Planar bilayer studies reveal multiple conductance states for synthetic anion transporters

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Compounds of the general type $R^1_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph insert in phospholipid bilayers and conduct ions. Different levels of activity were observed when R^1 was either decyl or octadecyl, as judged either by Cl^- release, detected by ion selective electrodes, or carboxyfluorescein dequenching, detected by fluorescence. Either method reports average behavior for all ionophores over all liposomes. These methods also show that at least two ionophores are involved in the formation of each pore. Planar bilayer experiments reported here confirm pore formation by these compounds but identify more than one conductance state for each. The pseudo-dimer, in which two molecules of the type shown above are covalently linked, shows only two conductance states, of which one is dominant. This state has been characterized by use of a current–voltage plot.

Introduction

The detailed mechanisms by which ion-conducting channels transport and gate have remained elusive despite more than a century of study.[1] The advent of solid state structures[2,3] has had a profound effect on the field but has not, to date, led to a definitive chemical mechanism for either phenomenon. Chemical models can be useful in this context and several markedly different approaches[4–7] have recently been taken in the development of synthetic anion transporters. Such compounds are referred to nearly interchangeably as anion channels and anion transporters. The distinction is a troublesome one that persists in the study of naturally occurring, chloride-transporting proteins.[8]

Efforts to develop synthetic cation channels paralleled the development of crown ethers for cation complexation and, more generally, supramolecular chemistry. By the turn of the century, however, anion complexation was developing in importance. Indeed, prior to late 2001, only synthetic cation transporters had been reported[9] and no example was available of an anion transporter or channel. This is logical both for historical reasons and because the selectivity of cation transport has been a dominant biological theme for so long. Notwithstanding the importance of the cations Na^+, K^+ and Ca^{2+}, Cl^- is also critical for cellular viability.[10] Like the proteins that transport cations through bilayers, the ClC family proteins are complex and far from being completely understood.[8,10] Model systems are, therefore, of considerable potential significance in understanding their action and may be useful as pharmaceuticals.

The first crystal structure of a ClC family protein did not appear until 2002,[11] so the design of a model system had to be based largely on chemical principles and biological function studies. We surmised that the earliest channels must have been structurally similar to the monomers that formed the membranes in which they functioned. There is no archaeological record of membranes in early organisms, so our anion transporter model was based on the structure of modern phospholipids. Our first successful compound was $(C_{18}H_{37})_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2Ph$, **1**.[4] The dialkylamine [$(C_{18}H_{37})_2N$–] was intended to mimic the phospholipid's fatty acyl chains. The diglycolic acid spacer mimicked the midpolar (glyceryl) regime. The heptapeptide replaced the phosphatidyl aminoester or phosphatidic acid. The *C*-terminal carboxyl was protected by a benzyl residue.

In concert, these elements produced an amphiphilic ionophore that transported Cl^- through vesicular phospholipid bilayers.[12] The structure–activity studies that followed the synthesis of the first successful artificial chloride transporter led to the creation of a family of compounds that we call synthetic anion transporters (SATs).[13–18] These compounds were studied using the established carboxyfluorescein release technique and also a method developed in our laboratory that involves the use of a chloride ion selective electrode (ISE) to detect ion release from vesicles.[19] In both cases, the ability of a specific compound to insert into the liposomal membrane and release encapsulated anionic species could be detected and quantified. Whether anion release is detected by fluorescence or ISE, only average behavior is observed. This is because more than one pore may form simultaneously in one or more liposomes and the average of all release will be observed over time. The number of pores formed in the experimental system is sufficiently large that very reproducible data are obtained. Such methods are extremely useful for characterization of ion release, but relatively few mechanistic details are revealed thereby. We, therefore, turned to planar bilayer conductance (BLM) methods so that we could more fully characterize these novel ion transporters.

The principle underlying the planar bilayer method is simple.[20] Two buffer-containing compartments are separated by a microscopic orifice, which is the only pathway that two electrodes immersed in the different buffers have to communicate with each other. When an ion-impermeable bilayer lipid membrane is "painted" into the hole, the two electrodes are insulated from each other. If an ionophoric species is added to the system, if it inserts into the membrane, and if it conducts ions, the current can be detected, amplified, and recorded. Depending upon the stability of the membrane, recordings may last several minutes to several hours. In the latter case, many thousands of transitions

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may be observed, although only a few are normally presented in the literature.

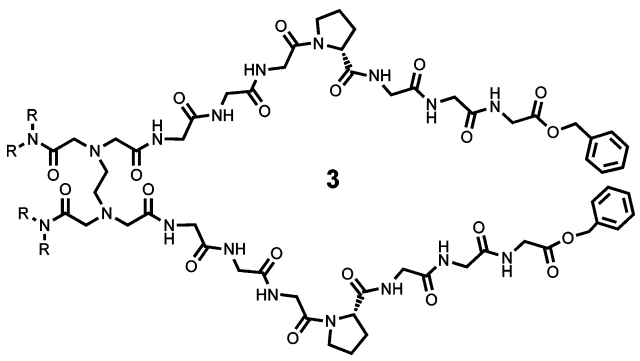
Our first BLM analysis of **1** detected the formation of voltage-dependent, chloride selective pores that showed open–closed behavior and had a conductance of about 1.2 nS.[4] We subsequently showed that the same compound could produce different pores that had a much higher conductance but no ion selectivity.[14] The work reported here is an effort to systematically characterize **1** and its relatives by using the BLM technique.

Results and discussion

Ionophores studied

The compounds studied for this report are all amphiphilic heptapeptides. They are either monomers of the general structure $R_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph, or a covalently linked dimer of such compounds. These amphiphilic heptapeptides are assembled in a modular fashion, as previously described. In short, the dialkylamine (R_2NH) is heated with diglycolic anhydride to form, in a single reaction, the unit $R_2NCOCH_2OCH_2COOH$. The reaction requires no catalysis and can be run without solvent. The monoacid monoamide is then coupled, using wet chemical peptide synthetic methods, to the amino acids or peptides required to give the desired product. This approach produced $R_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph derivatives **1** and **2**, in which R is octadecyl or decyl, respectively.[14]

A more elaborate approach was required to prepare **3**, which is pictured. Both *C*- and *N*-terminally linked dimer amphiphiles were prepared. Both compounds have been previously reported.[21] Only the *N*-linked dimer was studied for this work. The critical difference between **1** and the *N*-linked dimer (**3**) is that ether oxygen in the diglycolic acid residue is replaced by nitrogen (in boldface) to give $(C_{18}H_{37})_2NCOCH_2\mathbf{NH}CH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2Ph$, which are linked by an ethylene ($-CH_2CH_2-$) unit to form the covalent pseudo-dimer **3**. Experimental details are reported in reference 21.



Planar bilayer recordings

The first compound investigated in this study was $(C_{10}H_{21})_2$-$NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph, **2**. Compound **2** is more water soluble than **1** and Cl^- and carboxyfluorescein anion (CF^-) release experiments had both shown that its ion transport activity was greater.[14] Planar bilayer studies showed that, like **1**, **2** exhibited multiple conductance states. Fig. 1 shows recordings

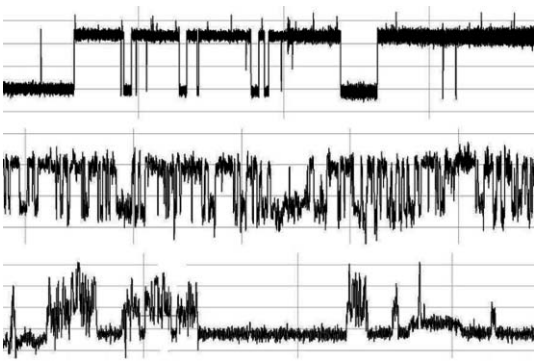


Fig. 1 Three conductance states observed by the BLM method for **2**. The conductance traces are shown on different scales; the average maximum currents are: top, 1.25 pA; middle, 3 pA; and bottom, 8 pA. The time spans shown are: top, 7.5 s; middle, ~10 s; and bottom, ~7 s.

obtained from three separate experiments in which **2** was the ionophore.

The top panel of Fig. 1 shows a well-behaved pore that exhibits current transitions between two well-defined and relatively long-lived states. The overall conductance of this pore is ~12 pS. The middle panel shows qualitatively similar, if less stable, behavior. The lack of pore stability is apparent in the pore's "flickering" behavior and the short transition lifetimes. The current levels are also much less stable or defined. The overall conductance is almost 100 pS in this case.

The bottom panel of Fig. 1 shows a conductance state that exhibits irregular, "bursting" behavior. In this case, the various current levels are not clearly defined and no useful information was obtained.

Fig. 2 shows recordings of the most common conductance state exhibited by **2**. In the majority of the experiments performed, several different levels of current were observed. These were not typically multiple openings of the same type of pore (see Fig. 3, discussed below). Instead, the conductance states appear to result from different pores, that probably have different stoichiometries. In principle, even pores of the same stoichiometry can organize in different ways. Of course, self-assembled pores formed from a different number of monomer elements would also be expected to form pores that have different conductances.

The most frequently observed conductance states shown in Fig. 2 suggest that these pores are favored in bilayers. Since this current level is predominant, an *I–V* plot can be obtained. This is shown in the lower panel of Fig. 2. Conductance values may be obtained from the *I–V* plot. However, these are representative of only one of the several conductance states that are simultaneously present. The *I–V* plot shown in Fig. 2 was obtained using **2** at a concentration of 0.67 μM. The two salt reservoirs contained 450 and 150 mM KCl in 10 mM HEPES at pH = 7. The calculated conductance for this predominant state is 96 pS. The current histogram is shown in Fig. 3.

Carboxyfluorescein release and Hill analysis

We have previously studied the release of carboxyfluorescein anion (CF^-) from liposomes.[14] The vesicles were prepared from a 3 : 7 (w/w) mixture of dioleoylphosphatidic acid (DOPA) and dioleoylphosphatidylcholine (DOPC). This is the same as

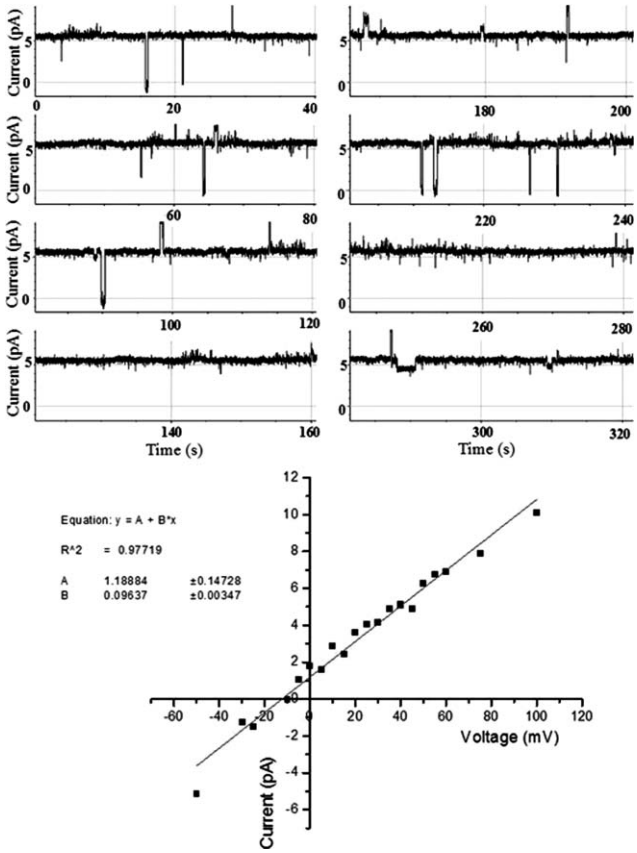


Fig. 2 (Top) Recordings of the different conductance states observed by the BLM method for **2**. The vertical scale in each case is 0 to 5 pA and the recordings run (abscissa) from 0–320 s. The data were acquired at +45 mV (compound concentration 0.7 μM). (Bottom) Current *vs.* voltage (*I*–*V*) plot of the most common conductance state for **2**.

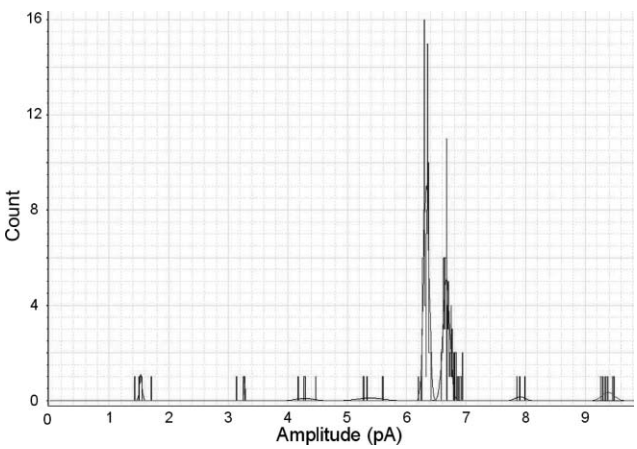


Fig. 3 Current histogram for different conductance states observed for **2**.

the membrane system used for the BLM experiments. Release of CF^- can be monitored by fluorescence, a technique that is both rapid and quantitative. This was done for both **1** and **2** and was described previously.[14] The fluorescence dequenching data can then be fitted to the Hill equation,[22] which identifies the average aggregation state of the pores involved in ion release. Fig. 4 shows Hill plots for **1** (open circles) and **2** (filled circles).[17] The lines shown are the calculated fits of the data. The correlation

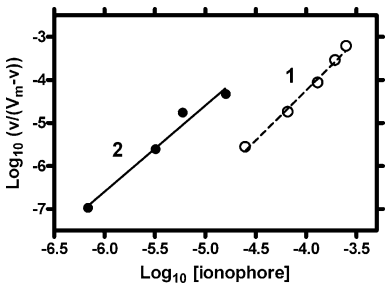


Fig. 4 Hill plots for carboxyfluorescein release from liposomes mediated by **1** and **2**.

factors (R^2) for the data fits of lines **1** and **2** were 0.99 and 0.97, respectively. The slopes for the lines were 2.3 and 1.97, respectively. These experimental values are too similar to clearly imply differences in pore organization. In both cases, we infer that at least two monomers are involved in pore formation. The differences in these two slopes may be within experimental error of each other, but it is clear that planar bilayer experiments with **1** have, indeed, proved more variable than those with **2**.

Carboxyfluorescein and chloride anions are vastly different in some respects. They are, however, both monoanions and they are more similar in size than might seem to be the case. A hydrated chloride ion is estimated to be a sphere of about 6.5 Å in diameter.[23] The xanthene unit of CF is ~10 Å but the molecule is no wider than ~6 Å. Considering that the pores formed by the compounds under study here are dynamic, passage of CF^- is less remarkable. In previous studies, we have observed a reasonable parallel in the release of these two different anions.

Studies with a covalently-linked pseudo-dimer

If the dimer state of compounds in the family containing **1** and **2** is the dominant pore-forming stoichiometry, then a covalent dimer should function as well or better than two monomers. Two such dimers were prepared and ion release from liposomes was reported.[21] Compound **3** is one of those pseudo-dimers and it was studied by the BLM method for this report.

Planar bilayer experiments were conducted with **3** using the DOPA : DOPC membrane system described above. Compound **3** showed a smaller degree of variability in these experiments than did **1**, its closest analog. In fact, only two different conductance states were observed. In most of the experimental recordings, the two distinct states were visible at different times. The recording shown in Fig. 5 (upper panel) shows both states in coexistence. The recordings were obtained when **3** was added at a concentration of 1.8 μM. The applied voltage was −63 mV and the two salt chambers both contained 450 mM KCl and 10 mM HEPES at pH 7.

The larger and less frequent transitions shown in the recordings of Fig. 5 correspond to a conductance state of about 32 pS. The smaller, but more frequent, transitions correspond to a conductance of about 14 pS. These values were determined by *I*–*V* plots, such as the one shown in Fig. 5 for the lower conductance state.

In a previously published study,[21] we found that addition of **3** (32.5 μM) to vesicles caused approximately 25% of encapsulated Cl^- to be released after 800 s. In contrast, Cl^- release mediated by **1** at twice the concentration (65 μM) of **3** amounted to only about 15% after the same time. The detection by the BLM experiment of only two significant conductance states suggests that the linked

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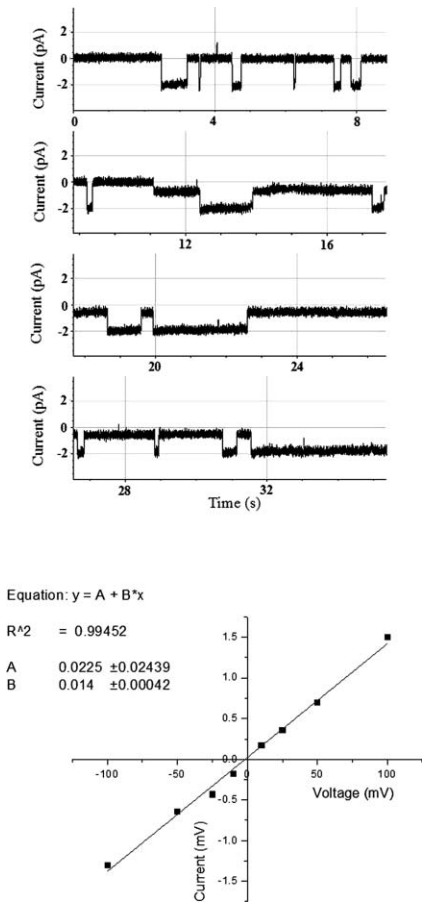

Fig. 5 (Upper) Planar bilayer recordings for **3** showing two conductance states (14 pS and 32 pS). The data were acquired at −63 mV (1.8 μM compound concentration). (Lower) Current–voltage (*I*–*V*) plot for the lower conductance state (14 pS).

dimer form gives a more stable pore than the individual, unlinked monomers could do.

Comparison of ion release and BLM experiments

The existence of more than one conductance state in no way contradicts previous results from which it was inferred that SATs formed pores that were at least dimers. The Hill coefficient values obtained in previous studies represent the *minimum* number of monomers involved in pore formation. The minimum number of two does not rule out formation of pores by aggregation of trimers, tetramers, *etc.*, although these are less likely statistically. Of course, preorganization such as effected in **3** is expected to lead to a dominant conductance state. The BLM experiments show that pores formed from monomers **1** and **2** exhibit a greater diversity of conductance states than does **3**.

Previous ISE and CF-dequenching experiments did not discern the behavior of individual channels. Such experiments show an average behavior for the macroscopic system. This is the statistical average of millions of different pores that form within the liposomal membrane. Planar lipid bilayer experiments detect a limited number of pores (ideally only a single channel). Thus, the BLM experiment reports differentiated conductance states whereas average behavior is observed in the liposome experiments.

Multiple conductance states have been observed previously.[24–31] A diverse range of compounds has shown several states that are not multiple openings of identical pores. In each case, the authors concluded that the behavior that they observed resulted from pores having different numbers of monomers or monomers that were differently organized. Montal stated thus: "[t]he heterogeneity of conductance species observed. . .both in amplitude and in the time that the channels remain open, is anticipated for an amphipathic peptide that self-assembles in the membrane to form noncovalent conductive oligomers of different sizes."[25]

Conclusions

The planar bilayer experiments reported here clearly confirm that compounds of the SAT family form pores that transport ions across a bilayer membrane. In some cases, these pores are well-defined, voltage-gated, and chloride-selective. At other times, pores having less selectivity have also been recorded. In most cases in which monomeric, amphiphilic ionophores were studied, multiple conductance states were in evidence. The transitions observed are clearly those typical of ion channels, with sharp jumps between specific current levels. These levels, however, do not necessarily result from multiple openings of the same unitary pore. In the present case, **1** and **2** exhibit multiple conductance states. Covalently-linked pseudo-dimer **3** is pre-organized and exhibits a dominant conductance state.

Planar lipid bilayer experiments serve as an important complement to ISE and CF-dequenching experiments. Together, they reveal both average and detailed behavior. The ISE and CF-dequenching methods permit a reasonably rapid assay of ion transport activity. The BLM experiment provides details that can be used to help understand the mechanisms at work in these dynamic, self-assembled pores.

Experimental

$(C_{18}H_{37})_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2Ph$, **1**,[14]
$(C_{10}H_{21})_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2Ph$, **2**,[14]
and pseudo-dimer **3**[21] were prepared as reported previously.

Planar bilayer conductance

BLM experiments were performed by using a Warner BC-525D bilayer clamp apparatus. Planar membranes were formed by painting lipids [DOPC–DOPA 7 : 3 w/w (2 : 1 mol/mol) 20 mg mL^{-1} in *n*-decane] over a 200 μm aperture on the side of a cuvette fitted into a chamber. Cuvette and chamber contained either a 150 mM or a 450 mM KCl buffer solution (10 mM HEPES, pH = 7) as specified in the single experiments reported (see text and figure captions). After membrane formation was confirmed (membranes with a capacitance lower than 100 pF were discarded), an aliquot of a trifluoroethanol solution of the compound was stirred into the buffer on the chamber side ["*cis*" side where the reference electrode is immersed ("ground")] to achieve the desired concentration. Records were filtered with a 4-pole Bessel filter (100 Hz) and digitized at a 1 kHz sampling interval per signal using Clampex 9.2 (Axon instruments). Data analyses were performed with Clampfit 9.2 (Axon Instruments).

Acknowledgements

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