Chapter 2

Hybrid In Silico/In Vitro Approaches for the Identification of Functional Cholesterol-Binding Domains in Membrane Proteins

Coralie Di Scala and Jacques Fantini

Abstract

In eukaryotic cells, cholesterol is an important regulator of a broad range of membrane proteins, including receptors, transporters, and ion channels. Understanding how cholesterol interacts with membrane proteins is a difficult task because structural data of these proteins complexed with cholesterol are scarce. Here, we describe a dual approach based on in silico studies of protein–cholesterol interactions, combined with physico-chemical measurements of protein insertion into cholesterol-containing monolayers. Our algorithm is validated through careful analysis of the effect of key mutations within and outside the predicted cholesterol-binding site. Our method is illustrated by a complete analysis of cholesterol-binding to Alzheimer's β-amyloid peptide, a protein that penetrates the plasma membrane of brain cells through a cholesterol-dependent process.

Key words Alzheimer's β-amyloid peptide, Cholesterol-binding motif, Langmuir monolayer, Molecular docking, Molecular dynamics simulations, Transmembrane domain

1 Introduction

Among eukaryotic membrane lipids, cholesterol (Fig. 1) is unique for several reasons. In contrast with other membrane lipids, which contain one (sphingolipids) or two (glycerophospholipids) acyl chains, whose variability may generate a high degree of biochemical diversity, cholesterol has only one molecular structure [1]. It contains two structural elements that are not found in other membrane lipids, i.e., carbon rings (the sterane backbone) and branched aliphatic groups (methyl and iso-octyl). The asymmetric distribution of these chemical groups defines two topologically distinct surfaces of the cholesterol molecule: one with reliefs, referred to as the "rough" face, and the other one devoid of this roughness, referred to as the "smooth" face (Fig. 1). According to the nomenclature of cyclic compounds proposed by Rose et al. [2], the smooth and rough faces are respectively identified as the α and β faces [1, 3].

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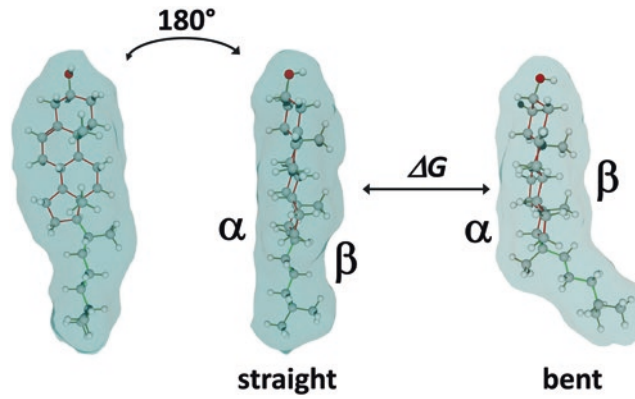

Fig. 1 Structure and conformational flexibility of cholesterol. In textbooks, cholesterol is often represented in such a way that the four rings of the sterane backbone are clearly visible (left panel). In this case, it is not possible to assess the distinct topologies of the α (smooth) and β (rough) faces. A 180 ° rotation of the molecule (middle panel) unmasks the two faces of cholesterol. Note that the iso-octyl chain of this particular conformer is not tilted with respect to the main axis of cholesterol, giving the molecule a "straight" structure that is compatible with an interaction with membrane lipids. Upon protein binding (a process that can be quantified by a variation in free energy, ΔG), cholesterol may adopt a "bent" shape due to the rotational flexibility of the iso-octyl chain (right panel)

Apart from its bifacial geometry, cholesterol has unexpected conformational flexibility properties that are conferred by the rotational movements of the carbon–carbon bonds at the level of the iso-octyl chain. Schematically, two types of cholesterol conformers have to be considered for studying protein–cholesterol interactions at the molecular level [4]. As shown in Fig. 1, these conformers differ by the angle between the sterane unit and the iso-octyl chain, which defines either "straight" or "bent" structures. Straight conformers are particularly adapted for interacting with the apolar part of sphingolipids, whereas bent conformers are generally bound to a membrane-spanning protein [5].

Finally, the amphipathic nature of cholesterol, with its polar OH group at one end and the iso-octyl group at the opposite, suggests a preferential orientation of the cholesterol molecule within a lipid bilayer, i.e., parallel to bulk membrane lipids with the OH group facing the polar-apolar interface. This thermodynamic constraint facilitates the search for a fit between cholesterol and a membrane-embedded domain of the studied protein because it significantly restricts the possibilities of forming a biologically relevant complex.

In this chapter, we describe a procedure for the prediction of a cholesterol-binding site on Alzheimer's β-amyloid peptide (Aβ). The choice of this particular protein is motivated by the fact that it lacks any predictable cholesterol-binding motif based on amino

acid sequences such as CARC or CRAC motifs [3]. Therefore, the molecular modeling study in this case has to start from zero ("ab initio" modeling). Nevertheless, we will also give some clues for generating a cholesterol-protein complex based on the detection of a consensus cholesterol-binding motif. Finally, we will describe the experimental procedure used in our laboratory for checking the validity of the models obtained in silico.

2 Materials

2.1 Computers, Websites, and Softwares

For modeling studies, we suggest using a high performance gaming computer (either Mac or PC) with a large HD monitor, a good video card, and at least 8 GB RAM. The websites that we regularly use are UniProt (http://www.uniprot.org) for protein sequence data and the Protein Databank (http://www.rcsb.org) for 3D structures. The software packages used for molecular modeling, structure analysis, and visualization are Hyperchem Professional (Hypercube, Inc., Gainesville, FL), DeepView - Swiss-PdbViewer (http://spdbv.vital-it.ch), and Molegro Molecular Viewer (CLC bio, Waltham, MA, http://www.clcbio.com). The surface pressure data were analyzed with the FilmWare X program (Kibron Inc., Helsinki, Finland). We developed our own software (NTB extractor) for transferring the FilmWare data (.ntb) to Microsoft Exel (.xls). The graphs are generated using Origin (OriginLab Corp., Northampton, MA).

2.2 Langmuir Trough

Surface pressure measurements are performed with a microtensiometer specifically designed for small working volumes (800 μL of the aqueous phase in which the protein or peptide is diluted), the MicroTroughX (Kibron Inc., Helsinki, Finland). A simple but reliable homemade setup for measuring surface tension has also been described by Fantini and coworkers [6].

3 Methods

3.1 Cholesterol Modeling

1. The first step is to obtain a workable file for the cholesterol molecule. Whatever the modeling program used, it should accept **.pdb** files, so that you can download the cholesterol molecule from the Protein Data Bank (cholesterol as ligand of cholesterol-binding protein) or by searching "cholesterol molecule pdb" in Google. The other solution is to generate cholesterol ab initio with your modeling software, but this may be painful because there are several asymmetric carbons that require special attention. As an example we have used the Swiss-PDB viewer program to extract a cholesterol molecule

from the PDB file 3D4S (cholesterol bound to the human β2-adrenergic receptor) [7].

2. Go to the 3D4S entry of the ProteinDatabank. Download the file in pdb format and save it on your computer desktop.
3. Open Swiss-PDB viewer, then open the 3D4S file. In the control panel window, you have the list of all amino acid residues and ligands. At the end of the list, there are two cholesterol molecules noted CLR402 and CLR403. You can create a .pdb file with one of these cholesterol molecules, e.g., CLR403. To do so, you can select all listed items other than CLR403 and delete these items with the "Remove selected residue" command of the "Build" menu. Then you just have to save the file now containing only CLR403 ("File" menu, "Save," "Current layer"). At this point, you have a cholesterol.pdb file.
4. Open this file with Hyperchem. Check the cholesterol molecule for atom valence, double bond (ring B of sterane), and hydrogen atoms (Fig. 2). Correct the structure if necessary.
5. Start an energy minimization process. In the Hyperchem program, geometry optimization is achieved using the unconstrained optimization rendered by the Polak–Ribière conjugate gradient algorithm. A typical process is shown in Fig. 2. In starting conditions, the value of the gradient is 2.3 kcal/(Å mol). At the end of the process (termination condition), the gradient is <0.01. These conditions can be changed

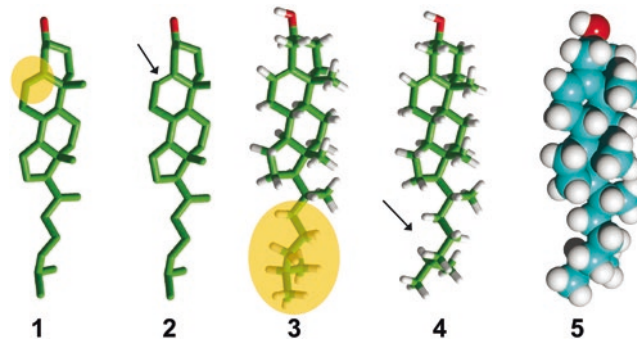

Fig. 2 Generating a workable cholesterol file. (1) Cholesterol downloaded from a PDB file (e.g., 3D4S). Note that it lacks the double bond (*orange disk*). (2) Cholesterol with the double bond (*arrow*). (3) Cholesterol with hydrogen, yet displaying a specific orientation of the iso-octyl chain (*orange disk*). (4) Cholesterol after geometry optimization with the Polak–Ribière algorithm (the change in the orientation of the iso-octyl chain is indicated by an *arrow*). (5) A sphere model of the cholesterol molecule shown in panel 4 (carbon in *blue*, oxygen in *red*, hydrogen in *white*). The molecule is viewed from the β face, in a typical "textbook" representation

in the software but are usually fine for small biomolecules such as cholesterol and peptides.

6. Save the file as a new file, e.g., "chol PR" (for cholesterol Polak–Ribière), not to be confused with the initial file you have downloaded from PDB or generated ab initio with Hyperchem. You can save this file with various extensions, but here we will use the pdb compatible **.ent** format. In this case, your file is named "**chol PR.ent.**"

3.2 Docking a Phenylalanine Tetrapeptide (Phe_4) onto Cholesterol: Looking for CH-π Stacking

1. As a first example of protein docking onto a simple protein motif, we will study the interaction of cholesterol with a minimal cholesterol-binding motif, e.g., a phenylalanine tetrapeptide (Phe_4). This modeling exercise will illustrate the process of formation of coordinated CH-π stacking interaction, a hallmark of protein–cholesterol interaction [8]. A workable structure of the Phe_4 tetrapeptide can be generated ab initio with Hyperchem by using the "Databases menu" and select four times the amino acid Phe. Apply the Polak–Ribière algorithm and save the file in the **.pdb** format.
2. Keep the Phe_4 file open and use the "Merge" function of the "File" menu of Hyperchem to insert cholesterol (**chol PR.ent**) in the same window. Now you can select cholesterol and Phe_4 independently.
3. Select the rendering method. For modeling purposes, "sticks" or "tubes" are suitable, but you may use the "sphere" rendition as well if you prefer. At this stage, you may also adjust the background (black or white) and the atom colors (carbon in green, oxygen in red, hydrogen in white).
4. Search for a potential geometric fit between Phe_4 and cholesterol. There are many possibilities for starting conditions, including totally random orientations. For instance, you can put two phenyl rings (Phe-2 and Phe-3) of the Phe_4 tetrapeptide onto the α face of cholesterol (Fig. 3a).
5. Apply the Polak–Ribière algorithm. The result is shown in Fig. 3b. Note that Phe-1 and Phe-4 are still in the same conformation, whereas the orientations of the phenyl rings of Phe-2 and Phe-3 have changed. In fact, both rings now form a flat structure that lies on the α face of cholesterol (Fig. 3c). The driving force of this process is the formation of CH-π stacking interaction between the first ring of sterane and the phenyl ring of Phe-3 (Fig. 3d). From this point, you can proceed for several rounds of molecular dynamics simulations to evaluate the robustness of this docking exercise. A typical example of molecular dynamics (MD) simulations of a protein–cholesterol complex with iterative snapshot and energy measurements has been published by Fantini et al. [9]. MD simulations

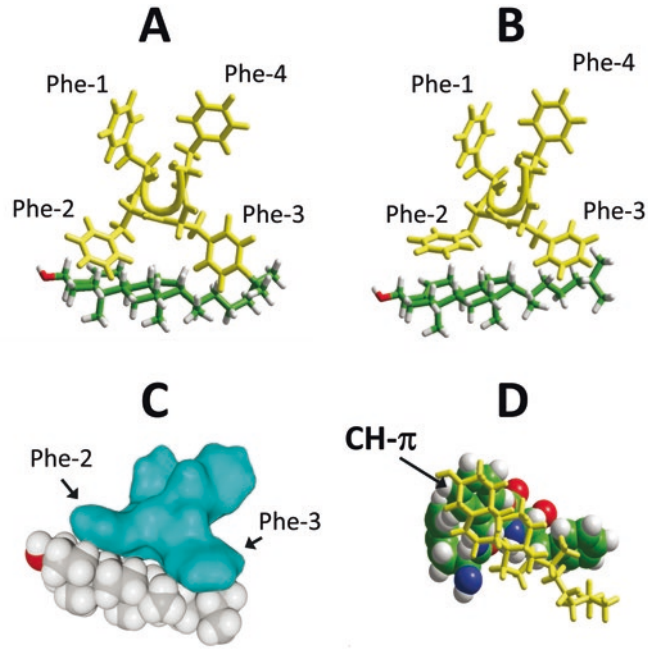

Fig. 3 Docking a phenylalanine tetrapeptide (Phe_4) onto cholesterol. (**a**) Starting conditions: the Phe_4 tetrapeptide is in *yellow* and cholesterol in atoms colors (carbon *green*, oxygen *red*, hydrogen *white*). (**b**) Obtaining a complex after applying the Polak–Ribière algorithm. (**c**) Evaluation of the surface of interaction between the Phe_4 tetrapeptide (in *blue*) and cholesterol. (**d**) Visualization of the CH-π interaction between the first ring of sterane (cholesterol in *yellow*) and the phenyl ring of Phe-2

are mandatory to check the robustness of the docking process before any experimental validation. Indeed, there are many examples of protein-ligand complexes obtained with docking programs that reached a high dock score but failed in MD simulations [10]. Such cases are particularly frustrating since the ligand literally "flies away" from its initial binding site as MD simulations are running. As emphasized by Chen in a recent review on potential docking caveats [10], the key "*difference between docking and MD is the variable, time.*" In essence, docking considers chiefly the binding affinity. In contrast, MD simulations calculate the movement of the complex and predict its evolution over the time. Unfortunately, due to hardware limitations, the simulation time of MD is usually less than 1 ms (and most often in the sub-ms range). Under these circumstances, further validation of docking results with appropriate bioassays is strongly recommended [10]. In the last part of this chapter, we will discuss how to assess the validity of in silico predicted protein–cholesterol interactions by experimental approaches.

3.3 Protein Modeling: Generating a α-Helix Structure from an Amino Acid Sequence

1. Open a window on your computer screen with the amino acid sequence. As an example we will study the 21–38 fragment of Aβ (21-AEDVGSNKGAIIGLMVGG-38).
2. Run Hyperchem. Use the "Databases" menu and the "amino acid" command to build the peptide fragment. Since you plan to generate an α-helix structure, check that "α-helix" is selected in the "Databases" window.
3. Once the peptide is built, apply the Polak–Ribière algorithm. Save the file.

3.4 Docking of Cholesterol onto a α-Helix (Aβ21–38)

1. Open the Aβ21–38 file with Hyperchem.
2. Merge with the cholesterol file (**chol PR.ent**).
3. Bring Aβ21–38 and cholesterol together in random or user-defined orientations and run the Polak–Ribière algorithm. You may try several possible starting conditions before you reach a good geometric fit. An example of a possible fit is illustrated in Fig. 4. In this case, the β face of cholesterol interacts tightly with the Aβ peptide. The binding process has significantly tilted the iso-octyl chain in a perfect example of a protein-bound cholesterol conformer. The amino acid residues that interact with cholesterol are chiefly Gly-25, Lys-28, and Ile-32 (Fig. 4). The identification of these residues is important for validating the model by physico-chemical approaches (i.e., test of mutant vs. wild-type peptides).

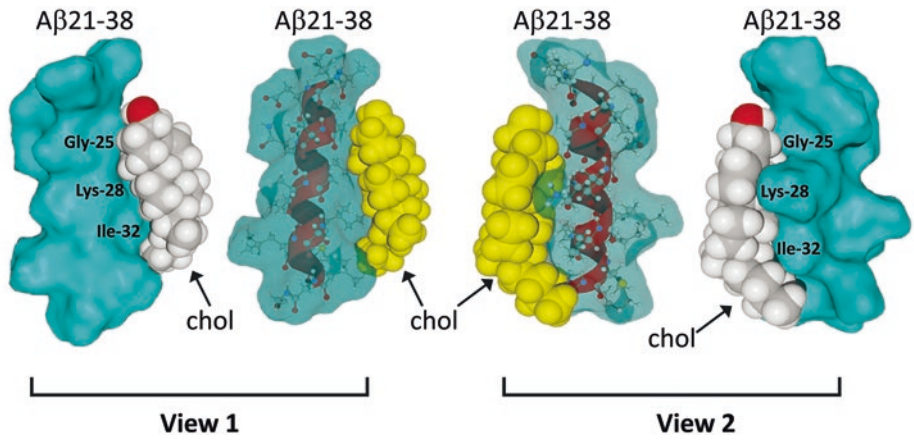

Fig. 4 Interaction of cholesterol with Aβ21–38. This model illustrates two major concepts of molecular interactions: geometry complementarity and chemical compatibility. Note that the β face of cholesterol interacts with the α-helical peptide. The complex is reinforced by the bending of the iso-octyl chain of the sterol which optimally spouses the peptide shape. Two distinct views of the complex are shown. In each case, a surface view is accompanied by a transparent rendition, allowing location of the α-helix (*red*) and the atoms of amino acid residues

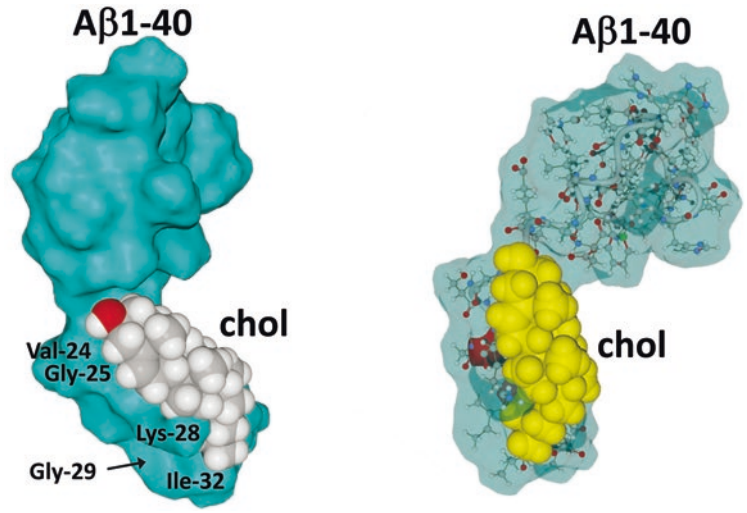

Fig. 5 Interaction of cholesterol with Aβ1–40. This model has been previously described by Di Scala et al. [4, 11, 14]. The location of Val-24, Gly-25, Lys-28, Gly-29, and Ile-32 is indicated on the model on the left. The model on the right is shown in a membrane compatible orientation with respect to cholesterol

3.5 Comparison of Data with a Published Model of the Aβ-Cholesterol Complex

The model of the Aβ21–38/cholesterol complex can be compared with published in silico studies of the Aβ/cholesterol interaction. As an example, we will analyze the data obtained with Aβ1–40 (Fig. 5). When merged with this longer peptide (compared with Aβ21–38), cholesterol spreads on a large region comprised between Phe-20 and Met-35 [11]. MD simulations of this complex allowed characterization of a very good fit, which involves a series of van der Waals interactions. Interestingly, the three amino acid residues of Aβ21–38 that were predicted to be in physical contact with cholesterol, i.e., Gly-25, Lys-28, and Ile-32, were also found to be important for cholesterol/Aβ1–40 complex [11]. In particular, in both cases the closest contact was with the methylene groups of Lys-28. Therefore, a first approach to validate both models is to assess the importance of this amino acid residue for the cholesterol/Aβ-binding reaction. For the sake of comparison, a residue that is not involved in the process (e.g., Gly-29) should be evaluated in parallel. We will now describe the way to measure the binding of cholesterol to wild-type and mutant Aβ peptides.

3.6 Langmuir Monolayer Technique

This technique is based on surface tension measurements of a simple system consisting of a lipid monolayer spread on the surface of a water phase [4, 6]. The surface tension of pure water is **72.8 mN/m**. When a surfactant (e.g., a lipid) is present at the water surface, it decreases the value of the surface tension proportionally to its amount. The surface pressure π is defined as the difference between γ_0, the surface tension of pure water, and γ, the surface tension measured in the presence of the surfactant:

$\pi = \gamma 0 - \gamma$. For instance, if a lipid monolayer decreases the initial surface tension to **56.3 mN/m**, the surface pressure for this monolayer is $\pi = 72.8 - 56.3 = 16.5$ **mN/m**. Increasing the amount of lipid molecules in the monolayer will further decrease surface tension, resulting in an increased surface pressure [12]. This rule applies as long as the monolayer is intact. If the area is maintained constant, the monolayer eventually collapses when the number of lipid molecules exceeds the available surface on water, resulting in a precipitous drop of surface pressure. For this reason, protein-lipid interactions measured this way are usually performed within the range of 10–30 mN/m. The injection of a protein (or a peptide) underneath a lipid monolayer induces an increase of the surface pressure when the protein (or the peptide) penetrates the monolayer. This process can be followed in real-time (kinetics studies) by dipping a platinum probe in the water bathing the monolayer [4].

1. Clean the platinum probe in the flame of a Bunsen burner (1 s) and hang it on its support.
2. Add 800 μL of ultrapure water into the tank, dip the microtensiometer probe at the air-water interface (about 1–2 mm is enough), and calibrate the apparatus to adjust the surface tension to 72.8 mN/m. Accordingly, the surface pressure π is 0 mN/m.
3. The purity of the aqueous subphase (pure water or buffer) can be assessed by following the surface pressure value over the time which should remain perfectly stable at the basal value of $\pi = 0$ mN/m.
4. Start again steps 1 and 2 and then inject 8 μL of peptide in the subphase to check its surfactancy. This control ensures that you are working with the appropriate concentration of peptide, and confirms that under these conditions, the molecule of interest does not modify the surface pressure by itself.
5. Start again steps 1 and 2 and spread a few drops (ideally less than 1 μL with a 10 μL Hamilton microsyringe) of lipid solution at the air-water interface. Wait 5 min for evaporation of the solvent. Check that the monolayer remains stable and note the initial surface pressure value (π_0).
6. Inject the protein or peptide (8 μL) in the subphase at the appropriate concentration. Do not worry that the needle of the microsyringe goes through the monolayer: once the needle is removed, the monolayer reseals instantaneously. Record surface pressure variations and note the final surface pressure value (π_{max}). The difference between the final and the initial surface pressure ($\Delta\pi_{max} = \pi_{max} - \pi_0$) is characteristic of the type of interaction. The kinetics of interaction of wild-type and mutant Aβ22–35 peptides with cholesterol monolayers are shown in Fig. 6.

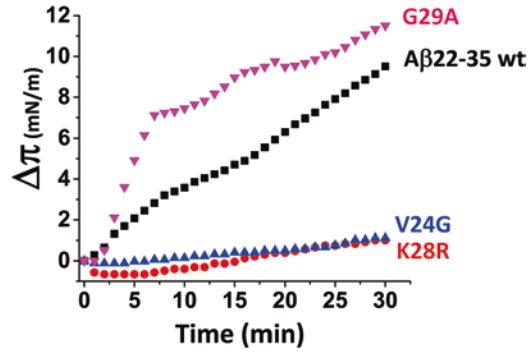

Fig. 6 Kinetics of interaction of Aβ22–35 wild-type (wt) and mutant peptides with cholesterol monolayers. Cholesterol monolayers were prepared at an initial surface pressure of 20 mN/m. After equilibration (5 min to allow solvent evaporation), the indicated peptide was injected underneath the monolayer at a concentration of 10 μM. The data show the evolution of the surface pressure as a function of time

In agreement with in silico studies, the K28R mutant did not interact with cholesterol. In contrast, a mutation at position 29 (G29A), which is not involved in cholesterol binding, had no inhibitory effect on Aβ–cholesterol interactions.

7. It is important to perform the experiments at various values of the initial surface pressure. In fact, the insertion of the protein into the lipid monolayer is expected to become more and more difficult as the initial pressure surface increases, i.e., with condensed monolayers containing a high number of lipid molecules. Indeed, the strength of lipid–lipid interactions is higher in a densely packed monolayer than in a loose monolayer. Thus, when the lipid-protein interaction is specific, the value of $\Delta\pi_{max}$ gradually decreases as π_0 increases. The extrapolated value of π_0 at $\Delta\pi_{max} = 0$ is referred to as the critical pressure of insertion π_c (Fig. 7). When the value of critical pressure of insertion is ≥30 mN/m (i.e., the mean surface pressure of the plasma membrane) the interaction is considered biologically relevant [12].

4 Notes

4.1 Docking Algorithms

Docking is becoming more and more popular, especially for drug screening and design. In a recent overview, Chen listed no less than 50 docking programs [10]. The strategy described in the present article does not use any of these programs. Instead, we propose an alternative process that combines both the search for an optimal protein-cholesterol fit and the possibility to run MD simulations with the same software. Our method takes into account the

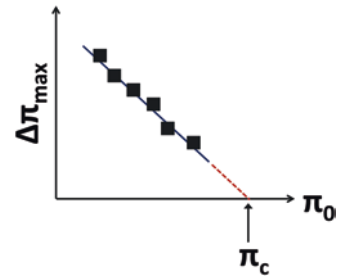

Fig. 7 Graphical determination of the critical pressure of insertion. Cholesterol monolayers are prepared at several distinct surface pressures (usually 10–30 mN/m). For each monolayer, the maximal surface pressure increase $\Delta\pi_{max}$ induced by the peptide (or the protein) is plotted against the initial surface pressure π_0. The critical pressure of insertion π_c is extrapolated as the theoretical value of π_0 at $\Delta\pi_{max} = 0$

mutual induced-fit mode of interaction, i.e., the conformational flexibility of both partners (protein and ligand). In our experience, the binding of cholesterol to a membrane protein generally proceeds through such mechanisms [3]. Since our method may lead to the characterization of several distinct cholesterol–protein complexes resulting from distinct starting conditions, it is of high interest to evaluate the affinity of each complex. The "ligand energy inspector" (Tools menu) of the Molegro Molecular Viewer software is a simple way to assess and compare the predicted energy of interaction of a series of molecular complexes. For each complex, the data are presented as a list of amino acid residues that physically interact with each atom of cholesterol [13, 14]. Finally, an important issue to consider is the environment of the ligand and the protein. The docking may be performed in vacuum to speed up the process, yet the introduction of water and lipid molecules is of course preferred, even if it will considerably increase the time of simulation, even at the docking step.

4.2 Consensus Cholesterol-Binding Motifs

In some cases, the membrane-spanning domain displays a consensus cholesterol-binding motif such as the CARC motif defined by the linear array (K,R)–X_{1-5}–(Y,F)–X_{1-5}–(L,V) according to Baier et al. [13]. The CARC motif is oriented in such a way that the OH group of cholesterol faces the cationic group of the basic residue (either Lys or Arg) of CARC, consistent with the establishment of a hydrogen bond [3, 12]. The aromatic residue may interact with one of the sterane rings of cholesterol through a CH-π bond (Fig. 3). Finally, the branched aliphatic residue of CARC (Leu or Val) may contact the iso-octyl chain of cholesterol, which could further stabilize the complex by a series of van der Waals interactions [3]. Overall, the basic principles that govern cholesterol binding to transmembrane domains fully apply to

CARC–cholesterol interactions. The search for a fit between cholesterol and a CARC motif is thus a good approach for testing in silico the biochemical logic of protein–cholesterol interactions, especially for membrane proteins.

4.3 Lipid Monolayer Assay

The Langmuir system has several advantages over other methods for studying lipid–protein interactions. On one hand, the actual molar ratio of lipids in the monolayer can be easily controlled. Accordingly, mixed monolayers containing several lipid species can be prepared. This point is important because in other reconstituted membrane lipid systems (e.g., liposomes or black lipid membranes), the lipid distribution in each monolayer is generally not determined. On the other hand, Langmuir monolayers can be probed with low protein amounts (nM–μM range) that may reflect in vivo conditions. Combined with in silico approaches, the Langmuir setup provides a robust and reliable method for studying lipid–protein interactions [12].

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