the leakage mechanism and leaves the requenching method as the most viable alternative to physical separation of vesicles from released contents.

We present below, in addition to the experiments just described, the extended theory of the requenching method, which accounts for the differential release of dye and quencher. We use the theory to reanalyze leakage induced by the antimicrobial human neutrophil peptide HNP-2 (Wimley et al., 1994), which, like all defensins (neutrophil peptides), has a β-sheet structure stabilized by three disulfide bonds. For the reduced and denatured form of HNP-2, which causes graded leakage from palmitoyloleoylphosphatidylglycerol vesicles, the rate of DPX release is almost twice as high as that of ANTS. In the case of native HNP-2, the new analysis is consistent with two possibilities: the mechanism is either all-or-none or it is graded with a very high preference of ANTS leakage over the DPX. The latter possibility is unlikely, however, because experiments with large dextrans show that pores of about 25 Å diameter are formed (Wimley et al., 1994).

By means of simulations, we show that for graded leakage, the observed fluorescence changes do not coincide with the release of the fluorescent markers, including self-quenching dyes. This means that the amount of released material will be overestimated and that kinetics will be nonexponential and have artificially high apparent rates. We show how the extended theory can be used to correct for such artifacts.

MATERIALS AND METHODS

All lipids were obtained from Avanti polar lipids (Birmingham, AL). ANTS and DPX were obtained from Molecular Probes (Eugene, OR). Melittin, the principal component of bee venom, was a gift of Dr. E. Habermann. The hydrophobic pentapeptide AcWLWLL was synthesized by batch solid-phase methods using standard FMOC techniques (Grant, 1992) and was purified by reverse-phase HPLC. The buffer composition was 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 3 mM NaN_3, pH = 7.0.

Large unilamellar vesicles of approximately 0.1 μm diameter were formed by extrusion under N_2 pressure through Nucleopore polycarbonate membranes (Mayer et al., 1986). To prepare large unilamellar vesicles with entrapped ANTS and DPX, the lipid was suspended in buffer containing the solute and was then frozen and thawed 20 times before extrusion. Lipid solutions were prepared at 100 mM to maximize entrapment. The total KCl concentration in ANTS- and DPX-containing vesicles was adjusted so that the entrapped solutions had the same osmolarity as the external 50 mM KCl buffer. Unencapsulated ANTS and DPX were separated from encapsulated material using Sephadex G-100 packed in a 2.5-ml Pasteur pipette. A more detailed description of the procedure has been presented by Wimley et al. (1994).

Steady-state fluorescence measurements were performed with a SPEX Fluorolog spectrofluorometer, which was upgraded and interfaced to a computer by OLIS, Inc. (Jefferson, GA). In the quenching experiment, the spectra of ANTS were recorded in the region from 420 to 670 nm with 380 nm excitation. Emission and excitation slits were 10 and 20 nm, respectively. After the spectrum for the blank was subtracted, the area under the curve was used as a measure of intensity. The excitation and emission wavelengths in kinetic experiments were kept at 380 and 520 nm with slits of 20 and 40 nm, respectively. The contribution of light scattering was negligible in all cases. The fluorescence lifetimes were determined by frequency-domain measurements using a SLM-48000 multifrequency phase-and-modulation spectrofluorometer (SLM/Aminco, Urbana, IL). The magic-angle configuration of polarizers was used to exclude artifacts associated with dynamic depolarization. In all cases the optical density of the sample was less than 0.1. Nonlinear least-squares analyses and data simulations were performed with the commercial software package Origin 3.5 (MicroCal, Inc., Northampton, MA).

The fluorescence quenching was analyzed according to two alternative models (Vaughn and Weber, 1970; Eftink and Ghiron, 1981):

$$I_0/I = (1 + K_d[\mathrm{DPX}]) \cdot \exp(V[\mathrm{DPX}]) \quad (1)$$

or

$$I_0/I = (1 + K_d[\mathrm{DPX}]) \cdot (1 + K_a[\mathrm{DPX}]) \quad (2)$$

where I_0 and I refer, respectively, to the ANTS fluorescence in the absence and in the presence of DPX at a concentration [DPX]. K_d is a dynamic quenching constant determined in separate lifetime experiments according to

$$\tau_0/\tau = (1 + K_d[\mathrm{DPX}]) \quad (3)$$

Equation 1 describes the sphere-of-action model and yields the volume element V within which the quenching is immediate. Equation 2 represents the weak-association model and yields the association constant K_a for ANTS/DPX nonfluorescent complex.

THEORY

The general idea of the requenching method (Wimley et al., 1994) is as follows. After partial leakage of vesicle contents has occurred, the vesicle solutions are titrated with DPX to determine the degree to which the dye molecules remaining in the vesicles are quenched. If the quenching inside is not changed by dye release, the mechanism is all-or-none. If the

quenching inside is changed, the release is graded. We present below a summary of the requenching method and then extend it to include the possibility of preferential release of ANTS or DPX.

The total fluorescence observed will be $F = F_o + F_i$, where F_o is the fluorescence originating from outside the vesicles and F_i is that from inside. If there were no quenching, the observed total fluorescence from ANTS inside and outside the vesicles would have the maximal value $F^{max} = F_o^{max} + F_i^{max}$. The addition of Triton X-100 causes lysis of the vesicles and dilution of the DPX to a negligible concentration so that the fluorescence observed in that case will essentially be F^{max}. We define quenching outside (Q_{out}) and inside (Q_{in}) the vesicles and the total quenching (Q_{total}) as follows:

$$Q_{out} = F_o/F_o^{max} \tag{4a}$$

$$Q_{in} = F_i/F_i^{max} \tag{4b}$$

$$Q_{total} = F/F^{max} \tag{4c}$$

Defined in this way, the Q parameters have a value of 1 when there is no quenching and 0 when there is complete quenching. The total fluorescence F is given by $F = Q_{total} \cdot F^{max} = Q_{out} \cdot F_o^{max} + Q_{in} \cdot F_i^{max}$. In the absence of quenching, the ratio of fluorescence coming from inside to fluorescence coming from outside the vesicle equals the molar ratio of dye molecules inside and outside. Therefore, the fractions of ANTS outside and inside the vesicles are $f_{out} = F_o/F^{max}$ and $f_{in} = F_i/F^{max}$, and it must be true that $f_{out} + f_{in} = 1$. The total quenching now can be expressed as

$$Q_{total} = Q_{out} \cdot f_{out} + Q_{in} \cdot (1 - f_{out}) \tag{5}$$

We are interested in the behavior of Q_{in} with the change of f_{out} because it reflects the state of the contents of the vesicles. Experimentally, one first disrupts untreated vesicle-encapsulated ANTS/DPX preparations with Triton X-100, incrementally adds DPX, and then measures the normalized fluorescence $F_{DPX}/F^{max} = Q_{out}$ to obtain a calibration curve for calculating the value of Q_{out} for a particular DPX addition. Then, the peptide (or other agent that induces leakage) is added to an ANTS/DPX vesicle solution and the system is incubated long enough to reach a plateau level of fluorescence. With this solution, the F_{total} as a function of DPX concentration is measured for incremental additions of DPX. After the last addition of DPX, Triton X-100 is added to determine F^{max} from Eq. 4C. Using those measurements and the Q_{out} calibration curve, one plots Q_{total} versus Q_{out} and thereby obtains a linear curve with slope f_{out} and intercept $Q_{in} \cdot (1 - f_{out})$, determined by using linear least-squares fitting procedures.

The requenching technique is based on the assumption that additions of DPX affect only Q_{out} but not Q_{in} or f_{out}. For graded release, however, the quenching of ANTS molecules remaining inside might be affected by externally added DPX because of the leakage of DPX back into the vesicles. This possibility is supported by experimental evidence presented below. Under these circumstances, the acquisition time for the requenching experiment should be as short as possible to reduce DPX back-flow effects. When the DPX back-flow is particularly strong, the titration of the same sample with consecutive additions of DPX is not recommended. Instead, experiments on several identical samples, one for each concentration of added DPX, should be performed, and the size of the initial drop in fluorescence intensity should be used for calculating F_{DPX} (Fig. 2).

In the case of all-or-none release, quenching of dye remaining inside will be independent of the amount of material released, i.e., Q_{in} will be independent of f_{out}. This is not true for graded release. Below we derive the dependence of Q_{in} on f_{out} for graded release. First, the dependence of Q_{in} on the concentration of the DPX inside the vesicles $[DPX]_{in}$ must be established. In general, the dependence of Q_{in} on $[DPX]_{in}$. is determined in a model experiment and is described by either Eq. 1 or Eq. 2. We will use Eq. 2, the model of weak-association of ANTS and DPX, which will be shown below to be more consistent with the model data. The overall conclusions of our analysis, however, are independent of the particular quenching mechanism and can be

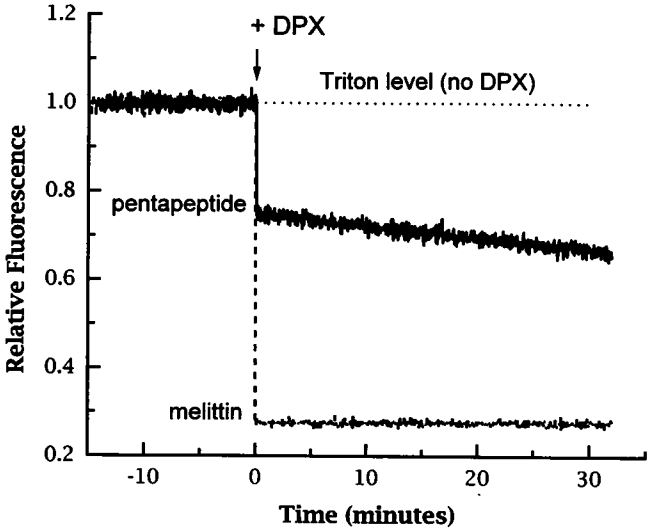

FIGURE 2 Requenching kinetics of POPC vesicles originally loaded with ANTS and DPX and treated with the pentapeptide Ac-WLWLL or melittin. Vesicle suspensions containing 0.5 mM POPC were mixed with 0.14 mM pentapeptide (*solid curve*) or 0.4 μM melittin (*dashed curve*). After the fluorescence increase due to leakage had reached a plateau, additional DPX was added at the time designated $t = 0$. Data are normalized to the fluorescence observed when the same amount of untreated vesicles where lysed with Triton X-100. Although in both cases DPX had apparently leaked completely as judged by fluorescence, the amount of released ANTS is clearly different, as indicated by the changes in the fluorescence levels for times greater than $t = 0$. The smaller immediate drop in intensity observed after addition of DPX for the Ac-WLWLL sample suggests that less ANTS was released compared to the amount released by melittin. The slow decline of fluorescence for $t > 0$ seen in the Ac-WLWLL sample may be a result of quenching of internal ANTS due to the back-flow of the externally added DPX into the vesicles. These results demonstrate the existence of graded preferential release of DPX over ANTS.

applied for systems other than ANTS/DPX. Rewriting Eq. 2 and substituting I_0/I with $1/Q_{in}$ yields

$$Q_{in} = [(1 + K_d \cdot [DPX]_{in}) \cdot (1 + K_a \cdot [DPX]_{in})]^{-1} \quad (6)$$

The concentrations of dye and quencher inside the vesicles are described by their initial values and the fractions that have leaked out:

$$[ANTS]_{in} = [ANTS]_0 \cdot (1 - f_{out}^{ANTS}) \quad (7a)$$

$$[DPX]_{in} = [DPX]_0 \cdot (1 - f_{out}^{DPX}) \quad (7b)$$

By definition, $f_{out} = f_{out}^{ANTS}$. If the release of DPX follows exactly the release of ANTS, then $f_{out}^{DPX} = f_{out}$. To account for the possibility of the preferential leakage of ANTS or DPX, we introduce the parameter α which is, in the simplest case of exponential release of markers, the ratio of the rates of release of DPX and ANTS: $\alpha = k_{DPX}/k_{ANTS}$. The individual rates are defined by

$$\frac{d[ANTS]_{in}}{dt} = -k_{ANTS} \cdot t \quad (8a)$$

$$\frac{d[DPX]_{in}}{dt} = -k_{DPX} \cdot t \quad (8b)$$

Equation 7b may consequently be rewritten as

$$[DPX]_{in} = [DPX]_0 \cdot (1 - f_{out})^{\alpha} \quad (9)$$

This relation could, in some cases, also be valid when effluxes of ANTS or DPX are not exponential. Equation 9 can be used as a definition of α in such cases.

Equation 6 can now be rewritten so that it contains only two unknown parameters, $[DPX]_0$ and α:

$$Q_{in} = [(1 + K_d \cdot [DPX]_0 \cdot (1 - f_{out})^{\alpha}) \cdot (1 + K_a \cdot [DPX]_0 \cdot (1 - f_{out})^{\alpha})]^{-1} \quad (10)$$

This is the basic equation describing the dependence of the internal quenching (Q_{in}) on fraction of dye released (f_{out}). It may be used to distinguish all-or-none from graded release and to estimate the parameter α.

RESULTS AND DISCUSSION

Demonstration of the graded preferential leakage of DPX over ANTS

Consider a suspension of vesicles loaded with dye and quencher that has been treated with a permeabilizing agent. The fluorescence increases in time to a maximum value that is equal to the fluorescence observed when the vesicles are fully solubilized by detergent. For all-or-none release, this maximum means that the contents of all vesicles have been entirely released. For graded release, however, two other possibilities exist: all the DPX leaked out while some ANTS remained inside (case A) or vice versa (case B). (The effect on fluorescence from the quenching by completely released DPX (case A) or from the quenching of ANTS remaining inside (case B) is negligible because, for the lipid concentrations that are used in practice, the total internal vesicle volume is negligibly small compared to the external volume.) In either of these cases, the addition of DPX to the external volume will reduce the fluorescence to different extents because the amount of ANTS immediately accessible for quenching is much higher in case B.

An experimental demonstration of the preferential leakage of DPX is presented in Fig. 2. POPC vesicles loaded with ANTS and DPX were initially treated with a model hydrophobic pentapeptide AcWLWLL (solid line) or melittin (dashed line). We chose melittin because it is known to induce leakage (Habermann, 1972; Schwarz et al., 1992; Subbarao and MacDonald, 1994), and its various membrane actions have been studied extensively (see review by Dempsey, 1990). The pentapeptide, synthesized for studies of binding to the membrane interface, was chosen because of its lack of secondary structure and the expectation that it would not form a structured leakage path. It partitions into the lipid membrane with a mole-fraction partition coefficient of about 30,000 (W. C. Wimley and S. H. White, unpublished observations). After incubation of ANTS/DPX-loaded vesicles with the peptides, the fluorescence stabilized at the level corresponding to that observed after Triton disruption. Additional DPX solution was added at $t = 0$ to bring the external concentration up by 4.5 mM (Fig. 2). Simple dilution in the absence of quenching causes a drop of intensity to approximately 0.9; and the remainder of the immediate change is due to the quenching of ANTS released by time $t = 0$. Although both peptides *appear* to have released most of the vesicle contents, it is clear that melittin released much more of the ANTS than the pentapeptide did. The slow kinetics observed for the pentapeptide after the DPX addition is possibly due to the back-flow of DPX into the ANTS-containing vesicles. These observations are consistent with graded and preferential release of DPX over ANTS. The absence of the slow kinetics in the case of melittin means that either all of the ANTS had leaked out by the time the DPX was added or that DPX cannot leak in. However, the data do not permit a definite conclusion to be drawn about the mechanism of release.

The quenching mechanism

To implement the requenching analysis, one must know ANTS fluorescence as a function of DPX concentration. Consequently, knowledge of the quenching mechanism for the ANTS/DPX pair is essential.

A Stern-Volmer plot for fluorescence intensity and lifetime is shown in Fig. 3. Because the steady-state intensity of ANTS (open symbols) decreases significantly more upon addition of DPX than the lifetime does (solid symbols), the quenching occurs primarily via a static mechanism. (By definition, static quenching reduces the intensity but not the lifetime.) The same conclusion is supported by the fact that quenching becomes less efficient with increasing tempera-

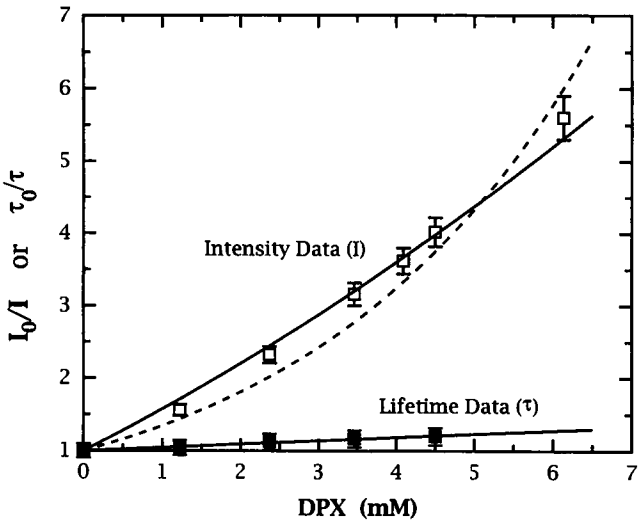

FIGURE 3 Stern-Volmer plot for the quenching of ANTS by DPX. The lifetime data (*solid squares*) were fitted with Eq. 3 to yield K_d = 50 M^{-1} and τ_0 = 4.0 ns. Steady-state fluorescence intensity data (*open squares*) were fitted according to two alternative models: Eq. 1 for the sphere-of-action model (*dashed line*, V = 270 M^{-1}) and Eq. 2 for the weak association model (*solid line*, K_a = 490 M^{-1}). Quenching is mainly static because the decrease in intensity is much greater than the decrease in lifetime.

ture (data not shown). Two models are used to describe static quenching. The model that assumes the weak association of fluorophore and quencher results in a more consistent data fit (Eq. 2; solid line in Fig. 3) than the more widely applied sphere-of-action model (Eq. 1; dashed line in Fig. 3). Neither of these models, however, provides a perfect fit, suggesting the possibility that both mechanisms contribute to quenching. However, our analysis depends only slightly on the exact model used to quantitate steady-state fluorescence quenching, and we choose to use the weak association model, which assumes that the interaction of ANTS and DPX results in a formation of a nonfluorescent complex in a ground state. We use K_d = 50 1/M and K_a = 490 1/M, derived from best fit of the data in Fig. 3, in Eq. 10.

All-or-none leakage can, in principle, be distinguished from graded leakage by the fluorescence lifetime measurements. In the case of dynamic quenching, for example, the two cases shown in Fig. 1 would result in the same intensity but different lifetime distributions. However, the dynamic contribution to quenching for the ANTS/DPX pair was found to be small, $K_d \ll K_a$ (Fig. 3). This prevents a direct resolution of graded and all-or-none release because changes in τ are too small to be useful. Therefore, the requenching method remains as the simplest method for determining the leakage mechanism. We use it in the next section to distinguish all-or-none from graded release and to estimate the parameter of preferential release, α, in the latter case.

Determination of the mechanism of leakage

We performed a set of simulations using Eq. 10 to explore the effect of $[DPX]_0$ and α on the Q_{in} dependence of f_{out}. The results are shown in Fig. 4. The solid lines in Fig. 4 *A* correspond to equal release of dye and quencher in a graded manner, and the dashed lines correspond to all-or-none release. Experimentally, we are limited to certain values of f_{out} (not more than 0.8) because the reduction in the fraction of molecules remaining inside increases the experimental error. The ability to distinguish the two mechanisms is affected by the choice of $[DPX]_0$ because the two curves become more similar at lower f_{out} values as $[DPX]_0$ increases. The optimal conditions are met when moderate concentrations of 4–8 mM DPX are encapsulated in vesicles.

The variation in α has a dramatic effect on determination of the leakage mechanism as shown in Fig. 4 *B*. Whereas preferential release of DPX ($\alpha > 1$) increases the difference between all-or-none and graded leakage, the preferential release of ANTS diminishes it. In the limiting case of

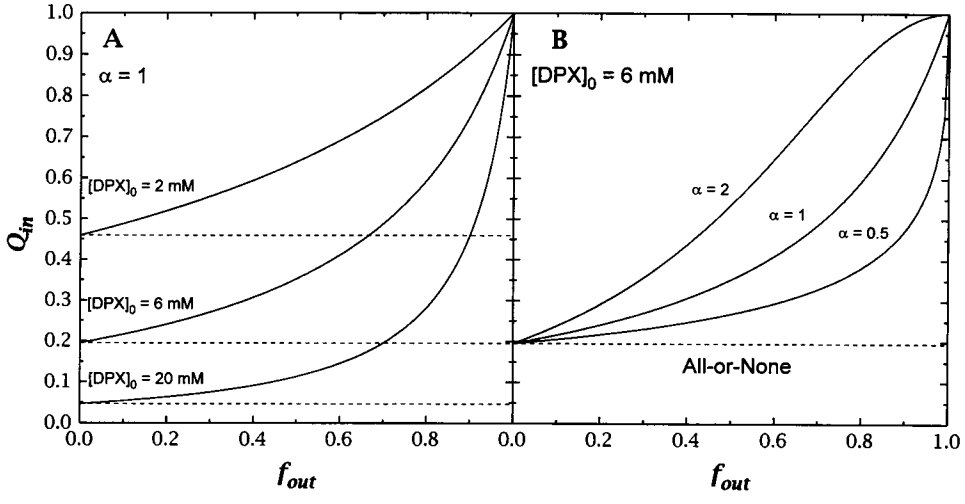

FIGURE 4 Simulations of internal quenching (Q_{in}) of ANTS inside vesicles as a function of the ANTS released (f_{out}). Dashed lines correspond to all-or-none release and solid lines to graded release simulated using Eq. 10. (*A*) The effect of initial DPX concentration for a nonpreferential release (α = 1). (*B*) The effect of preferential release. Because one is experimentally limited to $f_{out} <$ 0.8, both $[DPX]_0$ and α affect one's ability to distinguish all-or-none from graded release in a requenching experiment.

complete absence of DPX release ($\alpha = 0$), the graded release of ANTS becomes indistinguishable from the all-or-none release of ANTS and DPX.

The internal quenching (Q_{in}) as a function of ANTS released (f_{out}), measured experimentally by using native and reduced human defensin HNP-2 (Wimley et al. 1994), is presented in Fig. 5. The results for reduced HNP-2 (open symbols) are unequivocal: the release is graded. The best fit of the data (solid curve) with Eq. 10 yields $\alpha = 1.7$. The preferential release of the positively charged DPX over negatively charged ANTS could be caused by the fact that negatively charged lipids were used in the preparation and that the local concentration of charged molecules near the bilayer is different from the bulk concentration. This would cause ANTS to be depleted near the membrane surface and DPX to be concentrated there. In the case of native HNP-2, the data are consistent with either all-or-none release (dashed line) or highly preferential graded release of ANTS (dotted line). The best fit analysis with Eq. 10 yields $\alpha = 0.12$, which seems unrealistic in light of the charge effects just discussed. In addition, the latter model is in conflict with the HNP-2-induced release of large dextrans (Wimley et al., 1994). We conclude that the mechanism for native HNP-2 is all-or-none. This is also consistent with the threshold nature of the release observed for HNP-2 with increasing concentration (Wimley et al. 1994). The fact that Q_{in}, at higher values of f_{out}, is slightly higher could mean that a small amount of graded release is occurring. This could be due to the effect of HNP-2 molecules that are not assembled into the regular large pores responsible for all-or-none release (Wimley et al., 1994). As our result with model pentapeptides indicates (Fig. 2 and unpublished data), graded release can be caused even by a small peptide bound to the membrane surface, possibly by causing destabilization of the bilayer. Therefore, a small amount of graded release is likely to be present in all systems.

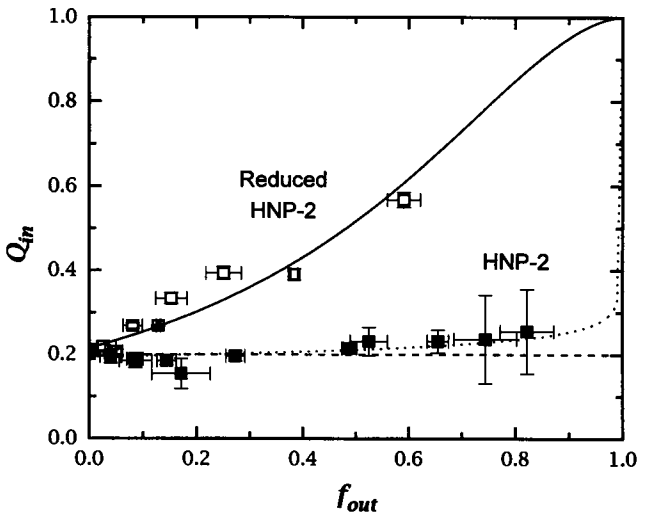

FIGURE 5 Internal quenching (Q_{in}) of ANTS inside vesicles as a function of the ANTS released (f_{out}) for leakage induced by the human defensin HNP-2 (data from Wimley et al., 1994). Solid squares (■) are for native HNP-2. Open squares (□) are for reduced HNP-2 (rHNP-2). The solid curve is a best fit of the rHNP-2 data with Eq. 10 ($[DPX]_0 = 5.5 \pm 0.4$ mM, $\alpha = 1.7 \pm 0.1$). It suggests that release is graded and that the leakage of DPX is preferential over ANTS. Two curves (dotted and dashed) have been fitted to the native HNP-2 data, which suggest two possibilities for release: the dotted curve is a best fit of data with Eq. 10 ($[DPX]_0 = 6.0 \pm 0.1$ mM, $\alpha = 0.12 \pm 0.01$), suggesting graded release with highly preferential release of ANTS over DPX. The dashed curve corresponds to all-or-none release. However, additional data on the leakage of large dextrans (Wimley et al., 1994) indicate that the correct mechanism is all-or-none.

Effects of the leakage mechanism on the results of kinetic measurements

How do the differences in the leakage mechanisms translate into fluorescence kinetic measurements? The kinetics of the fluorescence increase, caused by leakage, can be quantitated according to the same basic equations developed for the requenching procedure. In the absence of the externally added DPX and infinite dilution of the DPX that had leaked out, $Q_{out} = 1$. Equation 5 may be rewritten in combination with Eq. 4C given that f_{out} and consequently Q_{in} are functions of the time t elapsed after the vesicles were mixed with leakage-inducing agent. The result is

$$F(t) = F^{max} \cdot [f_{out}(t) + Q_{in}(t) \cdot (1 - f_{out}(t))] \quad (11)$$

where Q_{in} as a function of f_{out} is constant for all-or-none release or given by Eq. 10 for graded release.

For simplicity, assume that the dye is released at the constant rate of $k_{ANTS} = k_{true}$, so that from Eq. 8a

$$f_{out}(t) = 1 - \exp(-k_{true} \cdot t) \quad (12)$$

The results of simulations of the fluorescence change using Eqs. 11 and 12 are presented in Fig. 6 (solid curves). The initial fluorescence $F(0)$ was subtracted and the final increase was normalized to 1 ($F^{max} - F(0) = 1$) for each curve separately. In all cases, ANTS was released completely with the same rate $k_{true} = 0.05$. However, only in the case of all-or-none release does the fluorescence change exactly follow the release of ANTS.

Graded release in all cases leads to nonexponential kinetics. The curves generated for different $[DPX]_0$ and α parameters were fitted to the following equation for a simple exponential association (dashed lines in Fig. 6):

$$\frac{F(t) - F(0)}{F^{max} - F(0)} = 1 - \exp(-k_{app} \cdot t) \quad (13)$$

The recovered apparent kinetic constants for graded release k_{app} were always found to be higher than k_{true}.

Increasing the starting concentration of DPX leads to a smaller difference between real efflux of material and changes in fluorescence signal (Fig. 6 *A*). Preferential release of dye or quencher will also have an effect on the apparent kinetics (Fig. 6 *B*). An increase in α leads to a stronger deviation from the real kinetics. Only in the limiting case of $\alpha = 0$ will the fluorescence changes for graded release follow the release of ANTS exactly. It is important

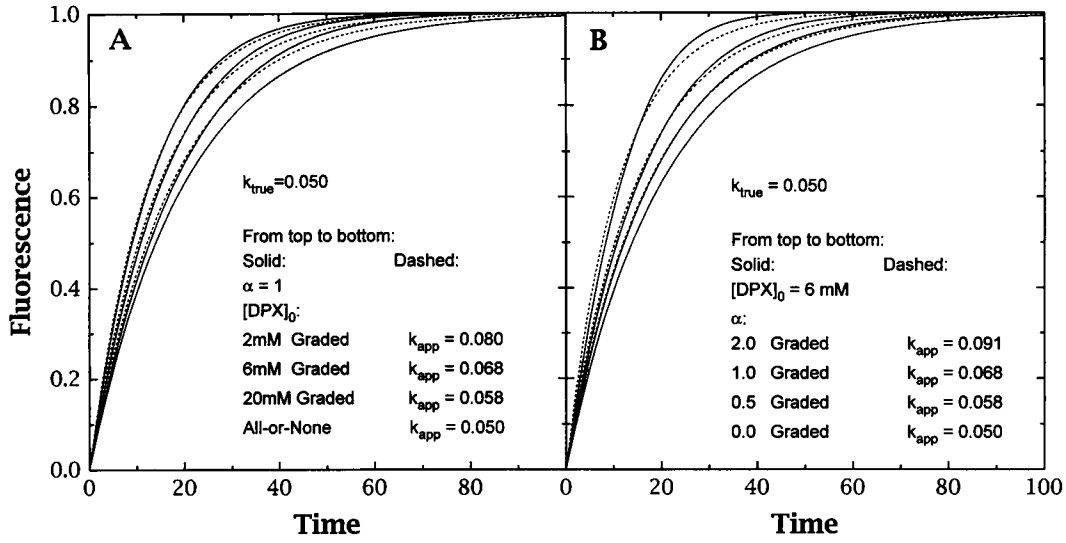

FIGURE 6 Fluorescence kinetics associated with the ANTS release with a rate $k_{true} = 0.05$. (*A*) Nonpreferential leakage. (*B*) Preferential graded leakage. The solid curves show the fluorescence increases simulated using Eqs. 11 and 12 for the all-or-none and the nonpreferential ($\alpha = 1$) graded leakage (*A*) and preferential graded leakage (*B*). The dashed lines correspond to the best fit of the exponential association model (Eq. 13), yielding an apparent rate k_{app}. In all cases of graded release, the kinetics of the fluorescence change became nonexponential. The apparent rate was always higher than the true rate of ANTS release, $k_{app} > k_{true}$. The fluorescence data follow the leakage exactly (lowest *solid* and *dashed curves* coincide) only when the release is all-or-none or when only ANTS is released in a graded fashion ($\alpha = 0$). Note that for the case of nonpreferential graded release (*A*), which is realized for self-quenching dyes, the nonexponential behavior of fluorescence kinetics and its deviation from true leakage of material increase with the decrease of concentration of loaded marker.

to realize that in the case of graded release, even in the absence of preferential release ($\alpha = 1$), the observed fluorescence change does not represent true leakage of vesicle contents. This means that even when leakage is studied with self-quenching dyes, such as carboxyfluorescein or calcein, one must know whether the mechanism is all-or-none or graded to interpret the results correctly. Consider, for example, two systems that have the same amount of dye being released with the same kinetic constant, but which have different leakage mechanisms. The one with graded release will *appear* to have a nonexponential leakage with a higher apparent rate.

In reality, even all-or-none leakage can have more complex kinetics than described by Eq. 12 (Schwarz and Robert, 1990; Schwarz et al., 1992; Wimley et al., 1994, and unpublished observations). Therefore, in practice, it is not possible to extract α directly from the nonexponential behavior of $F(t)$ in kinetic experiments. However, α can be estimated from the requenching experiment as shown in Fig. 5. Equation 11 will become suitable for fitting of the real data, when a more realistic model of leakage (as compared to Eq. 12) is introduced to describe the time dependence of $f_{out}(t)$. This, however, will be a subject of future studies. Clearly, the mechanism of release must be established before any attempt is made to fit a kinetic model for membrane leakage to experimentally observed fluorescence kinetics.

How to determine correctly the amount of leakage from the fluorescence data

The leakage caused by various peptides quite often does not go to completion, and fluorescence is still below the F^{max} level observed with Triton, even after relatively long incubation (Schwarz et al., 1992; Subbarao and MacDonald, 1994; Wimley et al., 1994, and unpublished observations). The relative increase in fluorescence calculated as $(F(\infty) - F(0))/(F^{max} - F(0))$ is often used as a measure of effectiveness of release. In the case of graded release, as we established, an additional correction to convert fluorescence change into efflux of material is required. For known values of α and $[DPX]_0$, such a correction can be performed numerically by using Eq. 11. The following procedure should be applied. The values of F/F^{max} as a function of f_{out} can be generated from Eq. 11. Those values are then compared to the experimentally observed F/F^{max} to find the corresponding values for f_{out}. We used this procedure to find the amounts of ANTS and DPX released by human defensins (Table 1) using the parameters of α and $[DPX]_0$ estimated from the requenching experiment (Fig. 5). Our

TABLE 1 Leakage of ANTS/DPX from POPG vesicles induced by reduced HNP-2 defensin (rHNP-2)

Concentration of rHNP-2	Fractional fluorescence change	Fraction of ANTS released (f_{out}^{ANTS})	Fraction of DPX release (f_{out}^{DPX})
55nM	0.22	0.15	0.25
92nM	0.78	0.58	0.77

Because the release is graded, the fractional fluorescence change $(F(\infty) - F(0))/(F^{max} - F(0))$ (data of Wimley et al., 1994) will not coincide with the fractions of dye ($f_{out}^{ANTS} = f_{out}$) and quencher ($f_{out}^{DPX} = 1 - (1 - f_{out})^{\alpha}$) released. The values of f_{out} are back-calculated from Eqs. 10 and 11 using the values of $[DPX]_0 = 5.5$ mM and $\alpha = 1.7$ estimated from the requenching experiment (Fig. 5).

results confirm that graded release introduces significant errors into fluorometric determination of leakage. However, knowledge of the parameters of preferential graded release allows those artifacts to be corrected.

CONCLUSIONS

We have shown that graded preferential release for ANTS/DPX-loaded vesicles can be observed experimentally for both neutral and anionic lipid vesicles. We demonstrated that graded release, if ignored, will lead to errors in fluorometric determination of both the amount of released material and the rate of release, not only for ANTS/DPX but for self-quenching dyes as well. The requenching technique described here allows the mechanism of leakage to be established, and in the case of graded release allows the parameters of preferential release to be estimated. This in turn provides a correction to both kinetic and steady-state measurements of leakage.

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