Fluorobenzyl Terminal Residues as Probes of Function in Tris(macrocycle) Channels: Evidence from NMR and Planar Bilayer Conductance Studies

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Most cation transport *in vivo* is thought to be mediated by proteins having multiple transmembrane helices that organize into pores. This process has been studied a great deal, but its inherent complexity has frustrated the elucidation of mechanistic details.[1,2] The fundamental biological importance of this area and the elusive nature of functional details have brought forth several synthetic organic chemical models for ion transporters; this work has recently been reviewed.[3]

In previous work, we have described a successful cation-conducting channel that functions in phospholipid bilayers.[4] We have inferred information about both the position of the headgroups in the bilayer and the active conformation of the channel-former from these studies. Information about the position of the molecule comes largely from fluorescence studies. It was found that the central macroring could be reduced in size from diaza-18-crown-6 to diaza-15-crown-5 without altering cation flux. We concluded from this and other data that the central macroring was not required to be parallel to the distal macrocycles. We wished to inquire similarly into the role of the distal macrorings which appeared to interact directly with Na$^+$ as judged from an application of the Hammett principle.[4c] We report here the results of that investigation.

Two tris(macrocycle)s were prepared for the present study: 4-F-$C_6H_4CH_2$<N15N>C_{12}<N18N>C_{12}<N15N>$CH_2C_6H_4$-4-F, **1**, and 4-F-$C_6H_4CH_2$<N18N>C_{12}<N18N>C_{12}<N18N>$CH_2C_6H_4$-4-F, **2**. 4-Fluorobenzyl bromide was treated with 1.1 equiv of 4,10-diaza-15-crown-5 to afford the monosubstituted crown, F-$C_6H_4CH_2$<N15N>H, in 47% yield. The central subunit was prepared by treating 4,13-diaza-18-crown-6 with excess 12-bromododecanoyl chloride to afford Br$(CH_2)_{11}$-CO<N18N>CO$(CH_2)_{11}$Br (89%) which was, in turn, reduced using B_2H_6·THF to Br$(CH_2)_{12}$<N18N>$(CH_2)_{12}$Br (43%). The crown dibromide was then treated with 2 equiv of the appropriate mono(fluorobenzyl)crown, FC$_6$H$_4$CH$_2$<N15N>H, to yield

(24%) channel **1** having two 15-membered distal macrocycles or FC$_6$H$_4$CH$_2$<N18N>H to produce (28%) **2**.[5]

1, n = 1; **2**, n = 2
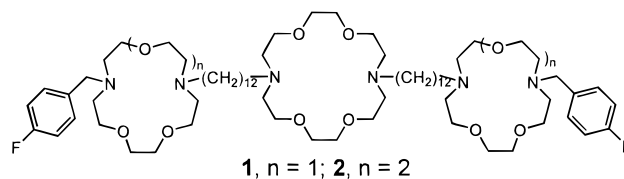
The activity of channel compounds **1** and **2** in phosphatidylglycerol/phosphatidylcholine bilayer vesicles was confirmed by using the dynamic ^{23}Na-NMR technique devised originally by Riddell and Hayer[6] and later elaborated for gramicidin.[7] The rates determined for Na$^+$-transport relative to gramicidin (k_{rel} = 100) for **1** and **2** were, respectively, 16 and 26.

The ^{23}Na-NMR method is an equilibrium experiment and does not involve a concentration gradient or electromotive pressure (see below). The results reflect overall transport efficacy of the system; single channels are not observed. It was inferred from experimental data for relatives of **2**[4c] that Na$^+$ passes through the distal 18-membered macrorings. There is currently no direct evidence that Na$^+$ also passes through the smaller macrorings of **1**, but the hole diameter of 15-crown-5 is sufficient to permit it.

An alternative to the ^{23}Na-NMR method for evaluating Na$^+$ transport is the planar lipid bilayer conductance (pbc) method.[8] The latter method measures current as a function of time in which the opening and closing of individual cation channels are observed directly rather than the overall transport phenomenon as determined by dynamic NMR. In principle, the pbc and NMR methods should produce similar information. A significant difference between the activities of **1** and **2** was observed by using the pbc method, but the calculated conductance of each channel was essentially identical: 13 pS. The latter point is illustrated by the superimposable "I−V" curves shown in Figure 1.

A difference between the two channels is apparent in the traces shown as Figure 2. The top panel (A) shows data for the 15-membered ring compound. In the lower panel, data for 18-membered ring structure **2** are shown. In each case, a 2 pmol sample of compound was studied at +100 mV applied potential in the presence of 500 mM NaCl. Each trace shows approximately 2 min of activity (of a total of several hours in each case). In the top pair of traces, four open/close transitions are apparent. The identity of the peak heights shows that each is a single channel event. The width of the peaks shows the duration (dwell) of the opening. The longest opening (top rightmost peak) has a duration of approximately 5 s. No multiple channel openings are apparent. In marked contrast, multiple channel openings are abundant in the lower trace.

We have analyzed a much more extensive data set than shown here by calculating open-close histograms for **1** and for **2** (Figure 3). The ordinate in the plot shows the number of transitions observed for each compound. For **1**, which has 15-membered

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(5) All new compounds were fully characterized by IR, NMR, and either combustion analysis (±0.4%) or HRMS (1 part in 10000).

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(7) Buster, D.; Hinton, J.; Millett, F.; Shungu, D. *Biophysical J.* **1988,** *53,* 145−152.

(8) The planar bilayer chamber was prepared with 0.5 M NaCl, 0.001 M sodium phosphate (pH = 7.0) solution on each side of the membrane. The membrane was formed by the painting method using a solution (30 mg/mL) of L-α-lecithin in decane. The channels were added (2 μL) as 1.0 μM solutions in trifluoroethanol and stirred for 5−10 min. After a 5 min equilibration period, a holding voltage was applied, and the channel responses were recorded using a Warner PC-505 patch clamp amplifier, a DigiData A/D converter and the acquisition software Axoscope.
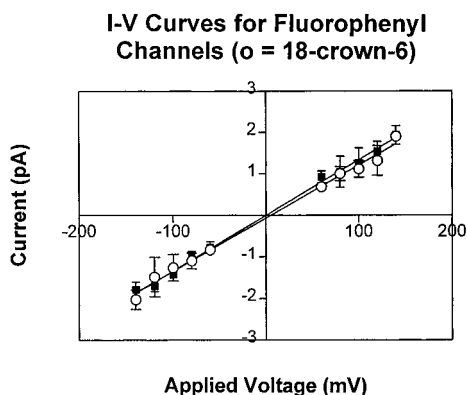
I-V Curves for Fluorophenyl Channels (o = 18-crown-6)

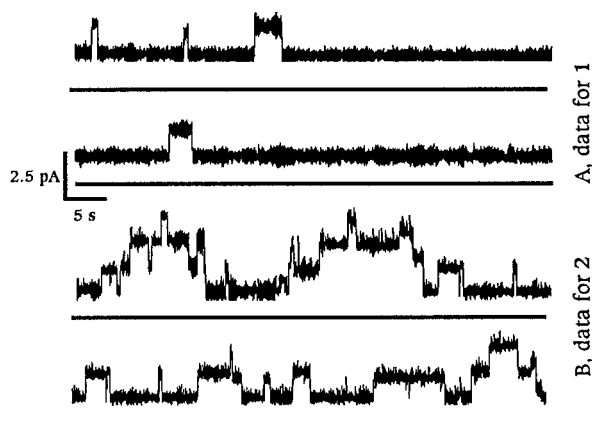**Figure 1.** Current/voltage ("I−V") curves for compounds **1** (○) and **2** (■).

Figure 2. Planar lipid bilayer conductance traces for 1 (panel A, 2 picomoles pH = 7.0; 0.5 M NaCl; +100 mV) and 2 (panel B, same conditions).

ring headgroups, single transitions dominate the profile; second level transitions are observed, but they are rare.[9] Two third level transitions were observed. For **2**, hundreds of multiple transitions were recorded and overwhelm the first level transitions apparent at ~1.3 pA. The fact that numerous multiple-channel openings are readily detected for **2** and not observed for **1** comports with the NMR results which also show that **2** is a more effective transporter than **1**.

Our current postulate about the conformation adopted by **2** has been illustrated.[4e] We believe that the distal macrocycles serve as both entry portals and headgroups for the amphiphile. As noted above, the central macroring appears to play primarily a structural role and is thought to be aligned with the bilayer's lipid axis. It was not anticipated that the change from benzyl to fluorobenzyl (**2**) would alter the situation. The change in ring size for the distal macrocycles is a more dramatic alteration.

(9) The low level transitions apparent at ~0.5 pA are often observed even in protein channels, and their origin is not understood.
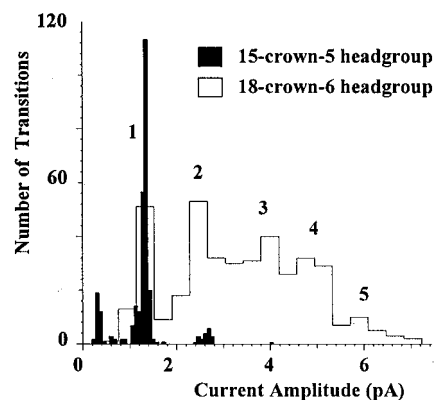
Figure 3. Open-close transition histogram for **1** (filled) and **2**.

The NMR data are in accord with a similar conformation and a restricted orifice. The fact that the conductances are identical (Figure 1) may mean that the electromotive pressure readily overcomes the presumed activation barrier difference between the two different ring sizes. Alternately, it may mean that when the distal rings are smaller, the channel adopts a different structural arrangement or perhaps aggregates. In the latter case, one plausible arrangement is dimer formation in which a column of sodium cations and water is sandwiched between two extended conformations of **1**. Of course, any inference about structure is speculative. The monomeric nature of analogs of **2** has been established.[4g] A separate set of experiments will be required to establish the aggregation state of **1**, and these studies are underway. We think that in either case, the channel structure defines the overall column of water and sodium which, in turn, defines the channel's conformation. The conformations of **1** and **2** are expected to be similar although the entry portals are of different sizes, and their conformations may not coincide. The size difference in macrorings likely affects the cation release rates which are critical to channel function.

The present model systems are like protein channels in that they open and close to gate cation passage; mechanistic details are unknown either for proteins or for these systems. A common "explanation" is that the phenomenon is induced by conformational changes. There is no doubt that our flexible systems can undergo intramolecular conformational or positional changes within the bilayer. The striking fact emerging from the present studies is the similarity in open-close behavior between these simple systems and much more complex proteins. We infer that the phenomenon known in the biological community as "gating" may be strongly dependent upon the chemistry of the cations involved and a much simpler chemical phenomenon than it is currently thought to be. We are investigating this latter issue and will report on those efforts in due course.

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