Interactions of Monomeric Rabbit Neutrophil Defensins with Bilayers: Comparison with Dimeric Human Defensin HNP-2[†]

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ABSTRACT: Human antimicrobial neutrophil defensin HNP-2 has been shown to form large multimeric pores in pure 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) bilayers that lead to all-or-none release of vesicle contents [Wimley et al. (1994) *Protein Sci. 3,* 1362−1373]. Because human neutrophil defensins form natural dimers in solution, the question arises as to the role of dimerization in pore formation. However, the dimers are so stable that this question is not easily answered directly. Rabbit neutrophil defensins, whose three-dimensional structures are very similar to those of human defensins, are monomeric in aqueous solution and thus provide an opportunity to test the hypothesis that dimerization may play a role in multimeric pore formation. We therefore examined the interactions of the six known rabbit neutrophil defensins with large unilamellar vesicles (LUV) under the conditions known to lead to stable pore formation by HNP-2. We find that the rabbit defensins bind strongly to LUVs formed from pure POPG or mixtures of POPG with neutral (zwitterionic) phospholipid but induce leakage of vesicle contents only from pure POPG vesicles. Rabbit defensin NP-4 does not cause leakage under any conditions examined. The remaining defensins, NP-1, NP-2, NP-3A, NP-3B, and NP-5, cause graded release of the contents of pure POPG vesicles as does a mixture of the six defensins. The graded release indicates that the rabbit defensins do not form stable pores in the membrane. This result thus suggests that the structural features of human defensins that permit dimer formation in aqueous solution are likely to be important in the formation of multimeric pores.

Defensins are small ($M_r \approx 4000$) antimicrobial peptides that have been isolated from the cytoplasmic granules of neutrophils of humans (Selsted et al., 1985b; Wilde et al., 1989), rats (Eisenhauer et al., 1989), rabbits (Selsted et al., 1985a), and guinea pigs (Selsted & Harwig, 1987) and from intestinal Paneth cells of mice (Selsted et al., 1992) and rats (Chow et al., 1993). They have also been cloned from human small bowel (Jones & Bevins, 1992, 1993). Neutrophil defensins are believed to kill phagocytosed cells intracellularly (Lehrer et al., 1991), whereas the enteric defensins probably function extracellularly (Selsted et al., 1992). These "classic" defensins are 29−35 amino acids long, have three disulfide bonds that stabilize a rigid β-sheet structure, and have a high content of arginine (4−10 per molecule). Three other classes of defensin-like peptides have been isolated from bovine tissues (Selsted et al., 1993), insect hemolymph (Hoffman & Hetru, 1992), and plants (Broekaert et al., 1995) and are designated β-defensins, insect defensins, and plant defensins, respectively. The structural features and modes of action of the three classes of defensins have been discussed in a recent review (White et al., 1995).

Available evidence suggests that all defensins act by permeabilizing the cell membranes of target microbes (Lehrer et al., 1985, 1989; Lichtenstein et al., 1986) but the details of the process are not yet clear. Their net positive charge causes them to interact readily with negatively charged model and, presumably, natural membranes (Kagan et al., 1990; Fujii et al., 1993; Wimley et al., 1994). A transmembrane potential may be required for antimicrobial activity (Lehrer et al., 1985, 1989). Wimley et al. (1994), however, observed permeabilization of large unilamellar vesicles (LUV)[1] formed from POPG by the dimeric human neutrophil defensin HNP-2 in the absence of a membrane potential. Upon binding, which is primarily electrostatic (Wimley et al., 1994), HNP-2 apparently breaches the bilayer by aggregating to form a pore about 25 Å in diameter (Wimley et al., 1994). This is consistent with tryptophan fluorescence and circular dichroism measurements which reveal that the secondary structure of human defensins does not change upon interaction with bilayers (Fujii et al., 1993). The leakage of the vesicle contents through the pores occurs in an all-or-none manner which indicates that the pores are sufficiently large and long-lived to allow full release of vesicle contents. The model for the pore proposed by Wimley et al. (1994) consists of a multimer of approximately six HNP-2 dimers. The question thus arises as to the exact role of dimerization in pore formation. Specifically, is dimerization in the aqueous phase required for pore formation? Unfortunately, the human neutrophil defensin dimer is so stable that this question

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[1] Abbreviations: POPG, 1-palmitoyl-2-oleoyl phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; LUV, extruded unilamellar vesicles of 100 nm diameter; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylenebispyridinium bromide; HNP, human neutrophil peptide (human defensin); NP, rabbit neutrophil peptide (rabbit defensin); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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Human HNP-2       CYCR IPAC IAGER RYGTC IYQGR LWAFC C

Rabbit NP-1     VVCACR RALC LPRER RAGFC RIRGR IHPLC CRR
       NP-2     VVCACR RALC LPLER RAGFC RIRGR IHPLC CRR
       NP-3A    GICACR RRFC PNSER FSGYC RVNGA RYVRC CSRR
       NP-3B    GRCVCRKQLLC SYRER RIGDC KIRGV RFPFC CRR
       NP-4     VSCTCR RFSC GFGER ASGSC TVNGV RHTLC CRR
       NP-5     VFCTCR GFLC GSGER ASGSC TINGV RHTLC CRR
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FIGURE 1: Amino acid sequences of rabbit neutrophil defensins and human neutrophil defensin HNP-2. Conserved residues are in bold type.

cannot be easily answered. The dimers are broken, of course, if the disulfide bonds stabilizing the monomer are reduced, but the mechanism of action of reduced HNP-2 is quite different (Wimley et al., 1994).

Rabbit defensins have a high sequence and structural simularity to the human defensins [reviewed by White et al. (1995)] but, conveniently, are monomeric in aqueous solution (Pardi et al., 1988). This difference in solution behavior raised the possibility that the membrane leakage mechanism of rabbit defensins might differ from that of human defensins if dimerization is important for pore formation. To explore that possibility, we have examined the leakage induced by the six known rabbit defensins (Figure 1) using the experimental conditions under which the human defensins cause all-or-none leakage. We find that the leakage mechanism is fundamentally different: Five of the six rabbit defensins cause graded release of the contents of POPG vesicles. The sixth defensin, NP-4, caused no measurable leakage. Graded leakage implies that the rabbit defensins do not form stable long-lived pores in the membranes. Because there is evidence for antimicrobial synergy between different defensins *in vitro* (Lehrer et al., 1986), we also examined the lytic properties of a mixture composed of the naturally occurring ratio of the six peptides to see if synergism occurred in the model system and if leakage changed to all-or-none, which would imply heteromeric pore formation. No synergistic effects were observed with this mixture which was also found to induce graded leakage of POPG vesicles. Taken together, these results suggest that the structural features of human defensins that permit dimer formation in aqueous solution are likely to be important in the formation of multimeric pores in POPG bilayers.

MATERIALS AND METHODS

Materials. All lipids were obtained from Avanti polar lipids (Birmingham, AL). The fluorophore ANTS and the fluorescent quencher DPX were obtained from Molecular Probes (Eugene, OR). Water was glass distilled. The buffer solution (pH = 7) contained 10 mM HEPES, 50 mM KCl, 1 mM EDTA, and 3 mM NaN_3. As described in detail elsewhere (Selsted et al., 1984), defensins were extracted from rabbit peritoneal granulocytes and purified by ion exchange and reversed-phase HPLC.

Vesicle Preparation. Lipids were mixed in chloroform which was subsequently removed under a stream of argon. Buffer was added to the dry lipids and the suspension frozen and thawed 2−3 times. Large unilamellar vesicles (LUV) of approximately 0.1 μm diameter were formed by extrusion under N_2 pressure through Nucleopore polycarbonate membranes (Costar, Cambridge, MA) (Mayer et al., 1986; Wimley et al., 1994). To prepare LUV with entrapped ANTS and DPX, lipid was suspended in buffer containing 9 mM ANTS and 25 mM DPX and then frozen and thawed 5−10 times prior to extrusion. Lipid solutions were prepared at 100 mM to ensure maximum entrapment (Wimley et al., 1994). 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. Free ANTS and DPX were separated from encapsulated material using Sephadex G-100 packed in 2.5 mL Pasteur pipettes. No detectable leakage of any solutes in the absence of defensins was observed for time periods equivalent to the duration of leakage experiments (about 5 h).

Binding and Aggregation. Binding of defensins to POPC/POPG vesicles was assayed by equilibrium dialysis and quantitative reverse-phase HPLC (Wimley & White, 1993). Dialysis cells (Spectrum Corp., Houston, TX) with 1.5 mL half-cells, separated by Nucleopore polycarbonate membranes with pore size 0.015 μm, were used for equilibrium dialysis. Experiments were begun with 5 or 10 mM vesicles in one cell and 15 μg of defensin in the other. The dialysis was run overnight at room temperature (equilibration occurred in approximately 10 h). Peptide concentration was assayed by HPLC (Wimley & White, 1993).

Fluorescence Spectroscopy. Fluorescence spectroscopy was performed using a SPEX Fluorolog fluorimeter interfaced to a computer by OLIS, Inc. (Jefferson, GA). Excitation and emission wavelengths for ANTS were 360 nm (slit 20 nm) and 515 nm (slit 50 nm), respectively. The contribution of light scattering was negligible in all cases.

Leakage of ANTS and DPX. The leakage of the fluorophore/quencher pair ANTS/DPX was assayed by measuring the fluorescence of ANTS in suspensions of ANTS/DPX-containing vesicles (Ellens et al., 1984). ANTS/DPX-containing vesicles (about 400 μM) were placed into an unstirred 1 × 0.2 cm quartz cuvettes of volume of 0.5 mL, and the ANTS fluorescence was measured as a function of time after addition of defensin. In order to compare results from different experiments, data were represented in terms of fractional fluorescence f_F defined by

$$f_F = (F - F_{initial})/(F_{max} - F_{initial}) \quad (1)$$

where F is the measured fluorescence, $F_{initial}$ the initial quenched fluorescence, and F_{max} the fluorescence corresponding to 100% leakage measured after addition of 0.4% Triton X-100. Wimley et al. (1994) have shown that DPX (an organic divalent cation) does not bind strongly to anionic POPG. In the absence of leakage, a fluorescence $F_{initial} = QF_{max}$ is observed where Q is the quenching factor (about 0.5 for POPG liposomes and 0.2 for POPG/POPC and POPG/POPE liposomes). Note that $Q = 0$ corresponds to complete quenching while $Q = 1$ corresponds to no quenching.

Leakage from vesicles can occur by one of two processes: graded, in which all vesicles release portions of their contents, or all-or-none, in which some vesicles lose all of their contents while others lose none. These two possibilities were distinguished by means of the fluorescence requenching method (Wimley et al., 1994; Ladokhin et al., 1995) that is based upon the fact that the ANTS molecules inside and outside the vesicles show different susceptibility to quenching with externally added DPX. The method as originally implemented (Wimley et al., 1994) assumed that the membrane permeabilities of ANTS and DPX were equal, but it was subsequently modified to account for the possibility of the preferential leakage of ANTS or DPX (Ladokhin et al., 1995).

The essential features of the requenching method (Wimley et al., 1994; Ladokhin et al., 1995) are as follows. The total quenched fluorescence recorded is $F_{total} = F_{QO} + F_{QI}$, where F_{QO} is the quenched fluorescence originating from outside the vesicles and F_{QI} is that from within. If there were no quenching, the observed total fluorescence from ANTS inside and outside the vesicles would be $F_{max} = F_{MO} + F_{MI}$. The addition of Triton X-100 to the system causes lysis of the vesicles and the fluorescent signal to reach its maximum value, F_{max}. If Q_{out} and Q_{in} are the quenching outside and inside the vesicles, respectively, the total quenched fluorescence $F_{total} = Q_{total}F_{max} = Q_{out}F_{MO} + Q_{in}F_{MI}$. The fractions of ANTS outside and inside the vesicles are $f_{out} = F_{MO}/F_{max}$ and $f_{in} = F_{MI}/F_{max}$ where $f_{out} + f_{in} = 1$. The total quenching is (Wimley et al., 1994)

$$Q_{total} = Q_{out}f_{out} + Q_{in}(1 - f_{out}) \quad (2)$$

To determine Q_{in} experimentally, a vesicle preparation with entrapped ANTS and DPX is first lysed with Triton X-100. DPX is then added incrementally and the normalized fluorescence $F_{DPX}/F_{max} = Q_{out}$ measured. Defensin is then added to a ANTS/DPX vesicle solution and incubated for a long enough time for fluorescence to reach its plateau level. As DPX is added incrementally to this solution, F_{total} is measured as a function of DPX concentration. Following the last addition of DPX, Triton X-100 is added and F_{max} measured. A plot of Q_{total} versus Q_{out} yields a linear curve with slope f_{out} and intercept $Q_{in}(1 - f_{out})$ determined by linear least-squares fitting procedures. An example of the fluorescence changes observed upon the incremental additions of DPX to vesicles permeabilized with NP-5 is illustrated in panel A of Figure 2. The arrows indicate the times at which the DPX was added to ANTS/DPX-containing vesicles.

If Q_{in} is independent of the fraction of ANTS that has leaked out, then the leakage is all-or-none. If it increases with f_{out}, then the leakage is graded. For graded release, Q_{in} depends on f_{out} as follows (Ladokhin et al., 1995):

$$Q_{in} = (1 + k_d[DPX](1 - f_{out})^{\alpha})/(1 + k_a[DPX](1 - f_{out})^{\alpha}) \quad (3)$$

where [DPX] is the initial DPX concentration inside the vesicles and α is the ratio of the rates of release of DPX and ANTS. The constant k_d is the dynamic quenching constant, and k_a is the association constant for the ANTS/DPX nonfluorescent complex. They were determined previously to be 50 and 490 M^{-1}, respectively (Ladokhin et al., 1995). α is estimated by fitting eq 3 to the experimental data. If $\alpha = 1$, then the leakage path is equally permeable to ANTS and DPX. If $\alpha < 1$, then ANTS leaks preferentially, whereas if $\alpha > 1$, then DPX leaks preferentially.

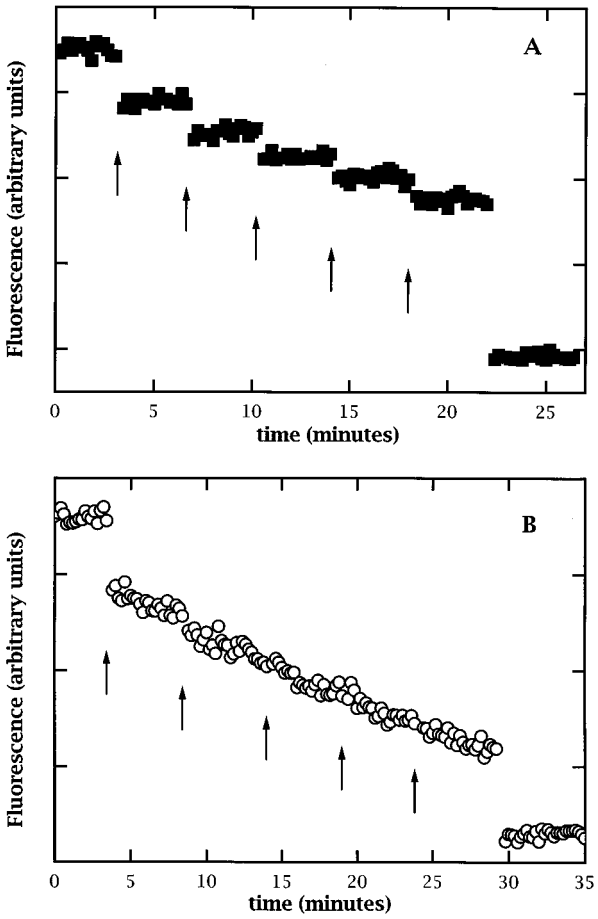

FIGURE 2: Requenching of vesicle suspensions treated with NP-5 (panel A) and NP-2 (panel B). (A) Samples were equilibrated after NP-5 addition for a time sufficient for fluorescence to reach its plateau level due to the loss of ANTS and DPX into the external solution where they are diluted. The arrows indicate subsequent 10 μL additions of a 45 mM solution of DPX. These additions quench the released ANTS causing the fluorescence level to drop to a new plateau level. (B) The behavior of NP-2 is distinctly different in that there is a steady drift downward of fluorescence that washes out the "stair-step" behavior seen in panel A. This effect probably occurs because the added DPX back-diffuses into the vesicles to quench the ANTS that remains inside (see text).

RESULTS

Binding of Rabbit Neutrophil Defensins to Lipid Bilayers. Initial studies were carried-out with NP-2 and NP-5, two defensins which have distinctly different spectra of antimicrobial activity (Lehrer et al., 1986; Cullor et al., 1991). Figure 3 demonstrates that neither NP-2 (panel A) nor NP-5 (panel B) binds to electrically neutral POPC vesicles. Additions of negatively charged POPG to the vesicles causes binding that is stronger for NP-2 (net positive charge of +8) than for NP-5 (charge +4): For NP-2, 100% binding is observed for 25 mol % POPG, whereas 50 mol % or more is required for 100% NP-5 binding. These results are entirely consistent with the findings of earlier studies which showed that the initial binding of small cationic peptides to lipid bilayers is predominantly electrostatic (Kim et al., 1991; Wimley et al., 1994).

Leakage of Contents of POPG Vesicles Induced by Rabbit Defensins. The leakage experiments were conducted by

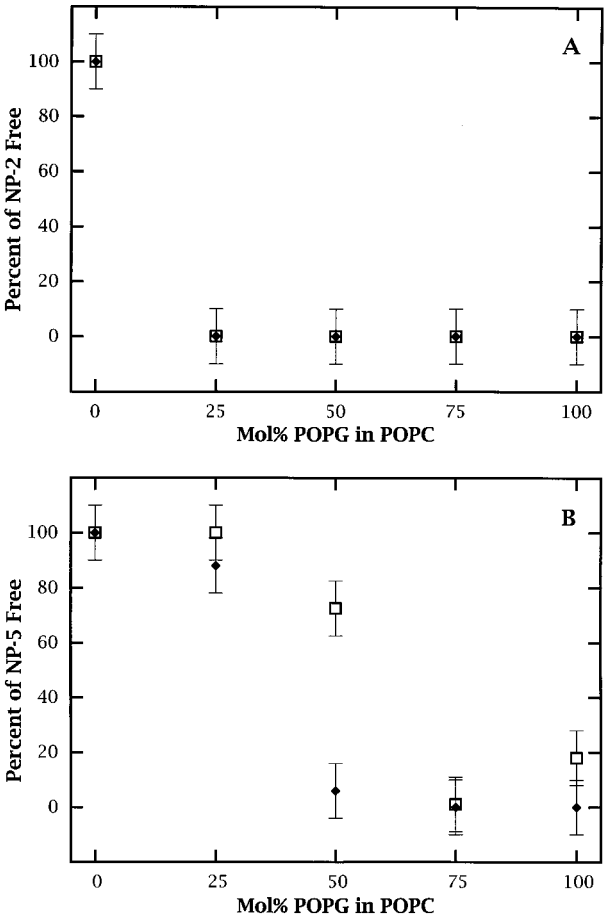

FIGURE 3: Binding of rabbit neutrophil defensins NP-2 (panel A) and NP-5 (panel B) to vesicles formed from mixtures of POPC and POPG. Binding was assayed by means of equilibrium dialysis and HPLC (Wimley & White, 1993). Experiments were begun with 15 μg of defensin in one half-cell of a dialysis chamber and 5 mM (□) or 10 mM (◆) of POPC/POPG vesicles in the other. The data show that neither NP-2 nor NP-5 binds to POPC vesicles unless negatively charged POPG is also present. Note that NP-2 (net positive charge +8) binds much more readily than NP-5 (charge +4).

adding defensins at time $t = 0$ to a 400 μM liposome solution with co-encapsulated ANTS and DPX. Leakage of vesicle contents was observed only for POPG vesicles; additions of neutral lipids (POPC or POPE) eliminated leakage. The fractional fluorescence changes due to leakage of ANTS from POPG vesicles caused by 40 μg of NP-2/mL and 40 μg of NP-5/mL are shown in Figure 4. The zero level corresponds to vesicle fluorescence in the absence of defensins. The maximum level of fluorescence, determined by lysis of the vesicles with Triton X-100, is assigned a value of 1. The increase in f_F for $t > 0$ demonstrates that both NP-2 and NP-5 induce leakage of the contents of the POPG vesicles. For both peptides, we observe that f_F increases strongly with peptide concentration (Figure 5) and always has a fast initial signal increase followed by a slower one (Figure 4). Although in the initial 50 min the fluorescence responses to a peptide addition are almost identical, NP-5 subsequently appears to be more potent in causing leakage: 40 μg of NP-5/mL leads to a fluorescent signal that reaches the maximal level, as determined by Triton X-100 titration, whereas the same concentration of NP-2 causes an increase to only about 65% of that level (Figure 5). Compared to human defensin HNP-2, we find that much higher concentrations of both

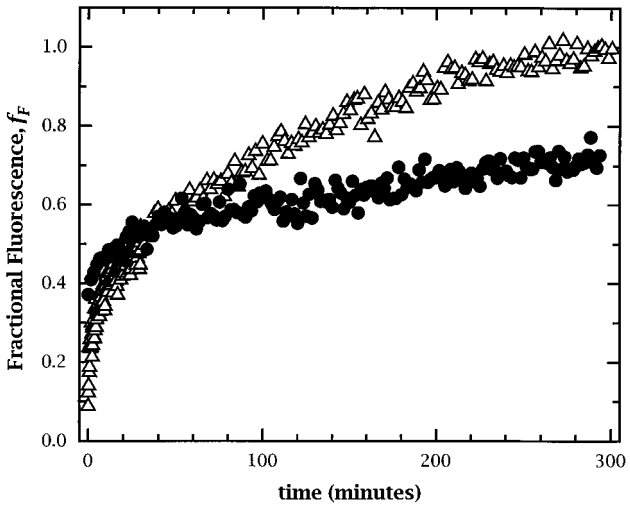

FIGURE 4: Kinetics of the leakage of contents from POPG vesicles induced by NP-2 (●) and NP-5 (△). Defensin at 40 μg/mL was added to 400 μM POPG vesicles with entrapped DPX and ANTS. Beginning at time $t = 0$, the fractional fluorescence (f_F) increases due to leakage of ANTS and DPX into the exterior solution where the dilution removes the quenching effect of DPX. The zero level corresponds to vesicle fluorescence in the absence of defensins (maximum quenching of ANTS by DPX). The maximum level of fluorescence (assigned a value of 1) is established by lysis of the vesicles with detergent. These results show that both NP-2 and NP-5 induce leakage of the contents of POPG vesicles but with different kinetics.

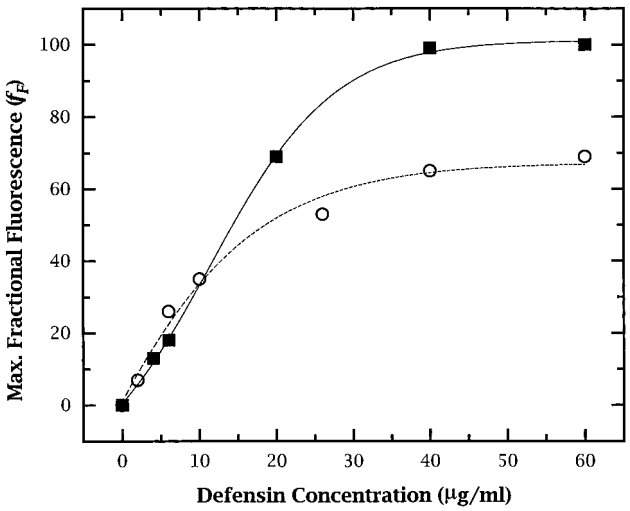

FIGURE 5: Dependence of maximum fluorescence increase upon aqueous concentration of rabbit neutrophil defensins NP-2 (○) and NP-5 (■). The maximum (plateau) level of the fractional fluorescence increase f_F (Figure 4) accompanying ANTS release from POPG vesicles was determined for a series of NP-2 and NP-5 defensin concentrations. The maximum values of f_F show a sigmoidal dependence on concentration (solid and dashed lines).

NP-5 and NP-2 are needed for a given fluorescence increase and that the release of contents is much slower than the release induced by HNP-2 [see Wimley et al. (1994)].

The defensins NP-1, NP-3A, and NP-3B were found to cause leakage of the contents of POPG vesicles in a manner similar to that of NP-2 (data not shown). Again, additions of neutral lipids such as POPE or POPC eliminated leakage. Remarkably, NP-4 caused no measurable increase in fluorescence and therefore no apparent leakage.

Mechanism of Leakage of Contents of POPG Vesicles Induced by Rabbit Defensins. We examined the mechanism

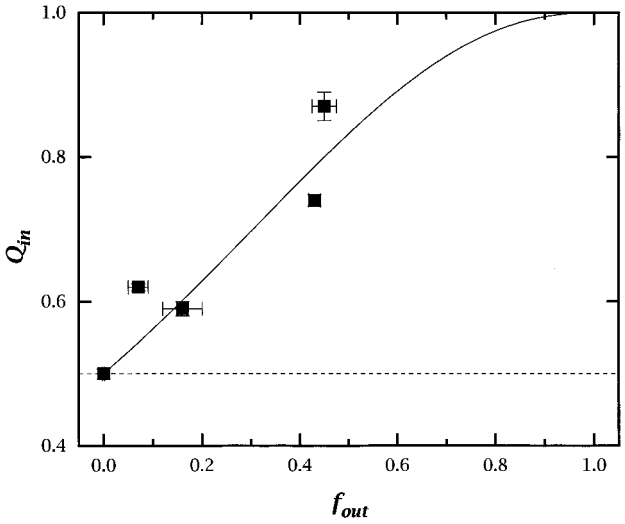

FIGURE 6: Determination of the mechanism of leakage from POPG vesicles induced by NP-5. The mechanism is established by measuring the quenching of ANTS fluorescence by DPX inside the vesicles (internal quenching, Q_{in}) as a function of the fraction of ANTS released from the vesicles (f_{out}) (Wimley et al., 1994). Release is graded because Q_{in} increases with the increase of f_{out}. Had release been all-or-none, Q_{in} would have remained constant at its initial level of 0.5 (dashed line). The analytical procedure of Ladokhin et al. (1995) (solid curve, see Materials and Methods) reveals that the rate of leakage of DPX is about 1.6 times the rate of release of ANTS.

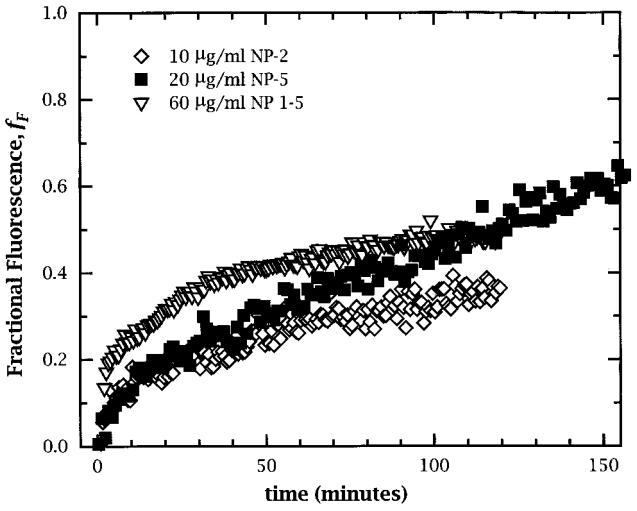

FIGURE 7: Comparisons of the leakage of ANTS/DPX from POPG vesicles induced by NP-2, NP-5, and the natural-ratio mixture (NP 1−5) of rabbit neutrophil defensins. The concentrations of defensins used are indicated in the legend. The NP 1−5 mixture contained 10 μg of NP-2/mL and 20 μg of NP-5/mL. The defensins were added to 400 μM POPG vesicles with entrapped DPX and ANTS at time $t = 0$. Although NP 1−5 causes a quicker fluorescence increase toward a plateau level in the initial moments, after about 2 h the signal has not reached the level achieved by NP-5 alone. No synergism between the six types of defensins in NP 1−5 is apparent.

of leakage of contents of POPG vesicles, induced by the rabbit defensins by performing requenching experiments as described in Materials and Methods. The results for NP-5, presented in Figure 6, show that the internal quenching of ANTS by DPX, Q_{in}, increases with the increase in fraction of ANTS released, f_{out}, and is well above the initial level of 0.5. This means that the release of contents is graded (Wimley et al., 1994). Had it been all-or-none, Q_{in} would have been independent of f_{out} (dotted line in Figure 6). Therefore, rabbit NP-5, unlike human HNP-2, does not cause all-or-none leakage from POPG vesicles. Application of the fitting procedure developed by Ladokhin et al. (1995) (solid curve) yields a value of α, the ratio of DPX to ANTS leakage, of about 1.6, which indicates that DPX leaks preferentially.

As shown in Figure 4, NP-5 at 40 μg/mL causes an increase in the fractional fluorescence signal f_F that attains the maximum value established by solubilization with Triton X-100. The requenching procedure reveals, however, that f_{out} is lower than f_F. This is probably due to dilution of both the ANTS that leaked out of the vesicles and the ANTS inside the DPX-depleted liposomes.

Unlike the requenching of NP-5-treated vesicles which showed a "staircase" decline following each DPX addition (Figure 2A), requenching of vesicles treated with NP-2 led to a gradual decline in the fluorescence signal following each DPX addition (Figure 2B). This behavior, which precludes calculating Q_{in} versus F_{out}, probably occurs because the externally added DPX leaks rapidly back into the vesicles and quenches the fluorescence of the ANTS remaining inside. Such behavior is consistent only with graded preferential DPX release (Ladokhin et al., 1995) because all-or-none release inherently involves massive non-preferential release of contents. Thus, we conclude that NP-2, like NP-5, does not permeabilize the POPG bilayer by means of multimeric pores. Comparisons of the initial drops in the fluorescent signal after the first DPX titration of samples of POPG vesicles incubated with NP-2 and NP-5 led to an estimate of about 3 for the ratio of DPX-to-ANTS leakage induced by NP-2. Strong DPX leakage and back diffusion similar to that observed for NP-2 (Figure 2B) was also observed for NP-1, NP-3A, and NP-3B, indicating that these peptides also cause graded preferential release of DPX.

After finding that none of the rabbit defensins can cause all-or-none release of vesicle contents in the manner the human HNP-2 defensin, and in search of synergy between the six rabbit defensins, we examined the combined action of a mixture (NP 1−5) corresponding to the naturally-occurring ratio of the six peptides (26% NP-1, 17% NP-2, 6% NP-3A, 6% NP-3B, 15% NP-4, 30% NP-5). As expected, we found that f_F increased upon the addition of the defensin mixture, thus showing that leakage of the contents of POPG vesicles was induced. To compare the permeabilizing activity of NP 1−5 with that of the individual components, a concentration of the mixture was used that had the same concentrations of NP-2 and NP-5 as when added separately. Figure 7 shows the early stages of the leakage of contents from POPG LUVs for 20 μg of NP-5/mL, 10 μg of NP-2/mL, and 60 μg of NP 1−5/mL (containing 20 μg of NP-5/mL and 10 μg of NP-2/mL). Although the mixture initially causes a more rapid fluorescence increase towards a plateau level, after about two hours the signal recorded when only NP-5 was present increases beyond the level induced by the mixture containing the same amount of NP-5. These data do not reveal any synergistic effects.

Figure 8 shows that, as for the individual defensins, the leakage caused by the natural mixture of rabbit defensins is graded. The dashed line in the figure corresponds to the result expected for all-or-none leakage and the solid line is the best fit of the data to eq 3 which yields a DPX-to-ANTS

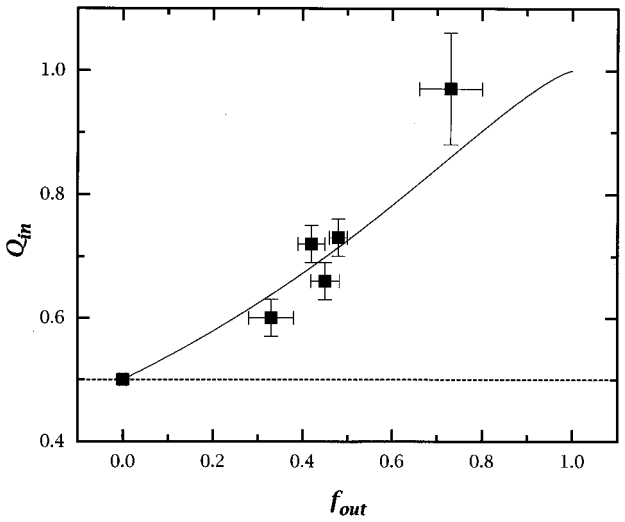

FIGURE 8: Determination of the mechanism of contents leakage from POPG vesicles induced by the natural mixture (NP 1−5) of rabbit neutrophil defensins. The experimental protocol used was the same as that described in Figure 6. These results indicate that the leakage mechanism is graded and that the ratio of DPX to ANTS leakage is about 1.3 (see Figure 6 and text).

leakage ratio of about 1.3. We conclude that there is no synergism between the different defensins that modifies the mechanism of release.

DISCUSSION

The binding assay shows that NP-5 and NP-2, especially, bind strongly to POPC/POPG bilayers. In fact, additions of POPC up to 75 mol % did not inhibit peptide binding which is still driven by electrostatic interactions with the acidic lipid. In the leakage assay, however, we observed that neither the individual defensins nor the natural-ratio mixture induced leakage from vesicles formed from equimolar mixtures of POPC and POPG. Similarly, we could not induce leakage from vesicles formed from mixtures of POPE and POPG (molar ratio 35:65). Thus, leakage of contents is induced by five of the defensins only if the LUVs are formed from POPG alone. The sixth rabbit defensin, NP-4, does not induce leakage under any conditions used.

In all cases examined, including the mixture of defensins prepared using the naturally occurring ratio, the leakage induced by the rabbit defensins is graded with DPX released preferentially relative to ANTS. This preferential release is not surprising because the positively charged DPX is expected to be in proximity to the negatively charged POPG membrane whereas the negatively charged ANTS should be depleted in this region (Ladokhin et al., 1995). The graded preferential release means that all of the vesicles loose some of their contents by a mechanism that can distinguish between ANTS and DPX. Therefore, the rabbit neutrophil defensins apparently do not form the large long-lived pores that are required for all-or-none release of vesicle contents.

The natural-ratio mixture of defensins induces leakage without any apparent synergism. Rabbit defensins NP-1, NP-2, or NP-3A or NP-3B can potentiate the action of NP-5 against *Candida albicans* (Lehrer et al., 1986). Although we did not examine specifically the potentiation of NP-5 leakage induction by those defensins, the lack of enhanced permeabilization by the mixture of six defensins suggests no equivalent effect in our model system. This is not surprising because our simple model systems do not mimic the complexity of biological membranes nor do they account for the likely important role of cell metabolism.

We have chosen to emphasize in this report the behavior of rabbit defensins NP-2 and NP-5 because the spectra of defensin bactericidal activity of the two are quite different. For example, NP-1, and presumably NP-2 which differs from NP-1 by only a single residue and behaves similarly to NP-2 in the induction of vesicle leakage, is far more effective against a panel of Gram-positive and Gram-negative organisms than NP-5 (Cullor et al., 1991). The activity of NP-5 was reported to increase significantly, however, when the pathogens were metabolically active (Cullor et al., 1991). Our results also reveal a significant difference in the effectiveness of leakage induction by NP-2 and NP-5. Contrary to the biological assay, however, NP-5 was more effective. Neither rabbit defensin was as effective as human defensin HNP-2.

The results presented in this paper indicate that the binding of the rabbit defensins is a necessary but not sufficient condition for permeabilization and that permeabilization depends crucially on the lipid composition of the bilayer as observed for HNP-2 (Wimley et al., 1994). This means that the lipid composition of membranes is an important aspect of defensin activity. Other factors may also be important. Kagan et al. (1990) showed that NP-1 forms channels in planar bilayers only in the presence of a transmembrane potential. However, we were not able to detect any changes in membrane susceptibility to leakage caused by potentials across POPG or neutral-lipid bilayers induced by standard means of transbilayer K^+ gradients and valinomycin (De Kroon et al., 1989; Ahmed & Krishnamoorthy, 1990; Medow & Lipkowitz, 1994). Another possibility is that some lipid, glycolipid, or protein component of microbial membranes may be important for defensin activity. Subtleties in defensin structure are also apparently important because the six rabbit defensins vary in the details of their bilayer permeabilization. The most striking example is seen with NP-4 and NP-5. Although these two defensins differ in only five amino acid residues, NP-4 has no effect on POPG vesicle leakage whereas NP-5 causes substantial leakage. This is consistent with previous findings that defensins differ dramatically in their *in vitro* antimicrobial spectrum despite their high degree of sequence homology.

In contrast with the rabbit defensins, the human defensin HNP-2 causes all-or-none release of vesicle contents by means of large-diameter pores (Wimley et al., 1994). There are two obvious differences between the defensins isolated from the two species that may determine the fundamentally different mechanisms of leakage. First, rabbit defensins bear a higher positive charge (+4 to +9 or higher depending on pH) than HNP-2 (+3). Second, the rabbit defensins exist strictly as monomers in solution, which may be related in part to their charge, whereas the human defensins form very stable dimers. Wimley et al. (1994) suggested that the dimeric state of the human defensins may be crucial for pore assembly. A possible reason for pore formation by HNP-2 is that the dimer has a strong amphiphilicity that is believed to be important for insertion into the bilayer (Hill et al., 1991). Because the rabbit defensins do not cause all-or-none release, we conclude that the structural features of human defensins that permit dimer formation in aqueous solution are likely to be important in the formation of

multimeric pores. The failure of NP-4 to cause leakage shows that small differences in monomeric structure can have very large effects.

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