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HOLE: A program for the analysis of the pore dimensions of ion channel structural models

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A method (HOLE) that allows the analysis of the dimensions of the pore running through a structural model of an ion channel is presented. The algorithm uses a Monte Carlo simulated annealing procedure to find the best route for a sphere with variable radius to squeeze through the channel. Results can be displayed in a graphical fashion or visualized with most common molecular graphics packages. Advances include a method to analyze the anisotropy within a pore. The method can also be used to predict the conductance of channels using a simple empirically corrected ohmic model. As an example the program is applied to the cholera toxin B-subunit pentamer. The compatibility of the crystal structure and conductance data is established. © *1996 Elsevier Science Inc.*

INTRODUCTION

More than a third of the proteins encoded by the yeast genome are thought to be membrane integral.[1] Ion channels are an important class of membrane proteins.[2] These proteins allow the translocation of ions across the hydrophobic barriers presented by lipid bilayers, and are often gated by the action of voltage gradients or the binding of small ligand molecules (such as neurotransmitters).

Color Plates for this article are on page 376

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Despite the obvious interest in ion channels there are only a limited number of structural studies currently available.[1] This is principally due to the difficulty in structure determination of integral membrane proteins, both by crystallographic and nuclear magnetic resonance (NMR) methods. In contrast to this, membrane proteins provide an attractive target for modeling studies. In part this is because of the limited possibility of main-chain groups forming hydrogen bonds with solvent in the lipid environment. As a consequence there is a restriction in the number of secondary structures available.[3] Modeling provides an attractive option in adding value to mutagenesis[4] or low-resolution structural studies such as by electron microscopy.[5]

The increasing number of experimental and modeling studies on channels led us to identify the need for dedicated methods for analyzing important structural features.[6] In particular, all current structures of channels have a cavity running through them. This cavity or pore is normally filled with solvent and provides the pathway for ion translocation.

Many methods exist for looking at the surface of proteins and cavities inside them. Most molecular graphics programs have routines for displaying the van der Waals surface of molecules and often can display the solvent-accessible surface, as pioneered by Connolly.[7] Specialized methods exist for isolating cavities within proteins, notably the commonly used Voido[8] method and Proact,[9] which introduces a powerful classification method. A method that concentrates on analyzing pockets on the surface of proteins, thus allowing easy identification of active sites, has been developed.[10] Although all of the methods could potentially be used to analyze ion channels, the specialized nature of the molecules makes a dedicated set of routines desirable.

This article summarizes work on the program HOLE, which concentrates on analyzing the dimensions of the pores running through molecules. Developments[11] allowing the analysis of anisotropy and prediction of conductance properties are described. As an example an analysis is then

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made of the pore properties of cholera toxin B-subunit pentamer.

METHODS

HOLE Monte Carlo simulated annealing

The HOLE method has been described in detail elsewhere.[6,11] The program requires the user to supply the coordinates of the ion channel of interest in Brookhaven Protein Data Bank (pdb) format. An initial point p, which lies anywhere within the central channel, is also needed. In addition the user specifies a vector **v** that is approximately in the direction of the channel (normally referred to as the channel direction vector). The program reads atoms from within the pdb file and sets up a van der Waals radius for each (various sets are available). It is normal to exclude solvent and ions during the read: the program allows this by the specification of a keyword stating the residue types to ignore.

It is easy to find the maximum radius $R(p)$ of a sphere centered at a point p without overlap with the van der Waals surface of any atom:

$$R(p) = \min^{N_{atom}}{}_{i=1} [|\mathbf{x}_i - \mathbf{p}| - \mathrm{vdW}_i] \quad (1)$$

where $\mathbf{x}_i$ is the position of atom number i, vdW_i its van der Waals radius, and N_{atom} is total number of atoms. The radius $R(p)$ can be regarded as an objective function of the point **p.** By using Monte Carlo simulated annealing, adjusting **p,** the radius of the sphere can be maximized within the pore of the channel. In all cases p is kept on a plane normal to the channel direction vector **v** (Figure 1A).

Note that all distance searches are conducted in three dimensions. Once the highest radius sphere center on a particular plane has been established a new search is initiated by taking a step of length s in the direction of the channel direction vector **v.** This results in searches being conducted for a series of parallel planes with a sphere of maximum radius being found for each (Figure 1B).

The net result can be thought of as producing the locus of a flexible sphere "squeezing" through the center of the ion channel. The use of Monte Carlo simulated annealing reduces the possibility of the routine becoming stuck in a local minimum. In addition it allows the mapping out of complex internal topologies (such as annexin V[12]) using multiple runs.

Visualization methods

There are a number of ways to analyze and visualize the results of a HOLE run. One of the most useful is to plot a graph of pore radius against coordinate z along the channel direction vector **v** (Figure 2).[13]

A graph can be used to examine issues such as how tight the central constriction of the channel is, whether water molecules could fit within the channel, and how large the mouth of the pore is. The HOLE package does not explicitly produce graphs but instead provides easily accessible numerical information so that the user can use the graph-plotting program of his/her choice.

Graphical presentation can be complemented by examination of HOLE objects using a molecular graphics pro-

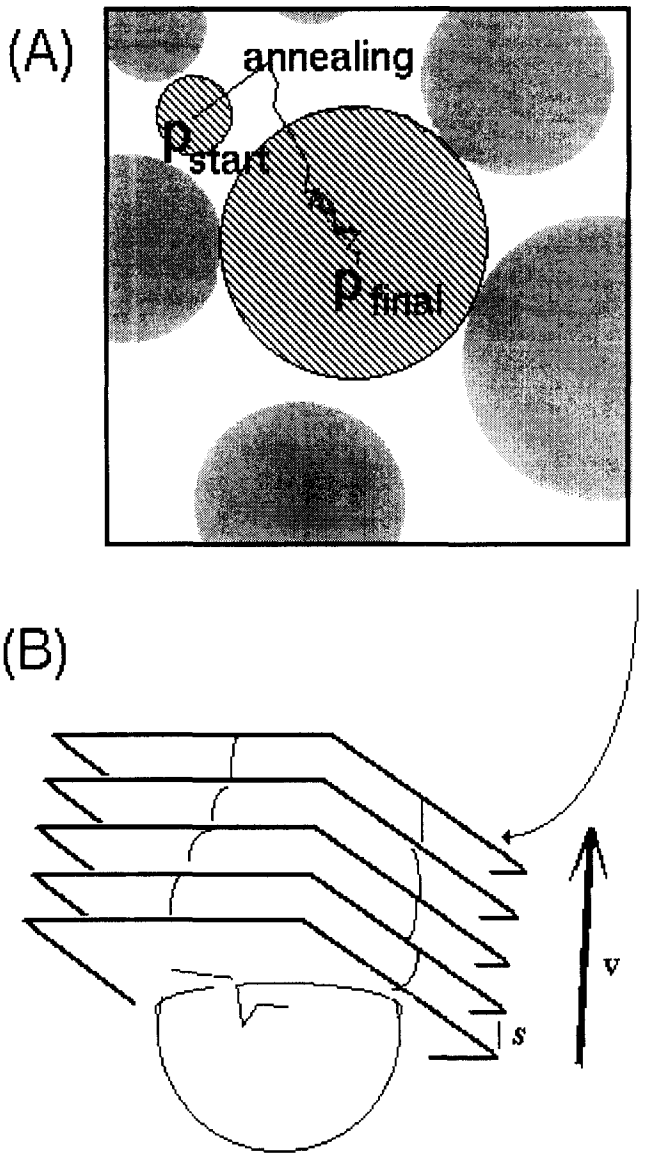

Figure 1. A schematic explanation of the use of Monte Carlo simulated annealing to determine the pore dimensions of a channel. (A) The process of maximizing the size of a sphere, centered at point **p** *(shown with diagonal lines), within the van der Waals volume of the atoms (open spheres). Note that* **p** *is kept on the plane normal to channel direction vector* **v.** *(B) The process shown in part (A) is repeated for a series of parallel planes normal to the channel direction* **v.**

gram. Two objects can be produced and displayed in conjunction with the ion channel structural model.

- The center line of the channel can be shown by plotting the locus of the center of the sphere as it proceeds through the channel.
- The locus of the surface of the spheres define the pore-lining surface. It is often useful to color code the surface. The standard scheme is to use red to indicate parts of the pore where there is insufficient room to accommodate a water molecule, green where a single water molecule could be placed, and blue for places sufficiently large for

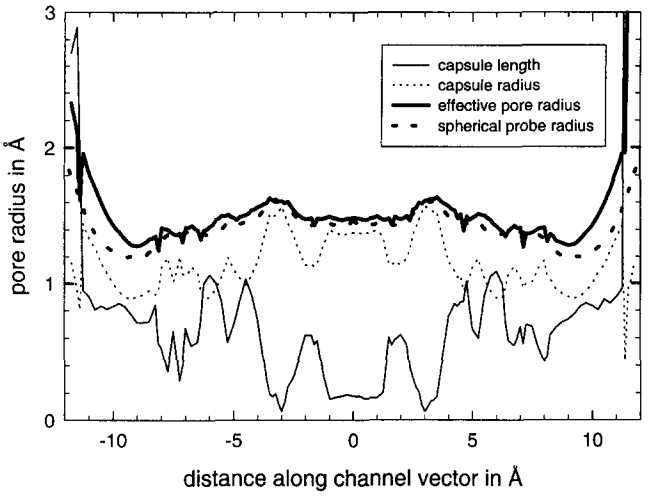

Figure 2. The graph shows the pore radius of the gramicidin A (channel form conformation) determined by Ketchem et al.[13] The dotted thick line shows the pore radius found with the spherical probe used in the original HOLE algorithm. The thick black line shows the effective radius found with a spherocylinder used as a probe. The difference between the two lines shows that the channel is only slightly anisotropic: as demonstrated by the low value of capsule radius (Figure 3) found (shown as thin dotted line). A radius of 1.15 Å is necessary to accommodate a water molecule—the channel is sufficiently large to allow water molecules along its whole length.

two water molecules to be placed side by side (see the electronic version of this article for an example).

The HOLE package is designed to be used primarily with the Quanta molecular graphics program. However, conversion routines are supplied to allow use with:

- Sybyl
- InsightII
- O'Alwyn Jones' crystallographic package: For further details see the O home page.
- Mage: Kinemage is a protein display and viewing language developed by D. Richardson. Kinemages can be viewed by the Mage program, which is available in versions for MS Windows, Mac, and various Unix machines. Mage is available by ftp from suna.biochem.duke.edu
- Work that is still in progress allows the use of virtual reality modeling language (VRML), which may become the standard way of representing 3D objects on the World Wide Web. The electronic version of this article includes examples of VRML files combining HOLE objects and molecules

An advance allows an alternative view of the internal surface of a pore from the inside. In this case we set up a cylindrical coordinate system working from the pore centerline (Color Plate 1A). The coordinate system can then be used to display properties of the pore lining in two dimensions. A simple way of imagining how the maps are constructed is to imagine that the internal surface of the pore is cut down a line and then rolled flat. A number of properties of the internal surface of the pore can then be displayed, for instance, whether the surface is lined by oxygen or nitrogen atoms. The versatile contour plotting program Surfer (see http://www.golden.com/golden/ for details) is used to process data produced by HOLE. This provides a different way of looking at the gramicidin channel (Color Plate 1B).[14]

Analyzing anisotropy

A spherical probe to analyze the pore dimensions of a channel has proved to be extremely useful.[6,11] However, the pores of many larger ion channels are clearly anisotropic, as exemplified by porins.[15,16] So as to analyze and consistently measure such structural aspects, an extension of the original HOLE method is introduced.

Instead of using a sphere to probe the internal surface of the channel in question a spherocylinder (capsule) is considered. This object can be thought of as a simple extension of a sphere, where the center is spread from a point onto a line section. It can be defined in terms of three properties: two centers and a radius, in comparison to a sphere, which has a single center and a radius (Figure 3).

In the HOLE method the axis of the capsule is held at right angles to the channel direction vector **v**. Only small adaptations are required to implement this: Instead of a single center being considered in the optimization, two independent centers are introduced. Each of these is constrained to move on a plane normal to the channel direction vector **v**. Instead of optimizing the radius of the sphere as in the original routine the area of the capsule on the plane normal to **v** is maximized (although this is converted in the program into an effective radius to ease comparisons). As the number of independent variables is doubled from two to

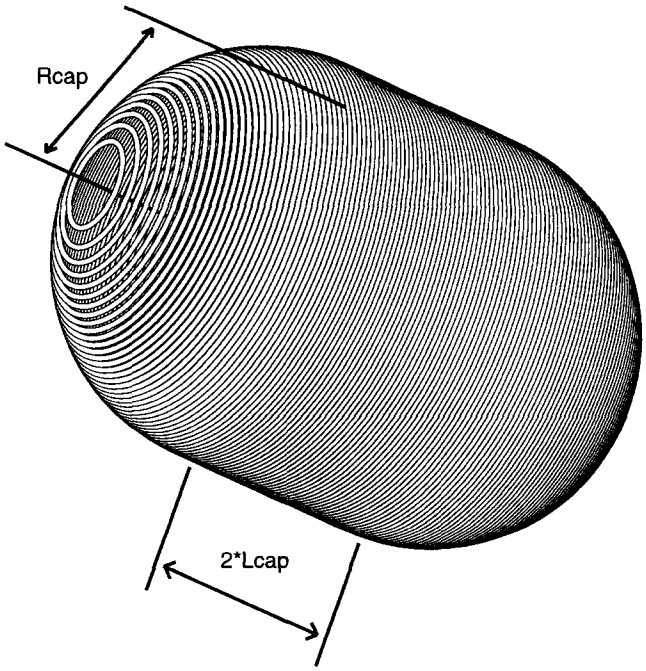

Figure 3. The spherocylindrical object (or "capsule") used in HOLE to measure the anisotropy of ion channels. R_{cap} is the capsule radius and L_{cap} is the capsule length. [Reproduced from Smart et al.[11]]

four, more steps of simulated annealing are required to achieve stability in results. Despite this increased CPU requirement most channels can be analyzed in well under 1 h of CPU time on a modern workstation. The result of optimizing a capsule inside the pore of gramicidin is shown in Figure 4.

The use of the capsule option allows the measurement of the anisotropy of a channel and properties such as the rotation of the capsule vector as it proceeds through the channel. Examples of this can be found in the section Sample Application to Cholera Toxin B-Subunit Pentamer. The more accurate delimitation of the cross-sectional area of the channel as a function of distance along the channel vector is also useful when making predictions of the conductance properties of channels, as discussed below.

Predicting conductance properties using HOLE

The difficulty in experimentally determining the three-dimensional structures of ion channels often make it necessary to resort to modeling techniques. Although modeling can lead to insights into the functional and structural properties of channels there is always the problem of knowing what level of confidence to place in the model. For this reason, tractable methods of validating a given model of a channel are required. HOLE has been adapted so that a reasonable prediction of the conductance of a channel can be rapidly made on the basis of its structure.[11]

The method is based on simple ohmic considerations. The HOLE program can be thought of as measuring the cross-sectional area $A(z)$ of a pore as a function of distance along the channel direction vector z. Consider the pore to be filled with an electrolytic solution of resisitivity ρ. A reasonable approximation of the resistance of the channel is given by

$$G_{\text{macro}}^{-1} = \sum_{z=\text{low}}^{z=\text{high}} \rho s / A(z) \qquad (2)$$

where s is the width between parallel planes used in HOLE (see the section HOLE Monte Carlo Simulated Annealing). Note that in ion channel patch-clamping studies it is normal to consider conductance (G) measured in siemens (S) rather than resistance. This type of approach was pioneered by Hille[2] and introduced into the HOLE methodology by Sansom and Kerr.[17]

Equation (2) assumes that the conductivity of an ionic solution within a channel is equal to that of the bulk solution. This would be true if ion channels had macroscopic dimensions (much larger than a water molecule). In practice it is found that real channels have a conductance that is around five times lower than that expected from Equation (2), giving the macroscopic limit G_{macro}. To make a reasonable estimate of the conductance of a channel, an empirically based correction factor is used, which is chosen to be dependent on the minimum radius of the channel:

$$G_{\text{pred}} = G_{\text{macro}}/C(R_{\text{min}}) \qquad (3)$$

The correction factor was parameterized[11] on the basis of results obtained for the gramicidin channel[14] and the *Escherichia coli* OmpF porin.[15] The prediction routine was tested by "predicting" the conductance found for all channel-forming proteins and peptides for which an experimental structure is available.[11] Results are summarized in (Figure 5.)[4,5,11,15,16,18–20]

Overall, the algorithm yields good results with predictions accurate to within an average factor of 1.8 relative to the experimental values. This accuracy is sufficient to make the method useful in validating model structures. Further work has been undertaken to reparameterize the correction function on the basis of all well-defined structures that were considered.[11] Future work will involve using molecular dynamics simulation procedures to find quantities such as the variation of the self-diffusion coefficient of water molecules within the channel and including such effects within the prediction methodology.

It is also possible to adapt the procedure to predict the effect of adding nonelectrolytes such as polyethylene glycol (PEG) to conductance measurements. Such experiments can be interpreted in terms of a radius profile for a channel. Encouraging preliminary results[11] have been obtained in comparing the expected profile calculated by HOLE from the X-ray structure of cholera toxin B subunit[19] with the experimental result[21] (see also Figure 7).

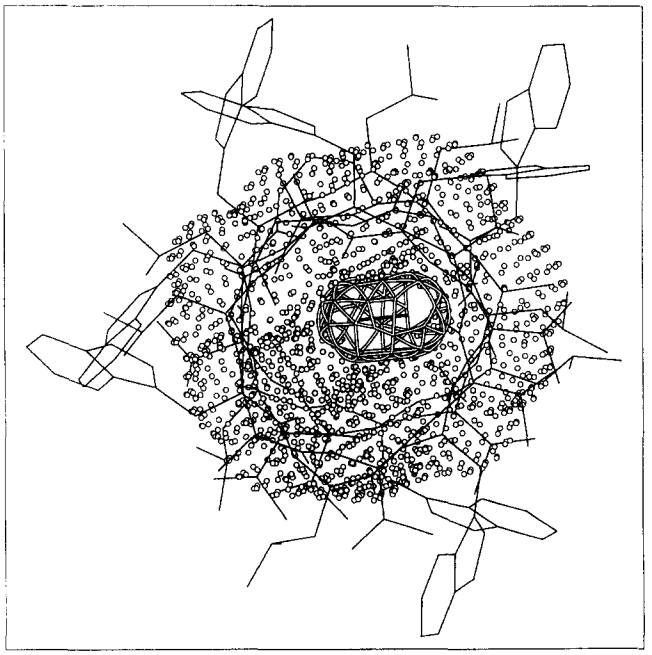

Figure 4. A capsule fitting inside the pore of gramicidin. The capsule is shown as a grid surface fitting inside the van der Waals surface of the pore lining residues, shown by dots. The axis of the capsule is visible inside. The view is almost exactly down the channel vector. [Reproduced from Smart et al.[11]]

SAMPLE APPLICATION TO CHOLERA TOXIN B-SUBUNIT PENTAMER

The protein cholera toxin (molecular mass ~84 kDa) causes the massive loss of fluids characteristic of the disease by altering the transport of salt and fluids across the intestinal epithelium.[22] The intact protein is a heterohexamer composed of five B subunits with a single A subunit.[23] X-ray

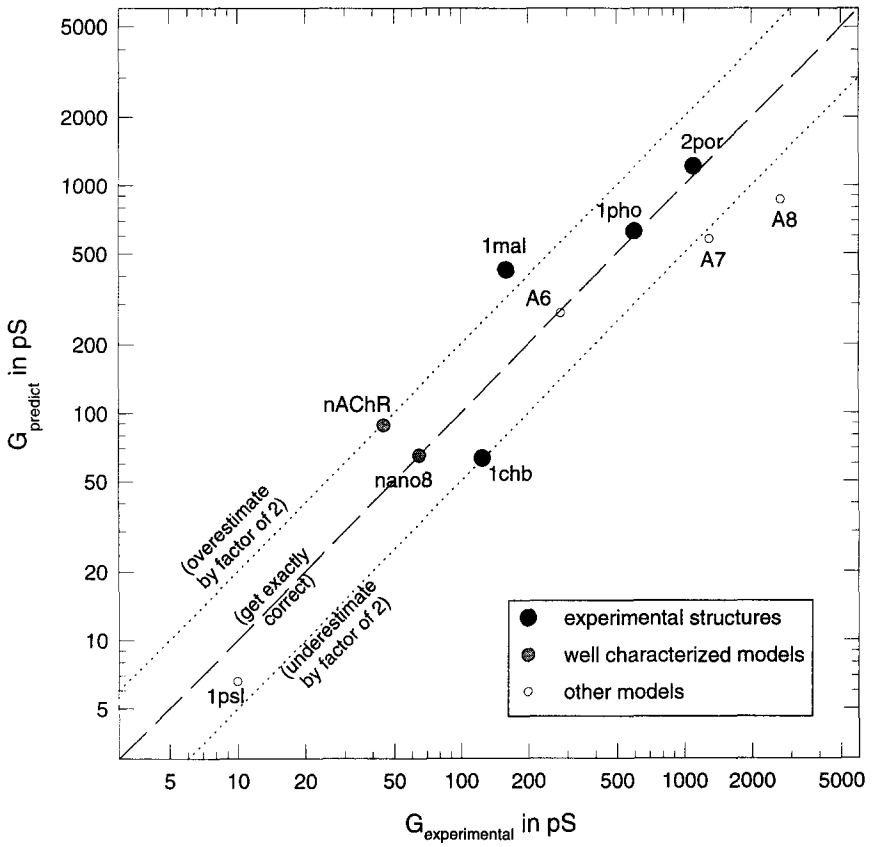

Figure 5. The results of using HOLE to predict the conductance of an ion channel with known structure.[11] Both predictions and experiments were for values using potassium chloride solutions. Results are shown in comparison to the published experimental values for conductance. The structures used to parameterize the empirical correction used in the calculation are excluded from consideration. The experimental (X-ray) structures considered are as follows: **1mal** *(*E. coli *maltoporin[18]),* **1pho** *(*E. coli *phosphorin 1PhoE[15]),* **2por** *(*Rhodobacter capsulatus *porin[16]), and* **1chb** *(cholera toxin B subunit pentamer).[19] Models with well-characterized structures are as follows:* **nano8** *(nano tube-forming cyclic octapeptide[20]) and* **nAChR** *(pore domain of the nicotinic acetylcholine receptor homopentameric α7 in the open state[5]). Other models (with less well-characterized structures) are* **1psl** *(transmembrane domain of phospholamban[4]) and* **A6, A7,** *and* **A8** *(alamethicin bundles with varying numbers of helices[11]).*

crystal structures of both the intact heterohexamer[19] and the B5 homopentamer[22] have been determined. Structures of the hexamer show that a ring of B subunits forms a central "donut hole" that is filled with the A2 α helix from the A subunit. However, the B subunits can form a stable complex that is similar except that the central hole is empty. Krasilnikov and co-workers have shown[21] that this pentamer is able to form ion channels in artificial membranes at low pH. It remains unclear as to whether the ability of the toxin to form ion channels has any role in its pathological mechanism of action.[23]

An examination of the pore dimensions of the crystal structure of the cholera toxin B-subunit pentamer was conducted using HOLE. It has been suggested that the A subunit, which must translocate through the membrane as part of its mode of toxicity may do so by passing through the channel formed by the B-subunit pentamer.[21] However, it has been noted that the pore found in the crystal structures is not sufficiently large to allow such a motion[23] and that there may be some rearrangement of the pentamer on incorporation into the membrane. It is therefore interesting to examine whether the crystal structure is compatible with the conductance data available—if it is, then such a rearrangement may be excluded.

The structure examined is the 2.2-Å resolution structure solved by Merritt et al.[19] The pore dimensions of the channel as calculated by HOLE show a pore that is sufficiently large to accommodate two water molecules side by side at any place within the channel, as shown in Color Plate 2. This can also be seen in Figure 6, which graphically shows the pore radius found versus distance along the channel coordinate.

Table 1 shows a comparison of the minimum effective pore radius found for cholera toxin B-subunit pentamer with those found for the porin family. Both the porins and the maltoporin allow translocation of molecules up to the size of a ketose carbohydrate.[24] As the minimum radius for cholera toxin B-subunit pentamer is in the same range it is expected that it would allow the translocation of solutes of around this size. In terms of being able to translocate a protein domain the size of the A subunit of cholera toxin, this confirms the observation[23] that the crystal structure is incompatible with this, unless the domain was unfolded prior to translocation.

This begs the question; is there a possibility of a conformational rearrangement of the B pentamer, with a widening of the pore, on incorporation into a lipid bilayer as postulated by Zhang et al.?[23] If the HOLE prediction of the conductance of the crystal structure were significantly smaller than the experimentally found conductance this would be indicative of such a change. In fact the predicted conductance is consistent with the experimental value,[11] so it would appear that a conformational rearrangement is unlikely. This conclusion is further strengthened by comparing the prediction for the expected profile of a PEG addition experiment with that found (Figure 7).

It can be seen that according to the HOLE calculation the crystal structure is in fact compatible with the effect on conductance data. Thus the possibility of a significant conformational arrangement accompanying the incorporation of cholera toxin B-subunit pentamer can be seen to be unlikely. This does not necessarily mean that the proposed translocation of the A subunit through the B pentamer does not occur, only that such a motion would involve a consid-

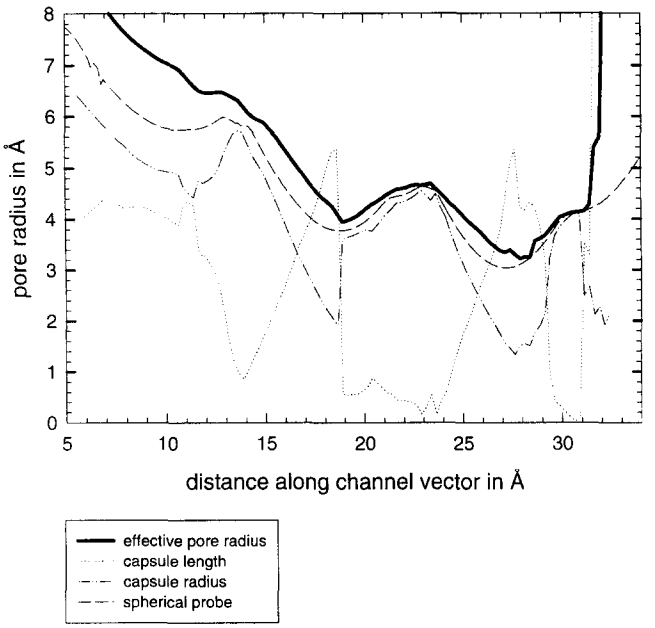

Figure 6. The pore dimensions of the X-ray crystal structure of cholera toxin B subunit pentamer.[19] The graph shows that the central pore is wide, with a minimum effective radius of 3.24 Å. Overall the pore has a reasonably isotropic cross-section—the use of a spherocylindrical probe only marginally increasing the effective radius.

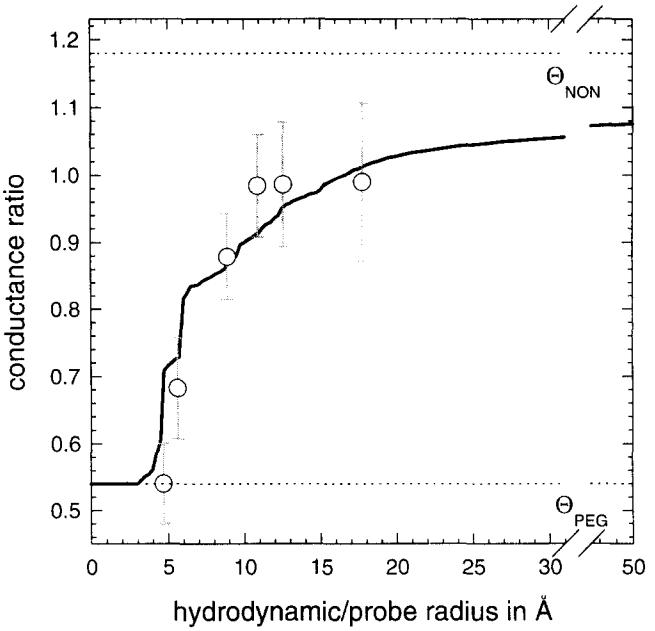

Figure 7. Comparing the experimentally found[21] effect of adding polyethylene glycols of differing molecular weights (marked by error bars) with that of the profile expected from HOLE calculations (line) on the basis of the X-ray crystal structure.[19] The ordinate shows the ratio of the conductance when 20% (w/v) polyethylene glycol is added to the medium in comparison to the polymer-free case. The molecular weight of the polymer can be related to its hydrodynamic radius (the x axis). The structure can be seen to be compatible with the data. [Adapted from Smart et al.,[11] where the calculations are described in detail.]

erable alteration to the structure of either or both the A subunit and the B pentamer.

DISCUSSION AND CONCLUSIONS

The field of ion channel structural studies is still in its infancy. However, the amount of available structural-information can be expected to steadily increase in the future. No doubt there are many surprises that await us and the current understanding will soon look remarkably primitive.

It is hoped that this work shows that an analysis of the pore properties of channels can lead to insights into their behavior. It describes initial attempts to link systematically structure with conductance properties. It is hoped that the methods developed will continue to be of use to other workers in the field.

Table 1. Pore radii found for porins and cholera toxin B-subunit pentamer

System	Minimum effective radius (Å)
Escherichia coli porin Ompf[a]	3.90
Escherichia coli phosphoporin 1PhoE[a]	3.39
Escherichia coli maltoporin[b]	2.85
Cholera toxin B5[c]	3.24

[a]See Ref. 15.
[b]See Ref. 18.
[c]See Ref. 19.

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