GRAMICIDIN AND RELATED ION CHANNEL-FORMING PEPTIDES
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Symposium on Gramicidin and related ion channel-forming peptides, held at the Novartis Foundation, London, 17–19 November 1998

This symposium is based on a proposal made by Bonnie Wallace

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Introduction: gramicidin, a model ion channel

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This symposium brings together a diverse group of people who are interested in gramicidin and related ion channels, with the goal of advancing our understanding of ion conduction across membranes. A large number of teams doing important work in this area are represented here, and as a result we can look forward to an exciting symposium.

This field has become increasingly interdisciplinarity, and one of the reasons for holding such a symposium as this has been to bring together experts in the fields of biophysics, physiology, synthetic chemistry, theoretical chemistry, biology and engineering. I expect that the book that results from it will become a valuable reference for those working in the field of ion channels. I would like to start by outlining some of the topics we originally had in mind for discussion when developing the concept for this symposium. I expect we will cover all of these areas, and more, during the course of the next several days.

(1) Structural studies, including X-ray crystallography and diffraction, circular dichroism, and solution and solid-state NMR spectroscopy. Comparisons between results obtained on gramicidin by these different techniques and under different conditions should better define the polymorphic nature of this molecule and the relationships between its different conformational states.

(2) Structure/activity relationships. Gramicidin has been engineered (using synthetic peptide chemistry techniques rather than by cloning due to the presence of D-amino acids) more than any other ion channel. The resulting 'mutant' molecules have helped us to understand structure/function relationships by examining functional consequences of changes in the sequence of the molecule and correlating these with the structural studies.

(3) Theory and simulation analyses. The small size of gramicidin has made it particularly amenable to these types of studies, maximizing the utility of currently available computing resources to examine complex multi-component systems over realistic time-scales.
(4) Biological function. This may be a bit awkward for our community, which has focused on gramicidin's role as a model ion channel, because we don't know what its true biological role is, but it may be an interesting area to pursue.

(5) Lipid interactions. Gramicidin represents a good model system for examining interactions between polypeptides and lipids with different fatty acid chains and head groups.

(6) Applications. One important example of the use of gramicidin is its newly created role as part of an analytical sensor system, as well as its old use as an antibiotic, perhaps with some new twists.

(7) Other ion channels. Contrasts and comparisons will undoubtedly be made with the recently solved *Streptomyces lividans* potassium channel structure and with other polypeptide ion channels.

This symposium will be interdisciplinary with respect to the fields covered and the approaches taken, as well as truly international, with participants coming from Europe, the US, Canada, Australia and Russia. There should be many exciting discussions, resulting from the presence of experts in so many different areas. Most of us know each other's names because we read all the gramicidin papers, but we don't necessarily know everyone's faces, so it will be great to put faces to names and get to know each other and initiate in-depth dialogues. I expect this symposium and our discussions will turn out to have important consequences in the field for many years to come.

This symposium is particularly timely because of the many technical advances that have taken place in the last few years which have ultimately increased our understanding of ion conduction. Advances in X-ray crystallography and diffraction, such as new instrumentation for data collection (including synchrotrons and detector technology), and computational developments in phasing and refinement and graphics have allowed us to learn more about the structure of this intermediate-sized molecule. Advances in solution and solid-state NMR spectroscopy have included magnet technology and computing methods that have permitted more accurate and facile structure determinations. Computing advances have also played a major role in theoretical and dynamic studies and simulations. Advances in peptide synthesis, chemistry, characterization methods and conductance measurements have provided important new insights into the functioning of this molecule. All of these advances have had an important impact on our knowledge of gramicidin, and we hope that by extension, these advances in understanding gramicidin have had an important impact on our knowledge of the process of ion conduction across biological membranes.

This symposium is also timely due to the number of controversies that currently exist in the field. These include ones that have arisen as a result of new X-ray and
NMR structures, conductance calculations and comparisons with the potassium channel structure. Many more controversies will undoubtedly arise during the course of this symposium. I hope that we will be able to resolve some of them during the discussions, and that others may lead to further studies that will advance the field in the future.
Correlations of structure, dynamics and function in the gramicidin channel by solid-state NMR spectroscopy

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Abstract. The high resolution structure of the gramicidin A channel has been determined in a lamellar phase environment using solid-state NMR spectroscopy. While the fold is similar to previous characterizations, channel function is exquisitely dependent on structural detail. There is essentially no structural change upon cation binding and no significant change in dynamics. The cations appear to be adequately solvated in their binding site by no more than two carbonyls and no fewer than three water molecules at any one time. The relatively large number of water molecules allows for geometric flexibility and little selectivity among monovalent cations. However, the dehydration energies of cations clearly explain the selectivity for monovalent versus divalent cations. Moreover, the binding site is shown to be delocalized, resulting in a shallow potential energy well so that efficient cation conductance can be realized. The potential energy barrier at the bilayer centre has been shown to be rate limiting under certain circumstances through a correlation between conductance and the electrostatic interactions between cations at the gramicidin monomer–monomer junction and the indole dipole moments at the lipid–water interface. The dynamics are functionally important. The time-scale for carbonyl fluctuations about the Ca-Cα axis and kinetic rates for cation movement in the channel are the same, suggesting a correlation between molecular dynamics and kinetics. These functional correlations will be described in light of the recent K+ channel structure and the biological challenge to achieve both selectivity and efficiency.


Gramicidin A has proven to be an exceptional model cation channel for exposing the principles by which cations can be transported across membranes by much more sophisticated proteinaceous channels. Although good models of the molecular fold have existed since 1971 (Urry 1971), it wasn’t until high resolution structural and dynamic detail was available (Ketchen et al 1997, North & Cross 1995) that many of the functional insights have been achieved.
This is not to belittle the knowledge from the fold that the channel supports a single file column of water molecules and that the polypeptide must provide much of the solvation environment for the cations. However, this does not explain the efficiency with which this channel conducts cations nor its selectivity, the issues of primary concern for the proteinaceous channels, such as the K\(^+\) channel from *Streptomyces lividans* (Doyle et al 1998).

Solid-state NMR spectroscopy has made it possible to collect high resolution structural and dynamic data (Cross 1997) from membrane proteins. Uniformly solid-state NMR spectroscopy has made it possible to collect high resolution structural and dynamic data (Cross 1997) from membrane proteins. Uniformly 

FIG. 1. Gramicidin A is a polypeptide of 15 amino acid residues having the sequence: formyl-Val1-Gly2-Ala3-D-Leu4-Ala5-D-Val6-Val7-D-Val8-Trp9-D-Leu10-Trp11-D-Leu12-Trp13-D-Leu14-Trp15-ethanolamine. This alternating sequence of L and D amino acids permits the formation of a \(\beta\)-strand-type structure with all of the side chains on one side, thereby encouraging the formation of a helix. This single-stranded helix of 6.5 residues per turn forms a pore of approximately 4 Å in diameter. The junction between the N-terminus of two monomers at the bilayer centre is supported by six intermolecular \(\beta\)-type hydrogen bonds. The monomer structure is defined by 120 precise orientational constraints from solid-state NMR of uniformly aligned samples in lamellar phase lipid bilayers (Ketchem et al 1997; protein data bank accession number 1MAG). The monomer–monomer geometry has been characterized by solution NMR in SDS micelles (Lomize et al 1992) and recently by solid-state NMR intermolecular distance measurements (R. Fu, M. Cotten & T. A. Cross, unpublished results 1999). The precision and accuracy of the structure is characterized by torsion angles defined to within an error of \(\pm 5^\circ\).
aligned samples with a mosaic spread of less than 0.3° are possible with gramicidin in dimyristoyl phosphatidylcholine (DMPC) bilayers. The observation of anisotropic chemical shifts, dipolar and quadrupolar interactions present spectral resolution that is comparable to solution NMR spectra. Unlike the isotropic chemical shifts, which are difficult to interpret, the anisotropic interactions have a simple orientational dependence with respect to the magnetic field axis, leading to precise structural constraints. The accuracy of the constraints is dependent on how well characterized the spin interaction tensors are defined and on how well the molecular motions are characterized.

Cation binding

For gramicidin, many of the most variable spin interaction tensors have been experimentally characterized both in terms of their tensor element magnitudes and their orientations with respect to the molecular frame (Mai et al 1993). Upon the addition of cations, some significant (> 1 ppm) changes in the observed 15N chemical shifts from oriented samples are observed (Fig. 2; Tian et al 1996, Tian & Cross 1999). Such changes could be due to a change in structure, a change in dynamics and/or a change in chemical shift tensor in the presence of cations. Because dipolar interactions are not significantly affected by the presence of cations it has been argued that there is little to no structural or dynamic influence by the cations on the polypeptide backbone. Indeed, the largest structural deviations as modelled by a change in orientation of the peptide plane about the Cα-Cα axis is just a 4° change in time-averaged orientation. This result suggested that the cation was influencing the electron density in the peptide plane containing a carbonyl that was providing cation solvation (Tian et al 1996). The chemical shift tensor is defined by the electron density surrounding the nuclear spin and it has since been shown that 15N tensors are significantly modified by the presence of cations in the channel (Tian & Cross 1998). Therefore, the changes in chemical shift reported in Fig. 2 define those peptide planes involved in cation solvation. The three carbonyls significantly involved are from Leu10, 12 and 14, whereas the carbonyls from Trp11, 13 and 15 and all others are not involved on a time-averaged basis to a great enough extent so that the average 15N chemical shift tensors are affected.

These results lead to several fundamental conclusions. First, there is no significant structural change upon binding a cation to this channel in a low dielectric environment. This might appear to be a surprising result, and many of the early molecular dynamics calculations had suggested large deformations leading to ideal or near-ideal solvation environments for the cation (Åqvist & Warshel 1989, Roux & Karplus 1993). However, we have argued that such changes in conformation could lead to a more substantial potential energy well
and hence barrier to cation translocation (Tian et al 1996). What is desired for the efficiency of the channel is adequate rather than ideal solvation of the cation in the channel. Here, adequate means that the equilibrium binding constant must be strong enough to attract cations into the channel away from the bulk aqueous solvent where six waters solvate the cation in the primary hydration sphere. However, the question of why the structure of gramicidin is not distorted by the presence of cations is not answered by these arguments that define why it is good to have little distortion.
Delocalized binding

Part of the answer comes from a realization that the cation-binding site is a series of subsites, i.e. cation binding is delocalized. This conclusion is necessitated because favourable interactions with the Leu10, 12 and 14 carbonyls cannot be achieved simultaneously (Fig. 3). Not only is there too great a separation of the carbonyl oxygens in the plane of the bilayers (end view, Fig. 3B), but the carbonyl groups are separated substantially along the helical axis (∼3 Å). Therefore, to induce the chemical shift changes observed in Fig. 2 it must be necessary for the cation to spend significant amounts of time in close vicinity to the individual carbonyls of Leu10, 12 and 14. This represents a delocalized binding site where the time-averaged influence of the cation is distributed over a significant spatial volume. The probability distribution is approximated by the histograms in Fig. 2 showing the influence of cations on the chemical shift tensors. Clearly, this probability distribution or time-averaged location for the cations in the channel is different for the various cations. The larger cations bind closer to the channel mouth and the divalent cation, Ba$^{2+}$, which is not conducted by the channel, shows no significant interaction with the Leu10 carbonyl, and induces only modest chemical shift changes in Leu12 and Leu14 carbonyl peptide planes despite its divalency. Not only does delocalized binding reduce the influence of the cation on the polypeptide structure, but it also generates a broad and shallow potential energy well for cation binding, ideal for displacing the cation from the binding site and moving it across the membrane. In addition, the entropic penalty associated with cation binding is minimized by allowing the cation to move within its binding site. Therefore, by avoiding such structural changes fast cation association rates can be achieved, as observed (Becker et al 1992).

Another question arises from this lack of structural change, and that is how does the channel provide adequate solvation for the cation without forming a constricted cluster of carbonyls about the cation? The dehydration energies for cations in the gaseous state are known (Dzidic & Kebarle 1970), and the energy required to remove each successive water is substantially greater than the removal of the previous water. Consequently, the removal of the last water prior to entry into the single file region of the channel (two waters per cation) has the largest energy barrier. This step appears to define the inner boundary of the cation-binding site. At the channel entrance the Leu14 carbonyl oxygen is the first site of interaction, displacing the first water molecule from the primary hydration sphere (Fig. 4). As the cation moves into the channel, more waters are stripped off through interactions with Leu10 and 12. Leu10 is the furthest into the channel that Na$^+$ can penetrate while maintaining at least three waters in the hydration sphere. These waters provide considerable flexibility for solvating cations and hence provide a lack of selectivity among monovalent cations.
FIG. 3. (A) Side view and (B) end view of the channel backbone in which the three carbonyl oxygens of Leu10, 12 and 14 are presented in van der Waals radii. These are three sites that significantly interact with cations. K⁺ (pale circles) is schematically shown interacting with the carbonyl oxygens (dark circles) through the peptide plane dipole moment dominated by a component parallel to the C-O bond direction. The three locations for the K⁺ ions are intended to illustrate the span of the delocalized cation-binding site through which the cation becomes significantly dehydrated. Note that this delocalized volume involves significant translation in both the radial and axial directions, suggesting a helical path for the cations.

(Christensen et al 1975, Cox & Schneider 1992). Such flexibility will not be anticipated in the K⁺ channel where specificity is exquisite. The stepwise dehydration, which had been previously suggested by molecular dynamics calculations (Åqvist & Warshel 1989, Jordan 1990, Roux & Karplus 1993), is important for generating an incremental pathway over large energy barriers associated with removing three waters from the cation. In this way, the association and dissociation rates are enhanced (Lehn 1973). To interact with the Val8 carbonyl in the second turn of the helix the hydration of even Li⁺ has to be reduced to two waters. However, carbonyls from the first turn of the helix, which have formerly not participated in solvation, such as the Trp15 carbonyl, now compensate for the fourth water which has been stripped from the cation.
Throughout the rest of the channel this solvation environment remains relatively uniform with no local large potential energy steps until reaching the symmetry-related, cation-binding site in the adjacent monomer.

Although the lack of selectivity among monovalent cations is explained by having three mobile ligands in the cation-binding site, divalent cations clearly do not penetrate even to the Leu10 site in the first turn of the channel structure. Dehydration of divalent cations requires substantially more energy than monovalent cations (Blades et al 1990), and there is insufficient compensatory

FIG. 4. End views (top) of the channel as in Fig. 3 showing the three individual carbonyl interactions with cations and a model of cation hydration for each configuration. The corresponding side views (bottom) are also shown. When the cation is interacting with Leu14 (left) as many as five waters can be found in the primary solvation sphere, whereas four are typically found when K⁺ is in the vicinity of Leu12 (middle) and only three when in the vicinity of Leu10 (right). When monovalent cations are in close contact with the Val8 carbonyl (not shown) additional stabilization is provided by the Trp15 carbonyl and only two waters solvate the cation.
interaction energy available from the polypeptide backbone. Divalent cations block the channel, and the interactions with the Leu14 and Leu12 carbonyls demonstrate that while enough solvent waters are not stripped off for the cation to pass through the channel the interactions with Leu12 and Leu14 are significant enough to initiate dehydration of the divalent cation resulting in binding of such cations. Anions show no influence on the spectra documented by monitoring the chemical shift changes for Na\(^+\) and Ba\(^{2+}\) salts of chloride and nitrate consistent with the known lack of anion binding to the channel. Although partial negative and positive charges are balanced in the peptide plane, the partial negative charge is focused on the carbonyl oxygen, whereas the partial positive charge is more distributed with much less charge density on the amide proton than oxygen, making the structure much less attractive to anions than cations. Furthermore, the refined structure has almost all peptide planes oriented with the carbonyl oxygens tipped in towards the channel axis and, therefore, the amide protons are oriented away from the channel and toward the lipid environment, making them inaccessible to anions (Ketchem et al 1997).

**Functional role for dipoles**

Solid-state NMR has also shed light on cation translocation through the channel. By accurately defining the orientation of the indole rings, Hu et al (1995) have determined the orientation of the indole dipoles with respect to the channel. Indeed, these dipoles are oriented such that the negative end is directed toward the channel axis at the bilayer centre. The influence of these dipoles has been assessed by comparing conductance with and without dipoles present (Becker et al 1991) to the sum of the monopole–dipole electrostatic interaction energy with the cation monopole located on the channel axis at the bilayer centre (Hu & Cross 1995). The elimination of dipoles was achieved by incrementally replacing the indoles with Phe. As shown in Fig. 5, a remarkable linear correlation is achieved between the natural log of conductance and the sum of these interaction energies. Such a correlation strongly suggests that the potential energy barrier at the bilayer centre is the rate-limiting step for cation conductance by gramicidin under these sample preparation conditions. Furthermore, the correlation also suggests that the structure of gramicidin in DMPC bilayers is similar to the structure in diphytanoyl phosphatidylcholine (DPhPC) bilayers used for the conductance measurements (Becker et al 1991, Busath et al 1998).

If the reduction in conductance by a factor of 20 (all Trp to all Phe) is equated with an increased potential energy barrier from an Arrhenius analysis then a dielectric constant for the 10–13 Å of intervening protein–lipid environment can be approximated as 5.1 (Hu & Cross 1995). However, the most important finding is that dipole–monopole electrostatic interactions are used by this molecule to enhance
the efficiency of channel conductance. It appears that in the K⁺ channel, a set of short helices are oriented such that the helix dipoles stabilize cations at the bilayer centre (Doyle et al 1998). Electrostatic interactions will be important over much longer distances in membrane proteins than typically observed in water-soluble proteins.

\[ E = \frac{\mu_i e}{4\pi \epsilon_0 \epsilon_r r_i^2} \]

\[ \ln(C) = A' + E/RT \]

**FIG. 5.** Natural log of conductance for various gramicidin analogues were obtained by Becker et al (1991). The interaction energy scale represents the summation of monopole (cation at the monomer–monomer junction)–dipole (various indoles) interaction energy. This interaction energy is dependent on both distance and dipole strength in the direction of the distance vector, \( r_i \), which are defined by the high resolution structure. This interaction is also dependent on the dielectric strength. Here, this scaling factor is estimated from the slope by first determining the energetic reduction in the potential energy barrier resulting in a factor of 20 loss in conductance. The \( E \) value is thereby determined to be 5.1 and a linear correlation is demonstrated between \( \ln(C) \) and the summed interaction energy.
Understanding the fundamental determinants of rates of reaction and transport is a major challenge in structural biology. It is generally accepted that such an understanding will not come without a detailed characterization of molecular dynamics. Experimentally, dynamics are often characterized by order parameters or thermal factors that do not provide the high resolution characterization needed for correlating dynamics and function. Solid-state NMR has the advantage that isotropic motions are absent and spectral parameters are far more sensitive to the local motions than in solution NMR. Powder pattern spectra of the polypeptide backbone in hydrated lipid bilayers have been obtained as a function of temperature. The axial rotation rate of the channel about the bilayer normal is less than 1 Hz at 6°C and 10^6 Hz at 36°C (Lee et al 1993, North & Cross 1995). Below 200 K local librational motions of significant amplitude (>5°) cease. As the temperature is increased the tensor elements for single amide ^15N sites in the backbone are unequally averaged as molecular motions occur. By modelling the spectra between 200 and 283 K it is possible to determine both the axis about which the motions are occurring and the amplitude (Lazo et al 1995). Throughout gramicidin, the backbone motions appear to be occurring about the Ca-Ca axis, presumably the result of compensating motions about ψ_i and φ_i+1 torsion angles. Unless the molecular motions are occurring in the millisecond and microsecond time-scale, the powder pattern spectra will not yield motional rates.

While powder patterns have characterized the motional axis and amplitude, relaxation parameters can now be used to determine motional frequencies in light of this experimentally defined motional model. The definitions of these frequencies require that either multiple relaxation parameters or relaxation parameters as a function of magnetic field strength are determined. For gramicidin T1 relaxation at two field strengths were obtained, yielding frequencies of approximately 10^8 Hz and amplitudes of approximately 6° throughout the backbone (North & Cross 1995). This amplitude is significantly smaller than that determined from the powder patterns, which were 15–20°. The reason is that all motional frequencies faster than 10^4 Hz will average the powder patterns; however, only motions in the vicinity of the Larmor frequency (10^8 Hz) will induce T1 relaxation. Relaxation parameters are highly non-linear detectors of motional frequencies, whereas powder patterns are linear detectors of motions greater the interaction strength represented by the powder pattern, in this case 10^4 Hz. Efficient relaxation rates clearly indicate the presence of motions near 10^8 Hz, and while these motions are of modest amplitude, motions in the picosecond time-scale are likely to be present and potentially account for the amplitude difference.

The 10^8 Hz rate is remarkably slow for a peptide plane motion. Even peptide plane motions one or two orders of magnitude faster are considered to be
overdamped motions, the result of correlated motions (Usha et al 1991). Here, correlations between the motions of one peptide plane and its adjacent, next nearest neighbour, etc. planes could result in severe overdamping of the motion (Chiu et al 1991, Roux & Karplus 1991, Elber et al 1995). It is known that the motion of cations and water molecules in the channel are correlated; in other words, the column of water molecules moves as a unit while a cation is transported across the bilayer. The question for gramicidin was whether or not the correlations extended from the small molecules in the pore to the polypeptide backbone lining the pore. Apparently, motions of molecules and ions in the channel are coupled not only to each other, but to the peptide planes lining the channel and they, in turn, are coupled together. Based on single-channel conductance measurements (Andersen & Koeppe 1992) it has been estimated that cations spend approximately 10 nsec with each carbonyl cluster along the pathway between cation-binding sites. This is in remarkable agreement with the measured dynamic frequencies for the polypeptide backbone. Therefore, a correlation between the motion of the peptide planes and the kinetic translation of the cations is suggested. If such correlations exist it further suggests that the cations do not follow a one-dimensional random walk through the channel, but rather they follow a ballistic motion through the channel, thus spending as little time as possible in the low dielectric environs of the bilayer centre.

Gramicidin has proven to be a remarkable model channel from which great insights into cation solvation, selectivity and conductance efficiency have been achieved. Moreover, the need for high resolution structural and dynamic information has been demonstrated and the ability of solid-state NMR to uniquely provide it.

Acknowledgements

The authors are indebted to the staff of the National High Magnetic Field Laboratory and Florida State University NMR facilities; T. Gedris, J. Vaughn and A. Blue for their skilful maintenance and service of the NMR spectrometers; and H. Hendricks and U. Goli of the Bioanalytical Synthesis and Services Facility for their expertise and maintenance of the ABI 430A peptide synthesizer and HPLC equipment. This work has been supported by the National Institutes of Health grant number AI-23007 and the work was largely performed at the National High Magnetic Field Laboratory supported by the National Science Foundation Cooperative Agreement DMR-9527035 and the State of Florida.

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**DISCUSSION**

_Roux_: You mentioned that the structure is not uniquely determined because of the second-order Legendre polynomial $P_2[\cos(\theta)]$. However, I would stress that what is measured is not $P_2[\cos(\theta)]$ but rather its average. One might wish to interpret the data in terms of a single structure, or in terms of an ensemble of structures symmetrically distributed around an average, but it may not be possible. Once it goes into the non-linear function $P_2[\cos(\theta)]$, the distribution becomes skewed, i.e. the average of the function is not equal to the function of the average. Therefore, there is some unavoidable ambiguity in the interpretation of the solid-state NMR data in terms of a unique structure. Although the molecular dynamics simulation is not perfect, it provides a nice illustration of the problem I am describing. In practice, if I take a trajectory and compute the average that corresponds to a backbone $^{15}$N solid-state NMR chemical shift, I may obtain the exact value that you measured, say 180 ppm. But if I look at the time course of the trajectory, I will observe fluctuations in the order of ±30 ppm. Just because the average is 180 ppm does not mean that the molecular conformation is such that it corresponds to this value all the time — the structure fluctuates. On the other hand, the chemical shift calculated from the average structure may not correspond to a value of 180 ppm.

_Cross_: I should have mentioned that the structure I presented is a time-averaged structure.

_Eisenberg_: Over what time-scale?
Cross: We average over the time-scale of data acquisition, which is a matter of milliseconds.

Eisenberg: This is much longer than the permeation times.

Cross: We have also looked at the dynamics of the backbone, and in particular at how the tensor is averaged from the point at which the torsion angles are frozen out (below 200 K) to 280 K. From this we obtain a clear idea of the magnitude of tensor element averaging, and it is sensitive to picosecond motions.

Jukobsson: I have another time-averaging question. In our molecular dynamics simulations we see many more side chain transitions than Tim Cross. I would like to ask Benoit Roux if he sees any side chains undergoing torsion angles transitions.

Roux: We see isomerizations of leucines and valines, but not many.

Jordan: What temperature are these experiments performed at?

Cross: They are all run at about 30°C, which is above the phase transition temperature of the dimyristoylphosphatidylcholine (DMPC) lipids.

Davis: Regarding the question of time averaging by NMR spectroscopy, I would like to point out that Tim Cross has used deuterium NMR spectroscopy. He has measured deuterium quadrupolar splittings of hundreds of kilohertz, and he is also using dipolar couplings of a few hundred hertz. He is going from a time-scale of 10 μs to a time-scale of 10 ms, and he is therefore not measuring the same time average in all of these different experiments, so some parts of his structure are averaged over one time-scale and other parts by another.

Eisenberg: That’s an important comment, but the currents are flat over that time-scale. We have direct experimental evidence that functionally there is only one potential mean force over that time-scale. When we’re talking about ensembles of averaging, we have to remember that for most of us the main interest is the function of the channel, and that it is necessary to recover the potential energy profile and the underlying charge that produces it. We have the advantage that we know it exists, and it doesn’t have to exist a priori for any molecular structure. The question is how to estimate it.

Davis: But you have to have a coherent view of what your structure is. We’re talking about a time-averaged structure, but this is not consistent because it’s averaged over several different time-scales.

Eisenberg: I don’t disagree with the fact that they’re different time-scales, but the observation that current is independent of time, and would change if there were a small change of potential barrier, proves that there is one time-independent structure from roughly a microsecond to seconds.

Cross: It is certainly true that the data are recorded with different time-scales. On the other hand, we have looked at the molecular motions occurring within those time-scales. We have observed global correlation times that occur within a 1 ms time-scale, and we have observed fluctuations of the peptide backbone that occur within a shorter time-scale. The only molecular motions that occur within the