Glycine position permutations and peptide length alterations change the aggregation state and efficacy of ion-conducting, pore-forming amphiphiles

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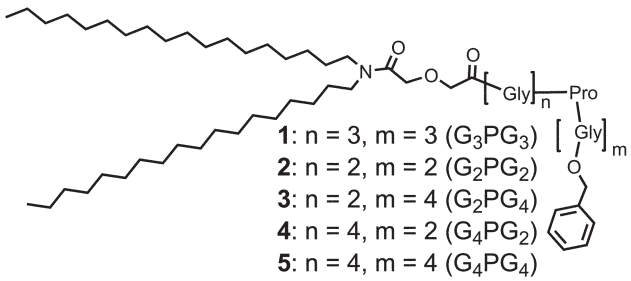
Changes in the peptide chain of amphiphilic heptapeptides known to form ion-conducting pores in bilayers dramatically alter transport efficacy and the aggregation number of pore formation.

The chloride-selective, synthetic ion channels that we developed[1] were based in part on the peptide sequence found in the ClC family of chloride conducting protein channels.[2–5] In addition to the heptapeptide, the initial design of these synthetic channels incorporated an *N*-terminal, twin-tailed hydrocarbon anchor and a linker intended to mimic the phospholipid midpolar regime. The *C*-terminal ester group was incorporated to prevent ionization of the carboxyl group but is now known to play an anchoring role as well.[6] The peptide sequence chosen for the initial studies was –$(Gly)_3Pro(Gly)_3$–. The presence of proline in this sequence was found to be critical for chloride transport. When the otherwise identical 6-membered ring of pipecolic acid replaced proline's five-membered ring, transport activity was profoundly affected.[7] Changes in the identity and chain lengths of both the *C*- and *N*-terminal residues have been studied in detail and found also to dramatically affect ion transport.[8] We now report that seemingly minor changes in the peptide chain length have profound effects on ion transport.

The first structure that was prepared and studied in this family was $(H_{37}C_{18})_2N$–$COCH_2OCH_2CO$–$(Gly)_3Pro(Gly)_3$–OCH_2Ph (**1**).[1] In the work reported here, we have maintained the central core of five amino acids of that first compound, –$(Gly)_2Pro(Gly)_2$–, but appended additional glycines either to the *C*-or *N*-terminus of the sequence. The five structures are shown as **1–5**. We refer to them hereinafter using shorthand: thus, **1** is G_3PG_3. The synthesis of **1** was previously reported.[7] Compounds **2–5** were prepared in an analogous fashion. All were colorless solids whose spectral properties agreed with the assigned structures. The melting points are: **1**, 116–118 °C;[1] **2**, 55–56 °C; **3**, 168–169 °C; **4**, 174–175 °C; and **5**, 206–208 °C.

Chloride release was assayed in liposomes prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) (20 mg, 7 : 3 w/w) by the lipid film



method. They were filtered through a 200 μm filter and their average size (2000 Å) was confirmed by use of a light scattering instrument. An Accumet chloride combination electrode was used to record the chloride selective voltage output. After 5 min equilibration time, the ionophore (~9 mM in 2-propanol) was added. Ion release was monitored for ~30 min (data shown for 1200 s) and the vesicles were lysed by addition of a 2% aqueous solution of Triton X-100 (100 μL). Data were normalized to the maximal value. The measured transport rates are shown in Fig. 1. Each curve shown is the average of 3–7 different experiments done with [lipids] = 0.31 mM and [compound] = 65 μM.

The data clearly indicate that lengthening the peptide increases chloride transport activity [**5** (G_4PG_4) >> **1** (G_3PG_3) > **2** (G_2PG_2)]. Shifting the proline position towards the *C*-terminal end of the peptide chain [**5** (G_4PG_4), **4** (G_4PG_2) > **1** (G_3PG_3) > **3** (G_2PG_4), **2** (G_2PG_2)] also enhances chloride ion transport. Little difference in activity is apparent in **1**–**3** compared either to **4** or **5**. The most active compound in this group is **5**, which has the longest peptide

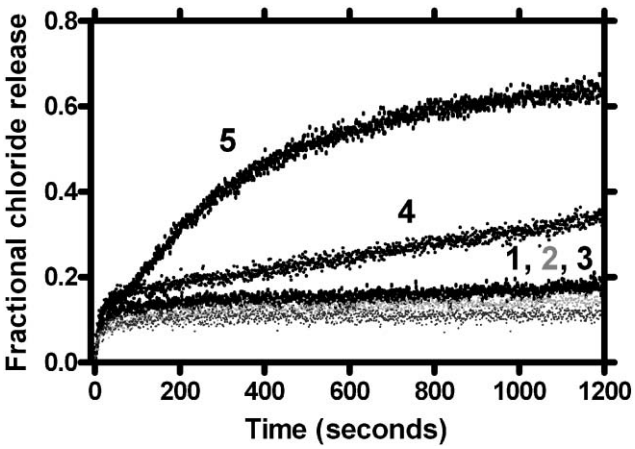


Fig. 1 Fractional chloride release from liposomes mediated by **1–5**.

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chain and has the proline four amino acid residues from the *N*-terminus.

The release of carboxyfluorescein (CF) from liposomes is a sensitive measure of anion transport that may be monitored by fluorescence. Both chloride and CF are anionic but the latter is larger. Hydrated Cl^- is reported to be a sphere of ~6.5 Å[9] and molecular models suggest that CF is about 10 × 10 × 5 Å. Previous studies confirm that CF can pass through the pore,[4] presumably if the two monomers that form the pore separate by only 1–2 Å at either end. Indeed, we have typically seen a reasonable correlation between Cl^- and CF release even though the liposome and ionophore concentrations differ for experimental reasons. Fluorescence detection of released CF is both a sensitive and rapid process, significantly faster than the response of the chloride electrode (10–20 s). This makes the assessment of pore formation kinetics possible.

Concentration dependent release of CF from 0.9 μM liposomes (3 : 7 w/w 1,2-dioleoyl-*sn*-glycero-3-phosphate and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, ~2000 Å) was studied using concentrations of **5** in the range 50 nM to 10 μM (~200-fold change).

In previous studies, anion efflux was characterized by exponential release followed by a linear phase. In the data shown for **5**, these two stages were preceded by an induction period (see Fig. 2), probably due to molecular organization and rearrangement after insertion into the membrane. We fit the data to a sigmoidal equation (using the software program OriginPro 7), *i.e.* $y = A \times [x^n/(k^n + x^n)]$, for which $R^2 > 0.99$. In this equation, k is the time constant and it is the inverse of the rate constant for the pore formation process. Pore formation is a pseudo first order process and depends only on the concentration of the pore-forming agent. Thus, $v = k^{-1} \times [\mathbf{5}]$. V_{max} is the largest velocity (v) value obtained with this equation. The logarithmic form of the Hill equation is $\log[v/(V_{max} - v)] = n \times \log[\mathbf{5}] - \text{constant}$. A plot of $\log[v/(V_{max} - v)]$ as a function of log [**5**] gives a straight line of slope n, which corresponds to the aggregation state of the active channel ("Hill analysis"). The plot is shown in Fig. 3.

The Hill plot has a slope of ~1.4, suggesting that G_4PG_4 (**5**) can form pores either as a monomer or as a dimer. The different aggregation states probably occur at different concentration ranges

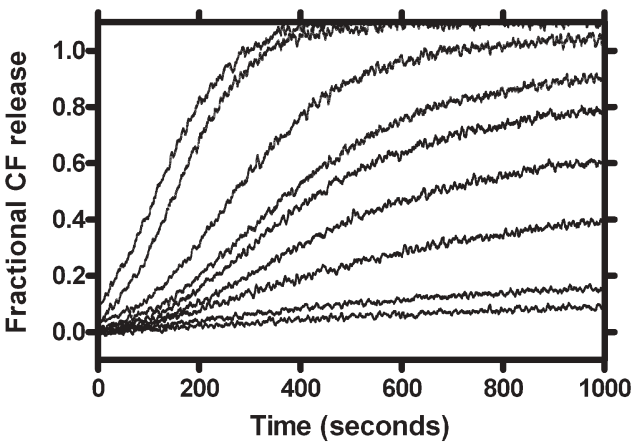

Fig. 2 Concentration dependent fractional release of carboxyfluorescein mediated by **5** from 0.9 μM DOPC : DOPA (7 : 3) liposomes *vs.* time during 1300 s. Concentrations of **5** are, from top to bottom, 9.90, 7.44, 4.97, 2.00, 1.50, 1.00, 0.37, 0.10, and 0.05 μM.

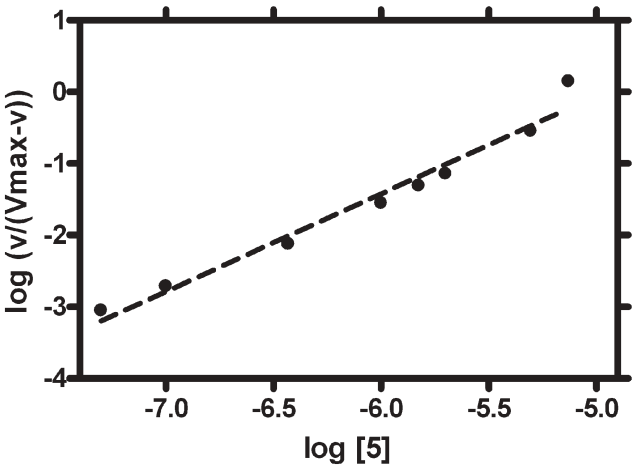

Fig. 3 Analysis of the CF release data fitted by the Hill equation (see text); the slope is ~1.4.

and both monomer and dimer pores likely exist at intermediate concentrations. In fact, the average line obtained has a slope of ~1.4, but it is clear from the graph that there is a range of concentrations where the compound is more diluted and works as a monomer. When the concentration increases, the curve gets steeper and the slope approaches 2. This also provides a plausible justification for the behavior shown in Fig. 2. At low concentrations, only the activity of the monomeric pore is observed. At higher concentrations, the dimeric pore can form quickly and its contribution to the observed level of release becomes predominant, even at early time points. At intermediate concentrations, the transition between the two mechanisms is apparent and the superimposition of the two release profiles engenders the biphasic time dependence that is observed. Such behavior was not apparent in the chloride release experiments, possibly because of the chloride electrode's slower response time.

The amphiphiles developed in this program have shown astonishing variations in transport and selectivity properties when seemingly minor changes were made to the *C*- or *N*-terminal ends of the molecule or to the central amino acid (*i.e.*, Pro → Xxx). We now show that equally dramatic changes are incurred by extending the glycine chain and/or by rearranging the position of proline within it. Extending the heptapeptide to a nonapeptide while maintaining symmetry leads to an ionophore (**5**) that readily releases carboxyfluorescein anion from liposomes at low nanomolar concentrations. It appears that pore formation is concentration dependent; the Hill plot gives a slope of ~1.4, which we interpret to mean both monomer and dimer pores are functional within the bilayer. In previous work with heptapeptide transporter **1**, dimer formation was confirmed by Hill analysis and by dextran sizing experiments.[10] Changes in the peptide chain clearly offer a significant pathway for altering pore formation properties and presumably sizes and selectivities.

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