

Molecular Properties of Amphotericin B Membrane Channel: A Molecular Dynamics Simulation

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SUMMARY

Amphotericin B is a powerful but toxic antifungal antibiotic that is used to treat systemic infections. It forms ionic membrane channels in fungal cells. These antibiotic/sterol channels are responsible for the leakage of ions, which causes cell destruction. The detailed molecular properties and structure of amphotericin B channels are still unknown. In the current study, two molecular dynamic simulations were performed of a particular model of amphotericin B/cholesterol channel. The water and phospholipid environment were included in our simulations, and the results obtained were compared with available exper-

imental data. It was found that it is mainly the hydrogen bonding interactions that keep the channel stable in its open form. Our study also revealed the important role of the intermolecular interactions among the hydroxyl, amino, and carboxyl groups of the channel-forming molecules; in particular, some hydroxyl groups stand out as new "hot spots" that are potentially useful for chemotherapeutic investigations. Our results also help to clarify why certain antibiotic derivatives, with a blocked amino group, are less active. We present a hypothesis for the role of membrane lipids and cholesterol in the channel.

AmB is a polyene macrolide antibiotic that is widely used in the treatment of systemic fungal infections (1) (Fig. 1, *top*). Despite its long clinical history, the molecular antifungal action of AmB is not well understood (2, 3). According to the most widely accepted mechanism, AmB molecules interact with membrane sterols to form channels (2–5). The channels are responsible for the leakage of monovalent ions, particularly K^+ , and other small molecules from the cell. The resulting irregular ionic distribution eventually causes cell death. The chemotherapeutic use of AmB is based on the higher affinity of this antibiotic for ergosterol (principal sterol in fungal cells) than for cholesterol (sterol in mammalian cells) (6). Because it also has high affinity for cholesterol-containing membranes, AmB is quite toxic, and its use in clinical treatment is limited to rather severe cases.

To reduce the toxic features of this powerful drug, its antifungal chemotherapeutic properties should be elucidated more precisely. Many experimental efforts (3, 7) designed to achieve a comprehensive understanding of the membranous

AmB/sterol channels have shown that these channels are difficult to study; thus, there is little information available for the molecular level, and the channel structure remains unknown. In addition to experiments, in several recent theoretical studies (8–14), the focus has been on the isolated molecular properties of AmB or sterols (8–12). In some of these studies, the channel structure was also considered (13–15); however, only simple molecular mechanics or electrostatic calculations without proper treatment of the membrane/water environment were used. Ultimately, experimental efforts together with molecular modeling approaches should lead to a more complete understanding of the molecular action of AmB.

According to a proposed model (4), the AmB/sterol channel consists of eight AmB and eight sterol molecules. Experimental data and data from previous molecular modeling studies suggest that the diameter of the channel is 7–10 Å (16). Because the length of the SLC is less than the membrane thickness, it was postulated (4) that two SLCs in a bilayer configuration are needed to form a channel (DLC) spanning the entire membrane. It is now accepted that both SLC and DLC types of channels can exist and function, depending on the membrane environment and thickness and the availability of AmB. The SLC channels are observed to transfer ions, which means that they are able to span the entire membrane. It might be argued that lipids around the AmB channel

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ABBREVIATIONS: AmB, amphotericin B; SLC, single-layer channel; MD, molecular dynamics; MDSI, first molecular dynamics simulation; MDSII, second molecular dynamics simulation; DMPC, 2,3-dimyristoyl-D-glycero-1-phosphorylcholine.

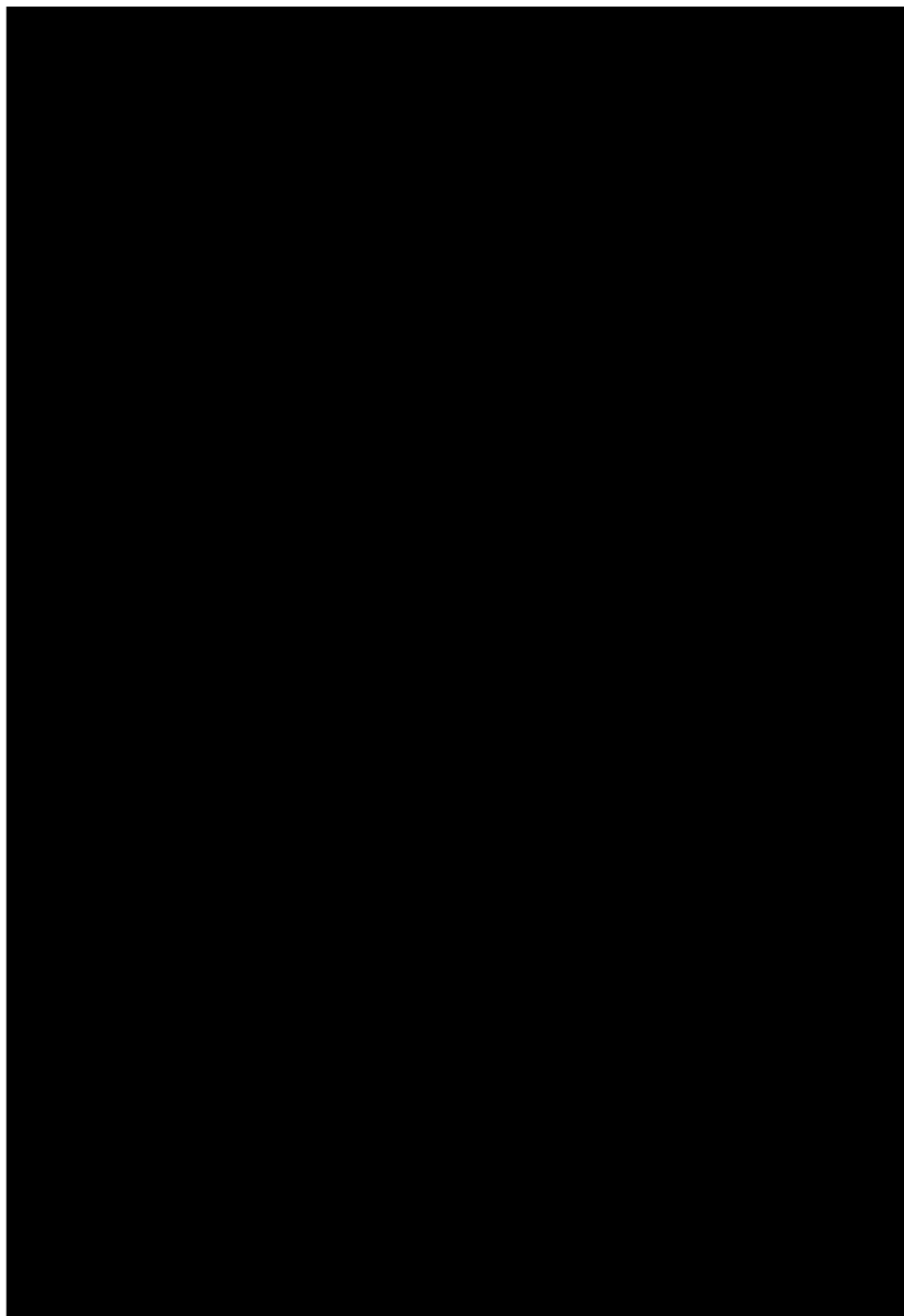


Fig. 1. *Top*, structure of AmB with partial numbering of atoms. *Bottom*, model of the AmB/sterol channel used as starting structure in the simulations. *Green*, AmB carbon atoms; *red*, oxygen atoms; *blue*, nitrogen atoms; *white*, hydrogen atoms; *magenta*, cholesterol molecules.

arrange themselves in such a way the lipid molecules are slightly pressed in to obtain a lipid bilayer thickness approximately equal to the length of the SLC. A similar situation is expected to occur for the DLC; the lipids surrounding the channel are expected to be separated slightly to accommodate the difference between the channel length and membrane thickness. Because AmB is an amphoteric molecule with two charged groups, polar interactions would, to a large extent, forbid AmB molecules from crossing through the cell membrane. Based on this assumption, it has been postulated that an SLC is formed when AmB is present only on one side of the membrane, but a DLC is formed when there are AmB molecules on both sides of the membrane. There is no evidence

yet for the channel formation mechanism, but Weakliem *et al.* (17) suggested that an SLC may form when a critical number of AmB molecules come together at a membrane surface.

In addition to being amphoteric, AmB is an amphiphilic molecule. The polar and nonpolar electrostatic potential patterns of the AmB molecule were studied semiquantitatively according to Poisson-Boltzmann methods (18). On the basis of Baginski and Borowski (18) and other studies (4, 14, 15), it can be assumed that the polar hydroxyl groups of AmB (Fig. 1, *top*) are placed at the center of the channel and the chain of conjugated C=C bonds interacts with the phospholipid/sterol environment. It is known from structure-activity stud-

ies that the polar head of the AmB molecule containing the amino sugar portion and the carboxyl group are very important for antibiotic activity; in particular, the presence of the protonatable amino group has been determined to be essential (19). On the basis of this observation, a model of interaction between AmB and sterol was proposed (20); however, the validity of this proposal is unconfirmed.

Because AmB has amino, carboxyl, and many hydroxyl groups, hydrogen bonding is expected to be very important in the interaction of AmB with molecules in its vicinity. For example, inwardly pointed hydroxyl groups can interact with the solvent and with the cations inside the channel (15). It has been observed that the charged amino and carboxyl groups of AmB are involved in hydrogen bonding (14, 20). Similarly, it has been suggested that hydroxyl groups of the macrolide ring can form intermolecular hydrogen bonds (14). Even though they are expected to play an important structural role, current knowledge of hydrogen bonding in the channel complex is very limited. Unfortunately, experimental studies cannot easily reach this level of detail, and past theoretical studies have used only simple representations such as isolated molecules or AmB/AmB or AmB/sterol molecular pair complexes (10, 12, 21).

Several groups have tried to explain the origins of the observed higher affinity of AmB for ergosterol by using arguments based on structural differences (20, 22–24). Baginski *et al.* (8) and Langlet *et al.* (10) suggested that the more rigid molecular shape of ergosterol, which better complements AmB, is responsible for this higher affinity. Nevertheless, the nature of AmB/sterol interactions and positioning of the sterol in the channel complex remains unknown.

To characterize and further understand the structure and molecular properties of AmB channel, two separate MD simulations were performed of a particular model of AmB/cholesterol channel. In this study, cholesterol rather than ergosterol was included as the sterol; thus, the simulated system corresponds to a mammalian system. This choice was based on the fact that it is the formation of AmB/cholesterol channels that is responsible for the undesirable toxicity of AmB in patients. Further knowledge of the molecular factors that stabilize the structure of AmB/cholesterol channel may help to eliminate or reduce drug toxicity. Comparative studies should also be performed on the AmB/ergosterol channel; this will be the subject of a future project.

The structural information obtained in simulations was compared with the available experimental data. Our analysis emphasizes structural properties that could be important for the functioning and stability of the AmB/sterol channel in its open state and for its formation. The chemotherapeutic implications of our results are also discussed. In particular, we comment on why certain previous modifications of the amino and carboxyl group of AmB might have led to lower antibiotic activity or higher selectivity. Similarly, the roles of some other groups that contribute to the stability of the channel are pointed out. Modifications of such groups might be used in future pharmaceutical investigations.

Materials and Methods

Molecular models. Our model for the AmB/cholesterol channel was built based on suggestions from the previous studies (4, 14). The

channel consists of eight AmB and eight cholesterol molecules. Other stoichiometries were also considered at the initial stages of the project, but we chose the model with eight pairs of molecules because it gives the most compact structure if the channel diameter is kept within the range of 7–10 Å. The SLC was preferred over the DLC for a couple of reasons. First, it is a simpler and computationally less demanding model to investigate. Second, as discussed, it is biologically more relevant because under physiological conditions, antibiotic AmB molecules enter the cell only from one side (i.e., the external side).

Because the cell membranes in which AmB/sterol channels are formed contain phospholipids, two separate MD simulations using different membrane environments were performed. The MDSI used an SLC solvated in such a way that the inside and both entrances of the pore are occupied by water molecules. To mimic the lipid membrane environment, the second simulation (MDSII) also included one or two layers of phospholipids surrounding the AmB/cholesterol channel. Again, the inside and both sides of the pore contained water molecules, representing an aqueous solution. The lipids in MDSII were modeled with DMPC because DMPC has often been used in experimental membrane studies (25–27), as well as in theoretical studies (28–30), including the gramicidin membrane channel (31). In both simulations, all hydrogen atoms, polar or nonpolar, were explicitly included as interaction sites. The structure of the cholesterol was taken from a previous conformational study, and the conformer with an extended side chain (i.e., B3) was chosen (8). Similarly, the structure of AmB in its zwitterionic form was taken from an earlier work (32). Initial dihedral angle values for the amino sugar position were ϕ (C42/O19/C19/C18) = -58° and φ (C43/C42/O19/C19) = 126° .

In MDSI, a 1:1 AmB/cholesterol complex was built by optimally aligning the sterol with AmB. Then, through appropriate replication, a symmetric channel with eight AmB/cholesterol pair fragments was constructed. The formed channel was energy minimized for 500 steps of steepest descent followed by 1000 steps of conjugate gradient algorithms. The resulting AmB/cholesterol channel (Fig. 1, *bottom*) was then solvated with 504 water molecules in such a way that the pore was filled with water molecules and both sides of the channel had approximately five solvation layers. This soaking was achieved by immersing the channel in an equilibrated box of water molecules and then deleting the solvent molecules that were within 2.6 Å from any of the channel atoms and deleting the water molecules that were not within a certain distance of the ends of the channel. The water molecules on the sides of the channel that would otherwise be occupied by the membrane were also deleted. Locations of the incorporated water molecules were later optimized by keeping the channel molecules rigid for 200 and 500 steps of steepest descent and conjugate gradient, respectively. During the equilibration MD runs (see the next section), 86 water molecules that were only loosely bound broke away from the channel complex. These “evaporated” water molecules also were deleted during the equilibration runs. Thus, there were a total of 2950 atoms in the MDSI simulation. In the molecular simulations, the AmB/cholesterol channel and solvating water molecules were treated as an isolated complex (i.e., no periodic boundary conditions were applied). Although it is a simple setup, use of the explicit water molecules inside the pore and as several solvation layers on both ends of the channel, as well as leaving the lipid region empty, roughly approximates the membranous cell environment.

In setting up the second simulation (MDSII), phospholipids and additional water molecules were added by using an equilibrated box of solvated bilayer membrane containing 72 DMPC molecules (36 in each layer). The latter coordinates were taken³ from a 100-psec MD simulation (28). The use of lipid coordinates from a thermally equilibrated system enabled us to not repeat portions of the equilibration simulation runs (33). In Damodaran and Merz (29), nonpolar hydrogen atoms of the DMPC were modeled as part of the carbons to which

³ Alan J. Robinson, personal communication.

they are bonded (i.e., the united atom model was used for those hydrogens). These missing hydrogens were added by using the CHARMM program (34). This solvated box of phospholipids was then overlapped with the starting configuration of the MDSI simulation. DMPC molecules whose heavy atoms are within 2.0 Å of any of the channel heavy atoms as well as the DMPC molecules inside the channel pore were deleted. A criterion of the short distance of 2.0 Å was preferred to avoid deletion of too many DMPC molecules because the spatial extent of the lipid acyl chains can be quite large. Similarly, water molecules that do not overlap with the AmB, cholesterol, or lipid molecules and are within approximately the first five solvation layers of the channel or the lipids were kept and the remainder were removed. The system with the remaining 1,666 water molecules, 8 AmB molecules, eight cholesterol molecules, and 34 DMPC molecules (17 in each layer) had a total of 10,706 atoms. The resulting initial structure of the channel complex was subsequently optimized for 500 and 1,000 steps of steepest descent and conjugate gradient, respectively. During this structural minimization, only the lipid and water molecules were allowed to move.

Computations. All minimization and MD simulations were performed using the CHARMM molecular simulation program (34). Only the bond, angle, dihedral, van der Waals, and electrostatic terms were included in defining the interaction potentials. The dielectric constant $\epsilon = 2$ was used for all electrostatic interactions. Interaction force field parameters were taken from the CHARMM potential library version 24b1, except the AmB and cholesterol site charges were taken from an earlier work. As described previously (18), AmB and cholesterol site charges were obtained by finding the charge sets that give the best fits to the electrostatic potentials generated using the MOPAC'93 program (35). The TIP3P model (36) was chosen to represent the water molecules, and its parameters were taken from CHARMM force field version 24b1.

A group-based cutoff was used to truncate the nonbonding van der Waals and electrostatic interactions. Each AmB and sterol was treated as a separate fragment. DMPC molecules were partitioned into 28 fragments using the CHARMM program topology grouping scheme. In the CHARMM program, group-based truncations are determined using the distance between the molecular centers of the groups. To incorporate the interactions between any two AmB and sterol molecules, a very large cutoff distance of 25 Å was used. The truncation was implemented using a smoothing switch function starting from the distance of 22 Å. The nonbonding interactions were updated every 25 and 50 steps in MDSI and MDSII simulations, respectively. To allow larger time steps, hydrogen atom covalent bond lengths were restrained using the SHAKE algorithm. Constant pressure and temperature conditions were used. The time step was 2 fsec.

Before data collection, the simulation systems were equilibrated in several steps. The system in MDSI was first heated slowly to 350°K in 2 psec, which was followed by a short equilibration of 1-psec duration at this high temperature. The system was then cooled to 300°K in 1 psec and subsequently equilibrated for 20 psec at this temperature. From the very beginning until the end of this equilibration, AmB and cholesterol molecules were constrained to better equilibrate the water before equilibration of the channel structure. During this final 20-psec run, 86 water molecules broke from the system and drifted away. These molecules were deleted before the next equilibration stage. A second equilibration stage, which lasted 20 psec, was run without any constraints. The data collection MD simulation was run for 50 psec, and trajectory was saved at 0.1-psec intervals for later use; overall, 500 snapshots were used in the analysis.

To maintain an adequate solvation pattern and keep the phospholipids in an appropriate membrane shape, cylindrical and planar restraints were imposed on the water molecules and lipids in the MDSII simulation; harmonic mean field restraint potentials as implemented in the CHARMM program were used for this purpose. An imposed mean field potential with a force constant of 30 kcal/mol

restrained the water molecules to stay inside a symmetrically placed cylinder with a diameter and height of 27 and 55 Å, respectively. Similarly, DMPC molecules were restrained with a force constant of 30 kcal/mol to stay within a cylinder with a 30-Å diameter. In this case, no height restraint was applied. The starting structure in MDSII was heated to 300°K in 2 psec, and then the lipid and water molecules were equilibrated for 32 psec while AmB and cholesterol molecules remained fixed. Later, all molecules were allowed to move, and another equilibration simulation was run for 60 psec. The final data collection run lasted 60 psec, during which configurations were saved at every 0.2 psec; thus, 300 structures were recorded for analysis of the results.

The time scale of our simulations, 60 psec, is much too short to allow observation of major changes, such as the formation or destruction of the channel or any large conformational changes. To observe such changes, the simulation should be ≥ 1 order of magnitude longer. Unfortunately, it is a large and complicated system, so the cost of running a simulation of this time scale would be at the order of 1000 CPU hr on 16 nodes of Cray T3D; such a high cost makes it practically impossible. However, the time scale of our MD simulations is sufficiently long to allow the study of certain structural properties of the AmB channel at the microscopic level. In this respect, what is reported here is the simulation of an already formed open channel that has a diameter sufficiently large (7–8 Å in this case) to transfer ions and small molecules (16). This, however, does not necessarily mean that the channel also accommodates a “closed form” and switches periodically between the open and closed forms, as is observed for many protein channels. Only long simulations would be able to show that the channel may have more than one state and switches among them.

The MDSI and MDSII simulations were performed on SGI R4400 and Cray T3D computers, respectively. In the latter case, 16 nodes were used. The final MDSII 60-psec simulation took ~60 CPU hr on Cray T3D.

Results

We focused our analysis of the MD simulation trajectories on investigating which intermolecular and intramolecular structural and dynamic properties of the AmB channel are important for the channel stability and its functional behavior. The MDSII simulation explicitly includes phospholipids and thus better represents the physical environment; results of this simulation are given. The results of MDSI are very similar to those of MDSII; existing differences are pointed out, and possible reasons for the differences are discussed.

Intermolecular properties. Because the sterols are required in the formation of AmB channels, intermolecular interactions of AmB molecules with other AmB molecules and with sterols are expected to be crucial for the stability of the channel. Visual inspection of the MD trajectories revealed that although the steric restraints are important, it is mainly the hydrogen bonding interactions that keep the channel intact.

Hydrogen bonds. On average, ~75% of the AmB/O8 hydroxyl groups form intermolecular hydrogen bonds with either O9 or O5 hydroxyl groups of the neighboring AmB molecules. The involvement of O8 hydroxyl groups in an intermolecular hydrogen bond chain (Fig. 2) is due to their favorable orientation, which is opposite the other hydroxyl groups in the polyhydroxyl chain (Fig. 1, *top*). This allows suitable alignment of this hydroxyl group toward the O9 or O5 atom of the adjacent AmB. As discussed below, all hydroxyls of the polyhydroxyl fragment either are involved in



Fig. 2. System of hydrogen bonds between some selected groups showing snapshot from the MDSII simulation after 20 psec. 51, Nitrogen atoms in the amino group; 53 and 54, oxygen atoms in the carboxyl group; 50, oxygen atoms in the O43 hydroxyl group; 55, oxygen atoms in the O15 hydroxyl group; 59–61, oxygen atoms in the O9, O8, and O5 hydroxyl groups, respectively.

forming intramolecular hydrogen bonds or interact with solvent molecules.

There also are intermolecular hydrogen bonds between the amino group of an AmB molecule and the carboxyl group of the adjacent AmB molecule. These hydrogen bonds form a hydrogen bond ring at the channel entrance on the extracellular side of the membrane (Fig. 2). This hydrogen bond chain is formed by seven of the eight AmB molecules (on average). It is very stable and exists throughout almost the entire simulation period. In MDSI, the equivalent chain is formed by all eight AmB molecules. In MDSII, one AmB molecule (on average) does not take part in this hydrogen bond ring and instead interacts with a nearby lipid molecule. Close ionic interaction of this AmB with DMPC takes place through the positively charged amino group of AmB and the negatively charged phosphate group of DMPC. While this happens, the trimethylammonium group of the same DMPC molecule interacts with the carboxyl group of the next AmB molecule, which also has an ionic character. Additional hydrogen bonds were observed between the O15 hydroxyl group of an AmB molecule and the O43 hydroxyl group in the sugar moiety of its neighboring AmB molecule. There were an average of five or six such hydrogen bonds. These hydrogen bonds have a typical O-to-O distance of 2.7 Å and form, to some extent, a secondary hydrogen bond chain at the entrance to the channel, further stabilizing the complex structure.

Although there are occasional hydrogen bonds between the hydroxyl group of cholesterol and the amino or carboxyl group of AmB, these are rather weak hydrogen bonds that do not make any significant contribution to the structural stability.

Fig. 3, *top*, provides the percentage of structures with a certain number of intermolecular hydrogen bonds between any two AmB, cholesterol, or lipid molecules. In analysis of the number of bonds at a given configuration, a pair was assumed to be hydrogen bonded if the distance between acceptor and donor atoms was <2.7 Å and the angle of the donor-hydrogen acceptor plane was $>140^\circ$. This is a somewhat strict criterion for defining hydrogen bonds and considers only the bonds that are strong or of medium strength.

Molecular structure of the channel. Visual analysis of the channel complex revealed that the smallest limiting di-

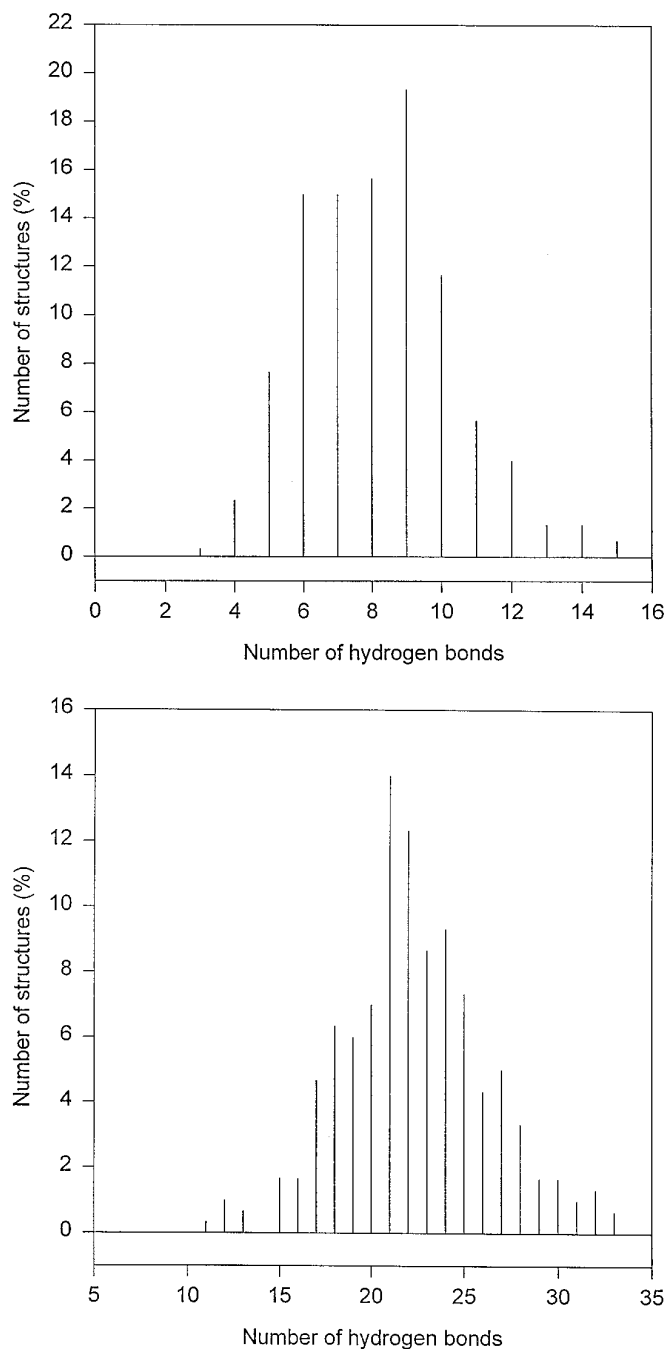


Fig. 3. *Top*, intermolecular hydrogen bonds in the channel complex, MDSII simulation. *Bottom*, channel-water hydrogen bonds, MDSII simulation. *Percentage of structures*, the total of the 300 snapshots obtained during the trajectory.

iameter of the channel pore is defined by the O8 and O3 hydroxyl groups of AmB. However, it should be noted that the channel is not quite cylindrically symmetrical (Fig. 4) and looks slightly elliptical. Judging from the closest distances between O8—O8 and O3—O3 hydroxyls of the oppositely located AmB molecules and from the pore solvent cross section, we estimated the diameter of the channel to be 8.5–10.5 Å. The time dependence of this diameter (defined as the distance between two opposite AmB/O8 hydroxyls) is shown in Fig. 5.

The AmB molecules move significantly (≤ 4 Å) in the direction perpendicular to the membrane, and their alignment is not necessarily at the same planar level. In addition, the vectorial direction of the pore axis fluctuates significantly as a function of the simulation time. The overall shape of the channel complex closely resembles a barrel formed by twisted and interlocking AmB molecules (Fig. 6). Molecular alignment of AmB molecules with respect to each other has almost a regular up-and-down pattern. This crown-type relative positioning of AmB molecules helps to establish the highly favorable intermolecular hydrogen bond ring formed by the O8 and O9 or O5 hydroxyl groups of adjacent molecules. As discussed, this hydrogen bond chain stays intact for almost the entire simulation and seems to be crucial in keeping the channel in its open state. Shifted placement of AmB molecules aligns the amino and carboxyl groups of two adjacent molecules in an orientation favoring the hydrogen bond formation between the two groups.

Cholesterol molecules also are very mobile and rotate $\leq 90^\circ$ about their long axis. The two simulations differed in this rotational motion of cholesterol molecules; sterol molecules

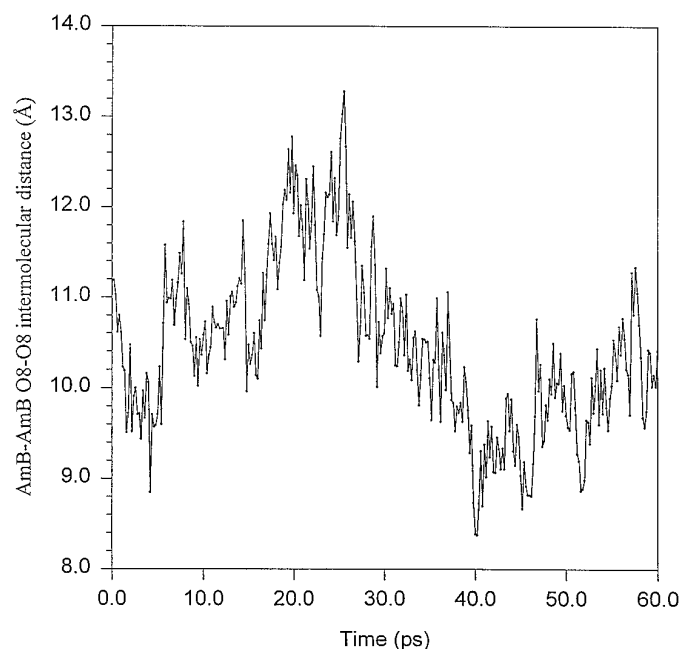


Fig. 5. Distance between two O8 hydroxyl groups of the opposite AmB molecules determines the approximate channel diameter.

were observed to rotate more often in the MDSI. This difference is due to the absence of phospholipids in MDSI. On the basis of the frequency of sterol rotations (and considering for MDSII the equilibration run lasting 60 psec as well as the data collection run), the estimated time scale of the cholesterol rotations about their long axes were 25 and ~ 100 psec

Fig. 4. A typical snapshot of the channel as viewed from the extracellular side, MDSII simulation, showing nonsymmetric structure of the pore. *Green*, AmB carbon atoms; *red*, oxygen atoms; *blue*, nitrogen atoms; *white*, hydrogen atoms; *magenta*, cholesterol molecules; *dark blue*, phospholipid molecules. For clarity, water molecules are not shown.

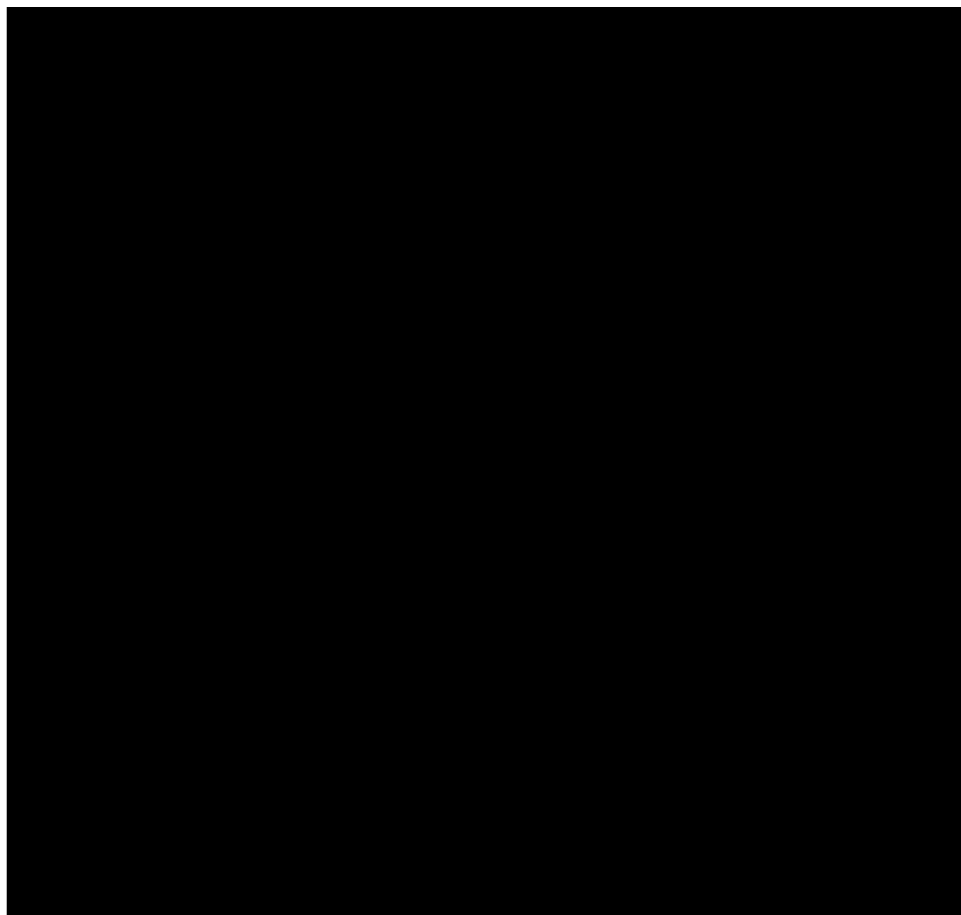


Fig. 6. Typical view of the channel; projection from the membrane side. Green AmB; magenta, cholesterol; red and white triangles, water molecules. For clarity, lipid molecules are not shown.

for MDSI and MDSII, respectively. Because the lipid environment was not incorporated, the MDSI result for this particular property should be taken with caution. An upper bound for the correlation time τ of the wobbling motion of cholesterol around its long axis was measured experimentally using NMR spectroscopy as $\tau \ll 10^{-9}$ sec = 1000 psec (37). The agreement with experiments for the estimated time scale of cholesterol rotational motion and the observed swinging-type rotations of cholesterol from $+90^\circ$ to -90° make us believe that regardless of its simplicity, the membrane model formed by one or two layers of phospholipids was fairly successful in mimicking the physical membranous environment.

The cholesterol molecules rotate quite freely, and there does not seem to be a specific way in which they interact with other molecules. Cholesterol molecules interact simultaneously with both AmB and lipid molecules, and it appears that their major purpose is to shield the interaction between the AmB channel and phospholipids. This interaction-buffering behavior of cholesterol was postulated previously on the basis of NMR data (27). MD simulations also showed that cholesterol molecules are rigid up to their side chains and rotate around their main axis as a rigid plane. Only the tail of the side chain of cholesterol extending from C22 (see Refs. 8 and 38 for carbon atom numbering) was observed to have a small bending motion. There is similar experimental evidence by Dufourc *et al.* (39), who reported the rigidity of cholesterol up to its C22—C24 atom.

Solvation of the pore. The AmB molecules are oriented such that the hydroxyl groups form the pore surface. There

are ~ 75 water molecules in the cylindrical part of the channel. If, as above, a hydrogen bond is defined as having a bond distance of ≤ 2.7 Å and a bond angle of $\geq 140^\circ$, there are 23 ± 7 hydrogen bonds between AmB and water molecules. These bonds are present during the entire MDSII simulation (Fig. 3, *bottom*). It was observed that water molecules quite freely enter and leave the channel. For example, during the 60-psec MDSII run, ~ 15 -pore water molecules were exchanged with the bulk water. Such high mobility of water molecules is due to the facts that as reflected in sizable fluctuations, the AmB channel is not very restrictive, and because the pore diameter is large (~ 10 Å), the pore water molecules can retain their bulk properties to a substantial degree.

Role of the lipids. In our simulations, only a thin layer of lipids was used. Therefore, the interaction between AmB and DMPC lipid molecules cannot be studied quantitatively, and the results must be used with caution. However, several interesting features were observed that might be responsible for the channel formation or destruction. Because the distance between the amino and carboxyl groups of AmB and the distance between the phosphate and amino groups of DMPC are almost equal, AmB and DMPC molecules can form different yet energetically favorable configurations. As a result, a DMPC molecule either interacts directly with one AmB molecule, or it can act as an electrostatic bridge in the AmB/DMPC/AmB configuration. Therefore, one might expect that AmB molecules would be anchored to the membrane surface through individual electrostatic interactions with phospholipids, and when the AmB concentration exceeds a

certain level, the bridging configuration becomes favorable and the channel starts to form. Such bridging configurations were actually observed in our simulations. By acting like a structural buffer, cholesterol molecules screen the interaction between AmB and DMPC molecules. This may slow down the AmB lateral migration or across the membrane and gives them the opportunity to regroup to form a channel. One may expect the observed interactions between AmB and DMPC molecules to not only help create the channel but also be responsible for destruction of the channel. Depending on the movement and positioning of the cholesterol molecules, either the formation of an active open channel is favored or an operational channel is destroyed.

Intramolecular properties. Because the construction and functioning of the channel depend on the dynamic behavior of constituent molecules, we also investigated the structural intramolecular properties of AmB and cholesterol.

Position of the AmB amino sugar. The amino sugar of AmB is the most flexible fragment of the antibiotic due to allowed rotation around C19—O41 and O41—C42 bonds. The amino sugar is positioned with regard to the aglycon part of AmB such that typical values of the dihedral angles ϕ and φ (Fig. 1, *top*) were -90° and $\pm 180^\circ$, respectively. Distribution of the ϕ and φ angles extracted from the MDSII trajectory for all eight AmB molecules is presented in Fig. 7, *top*. The angle (ϕ , φ) distribution was very similar in the MDSI simulation. This spatial positioning of the amino sugar helps to preserve the intermolecular AmB/AmB hydrogen bonds between the amino group of one AmB molecule and the carboxyl group of another AmB molecule or between hydroxyl group O43 of one AmB molecule and hydroxyl group O15 of another AmB molecule (Fig. 2). It should be mentioned that the position of the amino sugar is slightly different than that found crystallographically or through molecular simulation of an isolated AmB molecule and its derivative (11, 39). The current conformation was predicted in a previous detailed conformational analysis of AmB (32), but data from different sources are not in conflict. As in its crystal state (39), an isolated AmB molecule tends to form an intramolecular hydrogen bond between amino and carboxyl group with $\phi = -88^\circ$ and $\varphi = 142^\circ$. In contrast, in the channel complex, an AmB interacts with its AmB neighbor and forms intermolecular hydrogen bonds using the same polar groups. In this case, the position of amino sugar is different, but its conformation in the channel complex is still sterically allowed (32).

Conformational change in the polyhydroxyl part of AmB. The hydroxyl groups in the polyhydroxyl part of AmB can form intramolecular hydrogen bonds that can stabilize the all-*trans* structure of the entire carbon atom chain (Fig. 1, *top*). However, this uniform polyhydroxyl chain configuration is altered at C6 and C7. Our simulations showed that the chain can flip around the C6—C7 bond. Typical dynamic behavior of the dihedral angle defined by four carbon atoms (C5, C6, C7, and C8) is presented for two different AmB molecules in Fig. 7, *bottom*. The conformational change shown in Fig. 7, *bottom* (solid line), is related to the formation of intramolecular or intermolecular hydrogen bond; when C5/C6/C7/C8 dihedral angle is close to $\pm 180^\circ$, the O8 hydroxyl group can form intermolecular hydrogen bond with either the O9 or O5 hydroxyl group of the neighboring AmB molecule. In contrast, when this dihedral angle is -90° , an intramolecular hydrogen bond between O8 and O9 hydroxyl

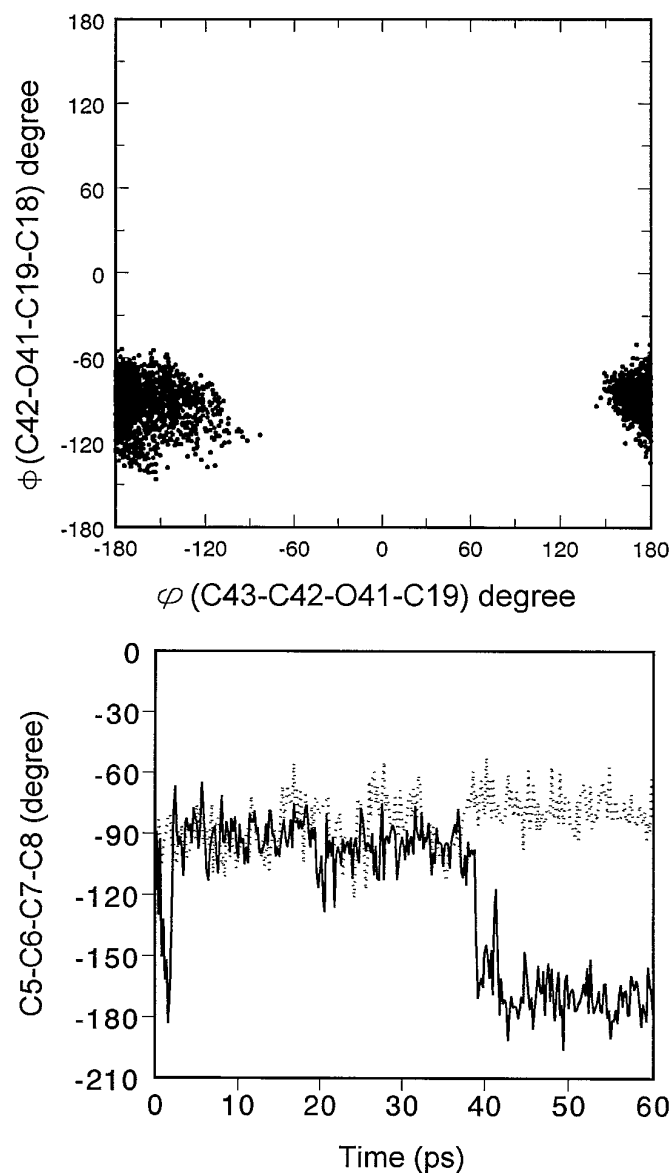


Fig. 7. *Top*, distribution of the ϕ (C42/O41/C19/C18) and φ (C43/C42/O41/C19) dihedral angles defining the orientation of the amino sugar with respect to the macrolide ring of AmB (see Fig. 1, *top*). Data are given for all eight molecules. Starting at ϕ , φ angles were -58° and $+126^\circ$, respectively. *Bottom*, dynamic behavior of C5/C6/C7/C8 dihedral angle of an AmB molecule for two different AmB molecules. The starting value of this dihedral angle (recorded before the equilibration stage) was 179° .

group is formed. For some of the AmB molecules, this torsional angle changed from *trans* to *gauche* during the equilibration stage of the simulation. Data in Fig. 7, *bottom*, show that this change is reversible, and the time scale of such conformational flip is ~ 100 psec.

Twisting of the conjugated double-bond system. The aglycon ring is the most rigid part of AmB. This rigidity is due mainly to the presence of seven conjugated C=C double bonds in the macrolide ring of AmB. However, this long conjugated system is not very planar, and it can twist. It was found in the MDSII simulation that the improper dihedral angle defined by C20, C25, C28, and C33 fluctuates and differs from ideal planarity by $\sim \pm 30^\circ$. The flexibility of this

conjugated double-bond system can be important for the interaction of AmB molecules with sterol molecules.

Discussion

Two MD simulations for a particular model of the AmB channel were performed to study the molecular properties of the channel in its open state. These simulations revealed certain structural and dynamic properties of the channel at the molecular level. Our findings are summarized below.

When the diameter of the AmB channel is kept in the range of experimental estimates (16), the channel formed by eight AmB and eight cholesterol molecules is stable, due mainly to strong hydrogen bonding interactions. On the basis of an analysis of the shape of the pore and its nonsymmetric behavior, it can be postulated that the stoichiometry proposed by de Kruijff and Demel (4) is not necessarily the only possibility. Channels formed by fewer than eight molecules can also exist. Even though the channel with seven AmB molecules would be the most likely alternative, because adjacent AmB molecules are shifted along each other, channels with an even number of AmB molecules have the advantage of forming a proper up-down-up crown-type structure. Therefore, with the current level of knowledge, six to eight would be the best answer to the question of how many AmB molecules are involved in the channel formation.

The existence of the intermolecular AmB/AmB hydrogen bonds in the central part of the channel was proposed by Khutorsky (14) and supported by our investigation of the dynamic behavior of these bonds (Fig. 2). It was found that the out-of-plane O8 hydroxyl group is responsible for the formation of the intermolecular bonds between AmB molecules. This intermolecular hydrogen bond is formed when adjacent AmB molecules are mutually shifted along their vertical pore axis. In addition, it was found that the formation of this bond correlates with the conformational change in the C6—C7 bond. Taking into account all the data, it can be stated that formation of intermolecular hydrogen bonds by O8 hydroxyl groups contributes to the stability in the central part of the channel.

The chain of hydrogen bonds between amino and carboxyl groups was also postulated previously (14). The results of the current study further support the finding that this hydrogen bond chain is very stable. These observations of the hydrogen bonding patterns are very important for chemotherapeutic studies because they indicate which amino and/or carboxyl group modifications might have pharmaceutical importance (7). The MD simulations further revealed that it is, again, the strong hydrogen bonding interaction between amino and carboxyl group that stabilizes the channel complex at the pore entrance. This observation helps to explain experimental data showing poor activity of some AmB derivatives with the modified amino group (19). Based on the current simulations, it can be explained that the derivatives of AmB with blocked amino groups cannot form appropriate hydrogen bonds that stabilize the channel structure and therefore a durable channel cannot be formed. Actually, the experimental data (19) suggest that substitution of the carboxyl group and alkylation of the amino group and/or shift of this group improve antifungal versus hemolytic selectivity. The result of our calculations can explain these experimental findings. When the carboxyl group is blocked [e.g., methyl ester or propyl

amide derivatives of AmB (19)], the amino group may still form intermolecular hydrogen bonds with other polar groups (e.g., the hydroxyl group O15 of the neighbor AmB molecule). When the amino group is shifted or substituted [e.g., dimethylglycyl or trimethyl derivatives of AmB (19)], the bulky substituents limit the hydrogen bond formation. Such suggestion may be derived from the analysis of mutual orientation of the AmB molecules (Fig. 2) and the trajectory animation of the channel (not shown). For these types of AmB derivatives, hydrogen bonds formed by the altered amino group are not as strong as the bonds formed between free amino and carboxyl groups of the neighboring AmB molecules. These weaker hydrogen bonds probably make the structure of the AmB/cholesterol channel less stable than the AmB/ergosterol channel that results in the improved selectivity; to confirm this, comparable AmB/ergosterol channels must be studied. Nevertheless, as discussed above, results of our simulation readily explain the observed effects of various modifications of AmB undertaken in other laboratories. The goal was to make new AmB derivatives with bulky substituents at the amino group that should still be able to form weak hydrogen bonds. These efforts seem to be very promising. A new, much more selective second generation of derivatives were obtained and tested (40).

Another modification worth testing would be to make ion channels composed of amphotericin B dimers formed by covalently bonding the amino group of one monomer with the carboxyl group of the other.⁴ This might be done using appropriate-sized linkers rather than directly bonding the groups, to allow freer molecular movement. The biophysical and biological studies in membranes with such dimers could show whether the channel forms faster or at lower concentrations. Such an experiment may confirm the mutual orientation of the amino and carboxyl groups in the channel, for which we derived at estimates on the basis of the current simulation.

A second chain of hydrogen bonds between hydroxyl groups O15 and O43 belonging to neighboring AmB molecules was detected at the entrance to the channel. These hydrogen bonds also contribute to the stabilization of the channel. Elimination of these bonds may disturb the structure of the AmB/cholesterol channel and help to reduce AmB toxicity, providing the same modification does not influence stability of AmB/ergosterol channel. This information can be very useful from a chemotherapeutic point of view. The hydroxyl groups O15 and O43 have not been modified previously, and this prediction of the simulations that indicates that they are new "hot spots" can be further tested experimentally. Concerning the interactions between AmB molecules in the channel, we suggest eliminating these hydroxyl groups or substituting related (e.g., methoxy or methyl) groups. However, we are aware that selective modification of the hydroxyl groups involves a very difficult chemical synthesis task. Other AmB hydroxyl groups are very similar; probably only through total synthesis from fragments can AmB derivatives be prepared with modified hydroxyl groups in certain positions.

The MD simulations also give information about the locations of the amino sugars that cause adjacent polar groups to reorient themselves to form as many hydrogen bonds as

⁴ This suggestion was made by an anonymous reviewer, to whom we are grateful.

possible. Torsional angles defining the position of sugar moiety agree well with previous studies (32). Moreover, it was found that these dihedral angles have different values in an isolated AmB molecule and in an AmB molecule that is part of the channel complex.

Analysis of the molecular simulation results shows that the cholesterol molecules do not interact with AmB molecules in any specific way; therefore, it can be stated that cholesterol is a rather nonspecific target for AmB. This property of cholesterol has been measured experimentally through NMR (27). The hypothesis given by Herve *et al.* (20) that predicts a specific interaction of cholesterol hydroxyl group with AmB amino or carboxyl groups is not supported by the current results. Thus, the explanation of why the affinity of AmB molecules to cholesterol is lower than that to ergosterol requires similar studies for the AmB/ergosterol/DMPC system. In addition to the theoretical simulations, the NMR solid state experiments can be undertaken; with NMR solid state measurements (27), comparative studies can be performed of AmB/cholesterol and AmB/ergosterol channels (in liposomes or lipid bilayers) to determine differences in mobility and orientation of both sterols along the AmB molecules in the channel, which can be simulated later using theoretical approaches. State-of-the-art NMR experiments can also be expected to determine the orientation of the AmB molecules in the channel. The latter results would support or reject the molecular simulations data.

The results of the AmB channel molecular simulations suggest that it is mainly the hydrogen bonding interactions that keep the channel in its open state. Results regarding the rotational motion of cholesterol and the lack of any specific interaction between cholesterol and AmB agree well with available experimental data. Our analysis also pointed out several hydroxyl groups that can have a role in the functioning of AmB, and we presented a hypothesis for the role of membrane phospholipids.

Our study is only a small step toward resolution of this complex system. The current understanding of the AmB/sterol channel and its molecular properties is only at a qualitative or semiquantitative level. Many important questions in this field concerning the structure and properties of the channel are unanswered; future experimental and molecular modeling studies are needed for a more comprehensive understanding.

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