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G protein coupled receptor interactions with cholesterol deep in the membrane

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Abbreviations: CGMD, coarse-grained molecular dynamics; EC, extracellular; GPCR, G-protein coupled receptor; IC, intracellular; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TM, trans-membrane.

Highlights

Simulations detect interactions between cholesterol and GPCRs deep in the membrane

These interactions change on agonist binding

Requirements for interaction are just a hydrogen bond partner and a hole or cleft

These interactions represent a new way in which cholesterol could affect membrane protein function

ABSTRACT

G-protein coupled receptors (GPCRs) are located in membranes rich in cholesterol. The membrane spanning surfaces of GPCRs contain exposed backbone carbonyl groups and residue side chains potentially capable of forming hydrogen bonds to cholesterol molecules buried deep within the hydrophobic core of the lipid bilayer. Coarse-grained molecular dynamics (CGMD) simulations allow the observation of GPCRs in cholesterol-containing lipid bilayers for long times (50 μs), sufficient to ensure equilibration of the system. We have detected a number of deep cholesterol binding sites on β_2 adrenergic and A_{2A} adenosine receptors, and shown changes in these sites on agonist binding. The requirements for binding are modest, just a potential hydrogen bond partner close to a cleft or hole in the surface. This makes it likely that similar binding sites for cholesterol will exist on other classes of membrane protein.

1. Introduction

G protein-coupled receptors (GPCRs) in mammalian cells are located in a membrane, the plasma membrane, rich in cholesterol with cholesterol making up between 25 and 50 mol % of the lipid in the membrane [1]. Crystal structures of some, but not all, GPCRs crystallized from cubic phases or detergent micelles containing cholesterol or cholesterol hemisuccinate show small numbers of resolved molecules of sterol bound to the GPCR surface [2-7]. All these resolved sterol molecules occupy sites which, in the native membrane, would be contiguous with the phospholipid bilayer around the GPCR, with the sterol polar group (-OH or hemisuccinate) adjacent to the headgroup region of the phospholipid bilayer.

No clear pattern of binding emerges from these crystallographic studies. A number of cholesterol-binding motifs have been identified, including the CCM, CRAC and CARC motifs, all involving hydrogen bonding to the cholesterol –OH group, but many resolved cholesterol molecules are not bound at any of these motifs and many examples of the motifs are not occupied by resolved cholesterol molecules [1, 8, 9]. In fact, full atomistic and coarse-grained molecular dynamics (MD) simulations of GPCRs in cholesterol-containing bilayers suggest that cholesterol molecules are not localised in well-defined deep energy wells on the protein surface but, rather, occupy a more or less continuous range of positions of similar energy, covering most of the

membrane-spanning region of the GPCR [10-13]. This parallels the arrangement of phospholipids on the protein surface, the majority of which also appear not to occupy distinct, highly localised sites [14]. A useful contrast can be drawn with the hydrophobic inhibitor thapsigargin that binds with high affinity to a specific site on the lipid-exposed surface of the calcium ATPase of sarcoplasmic reticulum, the specificity being attributed to an extensive hydrogen-bonding network between the protein and the eight oxygen atoms on thapsigargin [15], an option not open to cholesterol with its single –OH group.

The fact that cholesterol contains only a single polar group makes it more hydrophobic than a phospholipid; the calculated free energy of partition for a cholesterol monomer between water and hydrocarbon is comparable to that of an alcohol of C20 chain length [16]. As a consequence, although most cholesterol molecules in a membrane are located with their –OH groups in the headgroup region of the lipid bilayer with their hydrophobic ring systems more or less perpendicular to the plane of the membrane [1], a proportion of the cholesterol molecules occupy positions with their –OH groups deep within the fatty acyl chain region of the bilayer, close to the bilayer centre, as shown by neutron diffraction studies and full atomistic and coarse-grained molecular dynamic simulations and consistent with the reported high rate of flip-flop of cholesterol across the membrane [17-20].

Here we investigate the possibility that cholesterol molecules deep within the lipid bilayer interact with the transmembrane (TM) surfaces of two GPCRs, the β_2 adrenergic receptor and the A_{2A} adenosine receptor, in agonist-free and agonist-bound states. An important component of the binding of sterol molecules to water-soluble sterol-binding proteins is the hydrophobicity of the sterol ring system [1], but hydrophobic effects cannot be important in the hydrophobic interior of a lipid bilayer. Any interactions will therefore be driven predominantly by hydrogen bond formation with the cholesterol –OH group and, indeed, all published structures for protein-bound cholesterol show the presence of such hydrogen bonds [1]. Measurements of dissociation constants for small hydrophobic molecules in CCl_4 interacting via a single hydrogen bond give K_d values of ca 0.2 to 0.5 M [21], corresponding to K_d values of ca 0.02 to 0.05 when expressed in mole fraction concentration units; K_d values in this range could result in significant binding of cholesterol to GPCRs, given the high concentrations of cholesterol in the plasma membrane. Possible hydrogen bond partners for the cholesterol –OH group on TM surfaces of these proteins include exposed polypeptide backbone carbonyl and amide groups and exposed polar residues. Of course, most backbone carbonyl and amide groups in a TM α-helix will already be involved in hydrogen bonding within the helix, but this will not be true near any proline-induced bends. For example, five of the TM α-helices in the β_2 adrenergic receptor contain Pro residues, and the X-ray crystal

structure of the agonist-free receptor [2] shows that this results in four residues having carbonyl groups that are both exposed on the protein TM surface and more than 3.5 Å from the nearest possible H-bond partner: Leu84 in TM2, Thr164 in TM4, Leu284 in TM6, and Gly320 in TM7, as shown in Figs. 1A, B for Gly320. In contrast to the carbonyl groups, all the backbone amide groups in the proline-containing TM α-helices have hydrogen bond partners, perhaps related to the fact that introduction of a proline residue introduces one extra carbonyl without an extra amide. Additional possible hydrogen bond partners are provided by exposed side chains. For example, the β_2 adrenergic receptor contains 8 Ser, 4 Thr and 1 Tyr residue with –OH groups exposed on the hydrophobic TM surface of the receptor (Fig. 1A). These residues all form separate spots on the surface, except for Ser161 and Thr164 in TM4 and Ser207 in TM5 that together form a single cluster.

Whether or not a deep cholesterol molecule will interact with any of its potential hydrogen bonding partners will depend on the partitioning of cholesterol between surface and deep locations in the lipid bilayer, on the strengths of any hydrogen bonds formed, and on steric interactions between the cholesterol molecules and the protein surface and lipid fatty acyl chains close to the site. Changes in entropy could be particularly important as the presence of cholesterol in a phospholipid bilayer results in ordering of the lipid fatty acyl chains with a consequent decrease in system entropy [22]. Binding of

cholesterol in a deep cleft in a protein surface would effectively remove that cholesterol molecule from the lipid bilayer resulting in an increase in entropy for the phospholipid fatty acyl chains, favouring binding to the protein, although at the cost of a loss of entropy for the cholesterol molecule.

A view of the surface of the agonist-free β_2 adrenergic receptor made using the Travel Depth program [23] shows a number of clefts between the TM α-helices large enough to accommodate the ring system of a cholesterol molecule bound in an orientation parallel to the bilayer normal, together with a number of holes in the surface large enough to accommodate the –OH group of cholesterol in an orientation more or less perpendicular to the bilayer normal (Figs. 1C, D). It is worth noting that, since cholesterol is the only hydrophobic, alcohol-like molecule to be found at high concentrations in biological membranes, even a site with a low structural specificity for the cholesterol –OH group would be occupied only by cholesterol under native conditions.

We have adopted a molecular dynamics approach to search for possible interactions of cholesterol with GPCRs deep in the membrane. This approach requires long simulation times to ensure proper equilibration of the system and at present excludes full atomistic simulations. We have, therefore, used a coarse-grained (CG) approach allowing 50 μs simulations of the systems under study. Efficiency is gained in the CG approach by mapping, on average, four non-hydrogen atoms into one interaction centre, referred to as a bead [24, 25].

By its very nature CG models may not be suited to the study of ligand binding in highly specific pockets where packing of ligand and protein is tight. However, as described above, binding of lipid molecules to membrane protein surfaces is generally less structurally specific than binding at a typical ligand binding site [14], and the CG approach has been used to study lipid-protein interactions at annular (boundary) lipid binding sites for a wide range of lipid molecules, including cholesterol [11, 25-30]. Hydrogen bonds are not modelled explicitly in most force fields used in molecular dynamic simulations, and in the Martini force fields used in CG simulations are present implicitly in the non-bonded interaction potentials between the beads [25]. Although this loses the directionality typical of a hydrogen bond, the loss will be relatively unimportant for the type of loosely fitting binding site that typifies most lipid binding to membrane proteins. The CG approach has been used with success to model specific carbohydrate structures and sugar binding to enzymes, cases where hydrogen bonding is important [31, 32].

2. Methods

Crystal structure coordinates were downloaded for the inactive state agonist-free β_2 adrenergic receptor [PDB ID 3D4S] and the active state complex of the agonist-bound β_2 adrenergic receptor with the stimulatory G protein Gs [PDB ID3SN6] and for the inactive agonist-free A_{2A} adenosine receptor [PDB ID 4EIY] and for the active-intermediate agonist-bound A_{2A} adenosine receptor [PDB ID 3QAK] from the Protein Databank and any inserts were removed. Residues missing from the agonist-free A_{2A} receptor were modelled using the Modeller program. For the other proteins, gaps were retained and the residues at the end of each gap were restrained to their crystal structure positions. Structures were coarse-grained using the martinize.py script downloaded from http://md.chem.rug.nl/cgmartini/. Simulations were performed using the Gromacs simulation package v. 4.5 or 4.6 [33] with the MARTINI 2.1 protein force field [24] together with an elastic network between all C_α atoms within 10 Å to harmonically restrain all C_α particles in the protein [34]. A bilayer consisting of 3000 (for the β_2 receptor) or 4500 waters (for the A_{2A} receptor) and 200 molecules of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was self-assembled around the protein using a 1 μs MD simulation. Thereafter 30 POPC molecules in each leaflet were replaced randomly with cholesterol molecules and the system was equilibrated for a further 0.5 μs. The temperature was held at 310 K with a weak-coupling algorithm and the pressure was held at

1 atm with a semi-isotropic weak-coupling algorithm. Shifted-force versions of the Lennard-Jones and Coulomb potentials were used with a 12 Å cut-off; the dielectric constant was 15. After the equilibration a constant NPT production simulation was carried out for 50 μs with a timestep of 20 fs; snapshots were taken every 50 ps for analysis.

Cholesterol molecules were represented by a polar-head group bead (ROH), five ring beads and two chain beads [24]. Molecules of POPC were represented by a choline, a phosphate, and two glycerol linkage beads, with nine chain beads, C1-C4 making up the saturated chain and C1, C2, C4 and C5 plus D3 making up the unsaturated chain [24]. The densities of cholesterol ROH beads around the protein were calculated as follows. The centres of mass of the ROH beads were located on a grid with 1 Å resolution throughout the simulation and the counts divided by the number of snapshots processed to give the raw probability density ρ of observing an ROH bead at each grid point. Deep cholesterol molecules were defined as those with ROH beads located in the central 8 Å core of the membrane. The probabilities ρ_o of deep cholesterol molecules in the bulk lipid bilayer were calculated as the numbers of ROH beads in the central core divided by the area of the membrane, excluding the area occupied by the protein. The –OH group of a cholesterol molecule was considered to have made a contact with the protein surface if the ROH bead was

within 5 Å of a protein bead. Dissociation constants at the identified clusters (Table 1) were calculated as:

$$K_d = (1- f)\, x_{chol} /f \quad (1)$$

where f is the fractional occupancy of the cluster by cholesterol and x_{chol} is the mol fraction of the deep cholesterol molecules not in contact with protein (Table S1). The free energy ΔG^o was calculated as:

$$\Delta G^o = -RT\ln K_d \quad (2)$$

The Travel Depth programme was downloaded from http://crystal.med.upenn.edu/software.html [23]. Where it was required to show the position of a particular atom on the travel-depth surface, that atom was plotted as a van der Waals surface with radius scaled by a factor of 1.1 to make the atom visible.

3. Results

3.1 Simulations

CGMD simulations of 50 μs length were performed with agonist-free and agonist-bound β_2 adrenergic and A_{2A} adenosine receptors in bilayers of POPC containing 30 mol % cholesterol, typical of the cholesterol content of a plasma membrane [1], in excess water, at a molar ratio of GPCR to total lipid (POPC + cholesterol) of 1:200.

3.2 Deep cholesterol binding sites on agonist-free and agonist-bound β_2-adrenergic receptor

A snapshot of a simulation of the agonist-free β_2-adrenergic receptor shows the majority of the cholesterol molecules oriented around the receptor with their –OH groups just below the planes defined by the phosphate groups of POPC (Fig. 2A). This is consistent with previous atomistic [12, 35] and coarse-grained [28] MD simulations of cholesterol binding to the β_2 adrenergic and A_{2A} adenosine receptors. However, an average of 4.0 cholesterol molecules are also found within the central 8 Å core of the membrane, of which 2.6 have their –OH groups within 5 Å of a protein residue and are defined as those in contact with the protein, with 1.4 not in contact (Table S1); these deep cholesterol molecules have not been reported in earlier papers [12, 28, 35]. The ratio of the number of deep cholesterol molecules not in contact with protein to the total

number of cholesterol molecules is 0.023 in the presence of the β_2-adrenergic receptor and 0.028 in the presence of the A_{2A} adenosine receptor (Table S1). Marrink et al. [18] reported values between 0.03 and 0.1 for the proportion of deep cholesterol molecules in lipid bilayers in the absence of protein, the value depending on the degree of unsaturation of the lipid fatty acyl chains.

The spatial distributions of the –OH groups of the deep cholesterol molecules around the agonist-free β_2-adrenergic receptor, projected on to the plane of the membrane, are shown as 2D probability density plots in Figs. 2B and C, viewed from the (intracellular) IC and EC (extracellular) sides of the membrane, respectively. Probabilities ρ have been normalized relative to the bulk density ρ_0 in the central 8 Å core of the bilayer and plotted as $\ln(\rho/2\rho_0)$, the factor two accounting for the fact that the bilayer is composed of two monolayers. High concentrations of deep cholesterol molecules are seen close to TM4 and TM7.

The probability that a particular amino acid residue is in contact with the –OH group of a deep cholesterol molecule is shown in Fig. 3A. A limited number of residues have higher than background probabilities (> 15 %) of contact and comparisons of snapshots from the simulations with crystal structures (Fig. 4) show that these residues fall into two main clusters (Fig. 3A, Table 1). Cluster A1 consists of Phe321 (58 % probability of contact) and Gly320 (34 %) in TM7, together with Ile43 (23 %) in TM1. In the crystal

structure the backbone oxygen of Gly320 is exposed in a cleft, together with the backbone oxygen of Ile43, with Phe321 to the side (Figs. 1C and 4A). Overlaid snapshots from the simulations show a cholesterol molecule lying in this cleft with its -OH group occupying a more limited range of positions than its alkyl tail (Fig. 4B). The occupancy of the cluster, calculated as the probability that at least one of Ile43, Gly320, or Phe321 makes contact with a cholesterol –OH group, is 72 % (Table 1). As shown in Fig. S1 the calculated occupancy of this cluster, and of all the other clusters to be described below, has converged by about 30 μs of simulation.

Cluster A2 is more complex and contains one residue from TM3, four from TM4, and one from TM5, with occupancies ranging from 44% for Val206 to 21% for Val57 (Table 1, Fig. 3A). The crystal structure shows all these residues lining a hole, with an exposed –OH group on Ser161 (Fig. 4C). Overlaying snapshots from the simulations show cholesterol molecules occupying a series of positions within this hole, with the cholesterol rings more or less perpendicular to the bilayer normal (Fig. 4D). The occupancy of the cluster is 69% (Table 1).

The pattern of interaction of deep cholesterol molecules with the agonist-bound β_2-adrenergic receptor is different from that with the agonist-free β_2-adrenergic receptor, the most obvious difference being that no interaction is seen with the equivalent to cluster A1 in the agonist-free form (Fig. 3B). Further,

although interaction is seen in the region of TM3 and TM4, as in the agonist-free structure, the residues involved are different; the crystal structures show that the hole into which the cholesterol –OH group had previously inserted has closed (compare Figs. 5A and 4C) and a new hole has opened (Figs. 5B and C). In the agonist-bound structure the major residues involved in interactions at cluster B1 are one from TM2, two from TM3, and two from TM4, with occupancies ranging from 36% for Glu122 to 19% for Phe71 (Table 1, Figs. 5B and C); the occupancy of the cluster is 60 % (Table 1). A second cluster, B2, is composed of just Phe208 and Tyr209 but the occupancy of the cluster is only 27% (Table 1, Figs. 3B and S2).

3.3 Deep cholesterol binding sites on agonist-free and agonist-bound A_{2A} adenosine receptors

Although the surface of the A_{2A} receptor contains a number of clefts, as for the β_2- adrenergic receptor, the overall shapes of the two surfaces are distinctly different (Fig. S3 and Figs. 1C, D). 2D probability density plots show lower densities of cholesterol –OH groups close to the surface of the A_{2A} receptor (Fig. S4) than for the β_2-adrenergic receptor (Figs. 2B-E), the highest densities being in the vicinity of TM3, TM4 and TM5. Contact probability plots show the presence of one main interacting cluster C1 in the agonist-free receptor (Fig. 6A, Table 1). The cluster consists of one residue each from TM2,

TM3, TM4, and TM5 with occupancies ranging from 37% for Phe93 to 14% for Cys185. These residues are located in a wide hole with Cys185 at the top of the hole (Figs. 6C and D). The occupancy of the cluster is 49% (Table 1); overlaid snapshots of the simulations show that –OH groups occupy a range of locations within the site, with the cholesterol rings more or less perpendicular to the protein surface (Fig. 6D).

A second cluster, C2, consists of just Trp129 and Phe133 and has an occupancy of 29% (Table 1, Fig. 6A). In the crystal structure these two residues are seen on the far side of the ridge making up one wall of the hole described above (Fig. S5A). A third cluster, C3, consists of Phe182 and Phe183 and has an occupancy of 24% (Table 1, Fig. 6A). Phe182 and Phe183 are close to a cleft exposed to the membrane surface where a cholesterol molecule is resolved in the crystal structure, with its –OH group close to the predicted location of the glycerol backbone region of the surrounding lipid bilayer (Fig. S5B).

The probability density plots for the agonist-bound A_{2A} receptor are similar to those for the agonist-free receptor (Fig. S4) with major contacts with Phe93 in TM3 and a number of residues in TM4 (Fig. 6B). However, in the agonist-bound form these residues fall into two separate clusters D1 and D2 either side of Phe93 (Fig. 7A). The first cluster, D1, on the EC side of Phe93, contains 7 residues and has an occupancy of 38% (Table 1, Fig. 7B). The second cluster, D2, on the IC side of Phe93, consists of Phe93 and two other

residues and has an occupancy of 38% (Table 1, Fig. 7C). As for the agonist-free A_{2A} receptor, contact is also observed with a cluster, D3, consisting of Trp129 and Phe133 on the far side of the ridge making up clusters D1 and D2 (see Fig. S5A) but the probability of contact with the cluster is only 32% (Table 1). A final cluster, D4, corresponds to Val283 and Phe286 and has an occupancy of 27% (Table 1, Fig. S6). These two residues are predicted to be close to the glycerol backbone region of the lipid bilayer on the IC side and, indeed, the OPM data base [http://opm.phar.umich.edu/] locates the backbone oxygen of Phe286 at the hydrophobic/polar interface [36].

4. Discussion

4.1 Cholesterol interactions with GPCRs

Most of the molecules of cholesterol found in a biological membrane are located with their –OH groups in the headgroup region of the lipid bilayer, with their hydrophobic ring systems in the hydrophobic core of the bilayer [1]. Correspondingly, all the cholesterol molecules that have been resolved in crystal structures of GPCRs have been found at positions on the protein surface which, in a biological membrane, would locate the cholesterol –OH group contiguous with the bilayer headgroup region, with the cholesterol ring system contiguous with hydrophobic core of the bilayer [8]. A review of these crystal structures by Gimpl [8] has shown that while some cholesterol molecules are located at specific binding motifs, many are not. In most cases the cholesterol –OH group is involved in hydrogen bonding to a protein residue, with the cholesterol ring system either interacting with a single TM helix or located in a groove between two or more helices [8]. Overall the impression is of a set of binding sites showing little similarity at the structural level. In all cases the number of resolved cholesterol molecules is low, the highest number being three [8].

MD simulations of GPCRs in lipid bilayers containing cholesterol suggest that many more cholesterol molecules can bind to the hydrophobic surface of a GPCR with their –OH groups in the lipid headgroup region than

have been resolved by X-ray crystallography [12, 28, 35]. Although fully atomistic MD simulations cannot be run for long enough to ensure full equilibration, a 5μs simulation of the β_2 adrenergic receptor in a cholesterol-containing bilayer has provided important information [12]. Seven possible sites of interaction with cholesterol were detected with the site showing the strongest interaction involving hydrogen bonding between the –OH group of cholesterol and a Lys residue [12]. However, cholesterol molecules were not observed on much of the membrane-spanning surface of the GPCR, this possibly being a result of the short simulation time. In contrast, CGMD simulations with their longer simulation times suggest that cholesterol molecules can make contact with most of the membrane-spanning surface of a GPCR [28], and as shown here in Fig 2A. These CGMD simulations suggest that cholesterol, like most phospholipids, is not highly localised on the protein surface but can occupy a range of positions of similar energy [14].

Although the majority of the cholesterol molecules found in a biological membrane are located with their –OH groups in the lipid headgroup region, a fraction are found buried deep within the hydrophobic core of the bilayer (Table S1) [17-19]. The presence of potential hydrogen bond partners for the hydroxyl groups of these deep cholesterol molecules on the TM surfaces of GPCRs (see Figs. 1A, B) suggests that deep cholesterol molecules might be able to bind to the GPCRs, a possibility now supported by CGMD simulations although not

reported in previous simulations [12, 28, 35]. 2D probability plots calculated for GPCRs in bilayers of mixtures of POPC and cholesterol identify regions on the protein surface where the probability of finding a deep cholesterol molecule is higher than in the bulk bilayer (Figs. 2 and S4). Amino acid residues having a higher than background (> 15 %) probability of being within 5 Å of a deep cholesterol –OH group fall into identifiable clusters on the protein surfaces (Figs. 3 and 6 and Table 1). Some of these clusters show probabilities of being close to a cholesterol –OH group of ca 50% or greater in a bilayer containing 30 mol% cholesterol, suggesting that they correspond to sites to which cholesterol could be bound in the native membrane.

4.2 Interactions of deep cholesterol molecules with the β2 receptor

Of the clusters identified here, that with the highest probability of being close to a cholesterol –OH group is cluster A1 on the agonist-free β2 receptor, consisting of Ile43, Gly320, and Phe321 (Figs. 3A, 4A and 4B). Gly320 in TM7 is unusual in that the presence of a Pro residue in the helix results in Gly320 having a non-hydrogen bonded carbonyl group exposed to the surrounding lipid bilayer and thus capable of forming a hydrogen bond with the –OH group of a deep cholesterol molecule (Figs. 1B and 4A). CGMD simulations show that, in a bilayer containing 30 mol% cholesterol, the probability that a cholesterol –OH group is close to Gly320 is 34% (Fig. 3A), corresponding to a free energy of

association of 10.8 kJ mol^{-1}. The free energy for association of two small molecules in a hydrophobic environment interacting via a single hydrogen bond can be estimated from the data given by Kyte [21] to be between 7.4 and 9.7 kJ mol^{-1}, calculated in mole fraction units, so that the calculated free energy for interaction at the cluster is consistent with the formation of a single hydrogen bond.

Another unusual feature of Gly320 is that it is located close to a cleft in the protein surface, long and deep enough to accommodate the rigid ring system of a cholesterol molecule (Fig. 1C). Indeed, snapshots taken from the simulations show cholesterol molecules lying along the protein surface, occupying this cleft (Fig. 4B). The relationship between a cholesterol molecule bound in the cleft and the surrounding annular (boundary) phospholipids is shown in Fig. 8A, where annular phospholipid molecules are here defined as those for which the central two (C2 or C3) of the four beads representing the saturated chains or the central three (C2, D3, or C4) of the five beads representing the unsaturated chains are within 6 Å of the protein surface, consistent with EPR studies of membranes using spin-labelled phospholipids where the best resolution between annular and bulk lipids was obtained with a spin label at a position close to the middle of the fatty acyl chain [37-39]. The simulations show a cholesterol molecule bound at cluster A1 to be effectively adsorbed into the protein surface and covered by the fatty acyl chains of the

annular phospholipid molecules (Fig. 8A). Given that the presence of a cholesterol molecule in a bulk lipid bilayer results in an ordering of the lipid fatty acyl chains [22] the removal of a cholesterol molecule in this way from the phospholipid bilayer will generate an entropy term favouring binding of cholesterol in the cleft although this will be offset by a loss of entropy for the cholesterol molecule. The cleft is not specific for the polycyclic ring system of cholesterol in that simulations in POPC alone show that, in the absence of cholesterol, the cleft is occupied by phospholipid fatty acyl chains (data not shown).

The crystal structure of the agonist-free β2 receptor shows that the receptor contains three lipid-exposed, non-hydrogen bonded helical residues in addition to Gly320: Leu84 in TM2, Thr164 in TM4 and Leu284 in TM6. However, none of these show greater than background contact with cholesterol –OH residues (Fig. 3A) and none are located close to large clefts. Other residues capable of forming hydrogen bonds to deep cholesterol molecules include Ser, Thr and Tyr residues, of which a number are exposed to the hydrophobic core of the lipid bilayer (Fig. 1A). The exposed –OH group of Ser161 makes up part of interaction cluster A2 (Fig. 4C; Table 1) and Ser161 has a 28% probability of being close to a cholesterol –OH group, the probability for the whole cluster being 69%. The –OH group of Thr118, also part of cluster A2, is exposed on the surface of the protein and has a 26% probability of being

close to a cholesterol –OH group. In this case the cluster is not close to a cleft capable of accommodating the rigid ring system of cholesterol, but, rather, forms part of a hole into which the –OH group inserts, with the cholesterol ring more or less perpendicular to the protein surface (Fig. 4D). This orientation of the ring means that it penetrates between the fatty acyl chains of the annular phospholipids to insert between the chains of more distant phospholipids, with a consequent entropy penalty (Fig. 8B). The cholesterol molecules interacting with cluster A2 are not well localized, occupying a range of positions (Fig. 4D). This is not unusual for binding in the TM region of a membrane protein and has, for example, been observed for hydrophobic molecules binding at the general anaesthetic binding cavity on a pentameric ligand-gated ion channel [40].

The pattern of binding to the active state agonist-bound β_2-adrenergic receptor-Gs complex is different from that to the agonist-free form, reflecting the changes in TM helix packing on receptor activation (Fig. 3, Table 1). The most obvious difference is the lack of a cluster equivalent to the main interaction cluster A1 in the agonist-free form. Changes in this region on agonist binding, apparent in the crystal structures, include some filling in of the cavity close to Gly320 with only a small movement of Gly320 on agonist binding (compare Figs. 8C and 4A); the presence of protein density in the cavity where cholesterol binds in the agonist-free state is clear in the simulations (Fig. 8D). Further, although interactions with clusters of residues from TM3 and

TM4 are observed in both the agonist-free and agonist-bound states (clusters A2 and B1 respectively), the residues involved are different. Comparison of crystal structures of the two forms shows that agonist binding leads to movement of Val160 closer to Val206, covering the –OH group of Ser161 and filling the hole into which a cholesterol –OH group was inserted in the agonist-free state (compare Figs. 4C and 5A). However, a new interaction cluster B1 is formed around a hole into which the cholesterol -OH group now inserts (Figs. 5B and C). The residue with the highest occupancy (36%) in cluster B1 is Glu122 in which the carboxyl group is surface-exposed and non-hydrogen bonded in the absence of cholesterol, again consistent with an important role for hydrogen bonding in the interaction with cholesterol; the occupancy of the whole cluster is 60%. Finally interaction is also seen with a cluster B2 of just two residues (Fig. S2A, B) but the overall occupancy of the cluster is only 27% making it unlikely to represent an important binding site.

4.3 Interactions of deep cholesterol molecules with the A_{2A} receptor

The pattern of interaction of deep cholesterol molecules with the A_{2A} receptor is comparable to that with the β_2 adrenergic receptor (Table 1), the surface of the A_{2A} receptor containing a number of clefts and holes, as for the β_2 adrenergic receptor, together with a number of potential hydrogen bond partners (Fig. S3). Four of the TM α-helices in the A_{2A} receptor contain Pro residues,

TM2, TM5, TM6, and TM7. In the agonist-free structure Val57 in TM2 and Ala184 in TM5 have non-hydrogen bonded carbonyl groups but in helices TM6 and TM7, although the carbonyl groups of Cys245 and Ser281 in TM6 and TM7 respectively form no hydrogen bonds within their respective helices, they form hydrogen bonds with the backbone amide of Ser277 in TM7 and the side chain amide of Asn24 in TM1, respectively. The pattern of helix hydrogen bonding in the agonist-bound state is very similar to that in the agonist-free state. There are many fewer polar residues in the TM α-helices of the A_{2A} receptor than in the β_2 adrenergic receptor, with one Ser residue (Ser47) and two Thr residues (Thr138 and Thr279) with surface exposed –OH groups. Three Cys residues contain surface exposed –SH groups, Cys128, Cys185 and Cys245; all of these are involved in hydrogen bonding with backbone carbonyls.

The density of deep cholesterol molecules around the A_{2A} receptor is lower than around the β_2-adrenergic receptor (Figs. 2 and S4). Three clusters of interacting residues are observed on the agonist-free A_{2A} receptor (Fig. 6, Table 1). At the first cluster the residue with the highest probability of being close to a cholesterol –OH group is Phe93 (37%), additional contacts being made with Phe44, Cys128 and Cys185, all, however, with contact probabilities below 20%. The cluster is located towards the centre of the lipid bilayer, at one end of a large cleft leading to the IC surface; in the crystal structure the cleft is occupied by a fatty acyl chain (Fig. 6C). The overall probability of contact with this

cluster, 49%, is lower than that for the most occupied clusters on the β_2 adrenergic receptor, possibly because hydrogen bonds to sulphur are generally considered to be weaker than those to oxygen [41].

The crystal structure of the active-intermediate state agonist bound A_{2A} receptor shows changes in the vicinity of Phe93, and the simulations now show two binding clusters D1 and D2 in this region rather than just one (Fig. 7, Table 1). The major cluster D1 involves seven residues with a total probability of interaction of 74%, the residue with the highest probability of interaction being Ser132 (35%). The second cluster, D2, which includes Phe93 and Cys128, has a slightly lower occupancy than that of the similar cluster, C1, in the agonist-free form (Table 1). A cluster composed of just two residues, Trp129 and Phe133, appears in both the agonist-free (cluster C2) and agonist-bound (cluster D3) structures, but with a probability of occupancy of only ca 30%. A final cluster, D4, observed in the agonist-bound state again consists of two residues, Val283 and Phe286, with an occupancy of 27%.

4.5 *A comparison between β2 and A_{2A} receptors*

Figs. 9 and 10 show that the differences in binding clusters between agonist-free and agonist-bound structures are greater for the β_2 adrenergic receptor than for the A_{2A} adenosine receptor, probably because the agonist-bound β_2 adrenergic receptor structure [PDB ID3SN6], determined in the

presence of Gs, is in a fully activated state whereas the agonist-bound A_{2A} adenosine receptor [PDB ID 3QAK] is in an active-intermediate state.

The eleven clusters described in Table 1 fall into five groups based on similar Ballesteros-Weinstein numbers for the residues within the cluster, corresponding, of course, to similar positions on the protein surface (Figs. 9 and 10). One group (coloured yellow) contains clusters A1 from the agonist-free β_2 adrenergic receptor and D4 from the agonist-bound A_{2A} receptor (Fig. 9). A1 is a strongly interacting cluster in TM7 containing $G320^{7.47}$ and $F321^{7.48}$, where the subscripts give the Ballesteros-Weinstein residue number, and D4 is a weakly interacting cluster containing $V283^{7.48}$ and $F286^{7.53}$. It was argued in Section 4.2 that a Gly residue at position 7.47 is important for strong interaction at cluster A1, consistent with the weak binding at cluster D4 in the A_{2A} receptor which lacks Gly residues in TM7.

A second group of clusters (coloured sky blue) contains A2 from the agonist-free β_2 adrenergic receptor and D1 from the agonist-bound A_{2A} receptor (Fig. 10). Both these clusters interact strongly with cholesterol and contain a Ser residue as a potential hydrogen-bond partner, at position 4.53 (Table 1). Other similarities between clusters A2 and D1 include the pairs of hydrophobic residues $V160^{4.52}$ and $V206^{5.45}$ in cluster A2 and $L131^{4.52}$ and $A184^{5.45}$ in cluster D1. A third group (coloured pink) contains three medium affinity clusters, B1 from the agonist-bound β_2 adrenergic receptor, and C1 and D2 from the agonist-

free and agonist-bound A_{2A} receptor, respectively. A pair of residues are common to all these clusters, E122$^{3.41}$ and V157$^{4.49}$ in B1, and F93$^{3.41}$ and C128$^{4.49}$ in C1 and D2; clusters B1 and C1 also contain a Phe at position 2.42. It should be noted that the distinction between the groups A2/D1 and B1/C1/D2 is somewhat arbitrary as they occur in similar locations on the protein surface with some overlap (Fig. 10). The low affinity clusters C2 and D3 in the A_{2A} receptor (coloured orange) contain the same two hydrophobic residues, W129$^{4.50}$ and F133$^{4.54}$. Finally, the two low affinity clusters B2 and C3 (coloured cyan) are located at similar positions in TM5 although the Ballesteros-Weinstein residue numbers for the two hydrophobic residues in each cluster (F208$^{5.47}$ and Y209$^{5.48}$, and F182$^{5.43}$ and F183$^{5.44}$, respectively) are not identical (Fig. 10).

The comparison between the binding clusters on the β2 and A_{2A} receptors emphasises the difficulty in identifying potential binding clusters on the basis of residue number alone. For example, although Ser at position 4.53 in the Ballesteros-Weinstein numbering system is an important residue in binding cholesterol at both cluster A2 on the agonist-free β2 receptor and cluster D1 on the agonist-bound A_{2A} receptor, it does not interact with cholesterol on either the agonist-bound β2 receptor or the agonist-free A_{2A} receptor (Figs. 3 and 6). Similarly, although residues at positions 3.41 and 4.49 are present in clusters B1, C1 and D2 on the agonist-bound β2 receptor and agonist-free and bound A_{2A} receptor, respectively, there is no corresponding cluster on the agonist-free β2

receptor (Fig. 9, Table 1). Local structural features are clearly of importance in determining binding.

4.6 The nature of the deep binding sites

The CGMD simulations reported here detect a number of regions on the TM surface of GPCRs to which cholesterol molecules deep within the lipid bilayer can bind. Five of the interaction clusters listed in Table 1 have an occupancy of ca 50% or higher at a bulk cholesterol content of 30 mol % and seem likely therefore to be at least partially occupied by cholesterol in the native membrane. The common feature of the clusters is a potential hydrogen bond partner for the cholesterol –OH group, exposed on the protein surface. At one, cluster A1 on the agonist-free β_2 adrenergic receptor (Table 1), the cholesterol ring system is buried in a cleft in the protein surface (Fig. 1C) resulting in good localization of the interacting cholesterol molecule, as in a 'normal' binding site. At the other clusters there is no such burying of the cholesterol ring system and cholesterol molecules occupy a range of positions at the cluster. Many other examples of poorly localised binding at hydrophobic protein cavities are now known; recent examples include the binding of oxygen and other gases in small hydrophobic protein cavities [42], the binding of small organic molecules in the hydrophobic cavity of bee odorant binding protein [43] and the binding of general anaesthetics to pentameric ligand-gated

ion channels [40], binding in all these cases affecting protein function. The fact that we find differences in the deep cholesterol interactions with the agonist-free and bound forms of both the β_2 adrenergic and the A_{2A} adenosine receptors (Table 1) suggests that interaction with deep cholesterol molecules could affect receptor function.

Direct experimental evidence for the functional significance of deep cholesterol binding will be difficult to obtain because of the presence of multiple binding sites for cholesterol on the receptors at the membrane-water interface, in addition to the deep sites that are the focus of this paper (Fig. 2A). The presence of cholesterol or cholesterol hemisuccinate increases the thermal stability of many GPCRs in detergent micelles and in lipid bilayers and, in some cases, increases the affinity for agonists; the mechanisms of these effects are unclear [2, 44-47]. Addition of cholesterol to the β_2 adrenergic receptor expressed in Sf9 insect cells, where the cholesterol level is low, led to a two-fold increase in affinity for the partial inverse agonist timolol but no change for the full agonist isoproterenol [2].

Mutational studies point to an important role for Gly320 (a component of cluster A1) in TM7 in the β_2 adrenergic receptor family. Gly320 in other family members is either Gly or Ala, suggesting a preference for small residues at this position, and mutation of Gly320 to Ala in the human protein results in a halving of the affinity for isoproterenol [48]. The sequence NPLIY in TM7 is

conserved in the β_2-adrenergic receptor family and, with minor variations, in all members of the GPCR superfamily [49]. Mutation of Pro323 in this sequence to Ala resulted in a ca fivefold decrease in affinity for isoproterenol [49]. Mutational experiments have also suggested an important role for Glu122, a component of cluster B1, in the agonist-bound β_2 adrenergic receptor, where mutation to Ala resulted in a decreased affinity for both agonists and antagonists [50]. Further, Glu122, together with Phe321, have been identified as part of a pathway linking allosteric changes on the EC and IC sides of the β_2-adrenergic receptor [51]. Less information is available about the A_{2A} receptor but crystallographic studies have pointed to an important role for Cys185, a component of clusters C1 and D1 in the agonist-free and bound states respectively, this being part of a bulge forming in TM5 when agonists bind to the receptor [52].

Although the presence of a potential hydrogen bond partner for the cholesterol –OH group is seen in all the high affinity clusters detected here (Table 1), many non-hydrogen bonded backbone carbonyl groups and side chains exposed on the TM surface of the GPCRs do not interact with cholesterol in the simulations. The high affinity clusters listed in Table 1 all take the form of clefts or holes in the TM surface but the TM surfaces of GPCRs are very rough (Figs. 1C, D and S3) and it is not obvious *a priori* which clefts and holes would be suitable for docking a cholesterol –OH group and which would not.

Other complicating factors include potential steric clashes between the cholesterol molecule and the protein surface and the lipid bilayer and the strength of the potential hydrogen bond. Although, therefore, potential hydrogen bond partners can be readily identified from a crystal structure, there is no obvious way at present to predict *a priori* which of these will actually partner with a cholesterol molecule. The power of the CGMD approach is that its speed makes it possible to search for such interactions with simulations of sufficient length to ensure system equilibration. Since the requirements for binding of deep cholesterol to a membrane protein are so simple it is likely that deep cholesterol molecules will interact with other GPCRs and with many other classes of membrane protein.

Conflicts of Interest

The authors declare no conflicts of interest.

Author contributions

S.G. performed the simulations. S.G., A.G.L. and J.W.E. designed the project and wrote the manuscript. J.W.E. supervised the project.

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FIGURE LEGENDS

Fig. 1. The membrane spanning surface of the agonist-free β_2 adrenergic receptor. (A) Protein surface with the position of TM7 shown in yellow. The horizontal lines indicate the approximate positions of the glycerol backbone regions of the lipid bilayer, taken from the OPM database [http://opm.phar.umich.edu/] [36]. Regions where polar side chains are exposed on the surface are shown in red. Regions where a non-hydrogen bonded backbone carbonyl group is exposed are shown in green; this view only shows that in TM7. In these, and all the following Figures, the extracellular surface (EC) is at the top and the intracellular surface (IC) is at the bottom. (B) The non-hydrogen bonded backbone carbonyl of Gly320 in TM7 is shown 4.8 Å from the –NH group of Leu324 that would be its hydrogen bonding partner in a normal α-helix. (C, D) Travel Depth views of the agonist-free β_2 adrenergic receptor surface showing the distance of travel from a point on the protein surface to a reference surface; the two views are related by a 180^o rotation around the bilayer normal: (C) shows the cleft adjacent to TM7 with the backbone O of Gly320 shown in yellow and (D) shows the hole adjacent to TM4 with the -OH of Ser161 shown in yellow and marked by the white ring. Depths from the reference surface are colour coded from 0 to 12 Å.

Fig. 2. Distribution of cholesterol molecules around the β_2-adrenergic receptor. (A) A representative snapshot for the agonist-free receptor with POPC phosphate beads shown in green and cholesterol –OH beads in red, except for deep cholesterol molecules within 5 Å of the protein surface which are shown in yellow. (B-D) Spatial

distributions of –OH groups of cholesterol molecules in the central 8 Å core of the membrane, for agonist-free (B, C) and agonist-bound (D, E) β2-adrenergic receptor, projected on to the plane of the membrane. The views in (B) and (D) are from the IC side, and those in (C) and (E) are from the EC side. The data are plotted as $\ln(\rho/2\rho_0)$ where ρ and ρ_0 are the measured and bulk probabilities for finding a cholesterol –OH group, respectively, and distances along the x (horizontal) and y (vertical) axes are given in Å. The backbone positions of the TM helices are marked.

Fig. 3. Contact probabilities for agonist-free (A) and agonist-bound (B) β2-adrenergic receptor. The plots show the percentage of time for which a particular amino acid residue is within 5 Å of the –OH group of a deep cholesterol molecule, plotted as a function of the residue number. The seven TM helices have been given different colours. Clusters are indicated, as described in the text and Table 1.

Fig. 4. Interaction clusters for deep cholesterol molecules on the agonist-free β_2-adrenergic receptor. Interaction clusters A1 (A, B) and A2 (C, D) are shown in the crystal structure (A, C) and in the simulations (B, D). In A and B residues are coloured: Ile43, green; Glu320, blue; Phe321, yellow; in A the backbone oxygens of Ile43 and Glu320 are shown in red, with VDW radii scaled by a factor of 1.1 for clarity. In C and D residues are coloured: Thr118, magenta; Val157, green; Val160, yellow; Ser161, dark blue; Val 206, sky blue; in C the side chain –OH of Ser161 is shown in red, with VDW radius scaled by 1.1. B and D show five overlaid snapshots at 10 μs intervals between 10 and 50 μs. Cholesterol molecules are shown as surface

meshes with different colours for each snapshot, except for the cholesterol –OH beads which are all coloured red. In (D), one snapshot shows two cholesterol molecules (coloured yellow) bound to the cluster at the same time.

Fig. 5. Interaction clusters for deep cholesterol molecules on the agonist-bound β_2-adrenergic receptor. (A) The crystal structure of the agonist-bound state showing the locations of the three residues Thr118, Val157 and Val160 making up cluster A2 in the agonist-free state, with the same colour coding as in Fig. 4C. (B) Cluster B1 in the crystal structure of the agonist-bound state. Residues are coloured: Phe71, orange; Glu122, magenta; Val126, green; Ile153, blue; Val157, yellow. (C) Five overlaid snapshots from the simulations showing binding at cluster B1, with residues coloured as in (B) and cholesterol molecules shown as surface meshes.

Fig. 6. Interaction clusters for deep cholesterol molecules on the A_{2A} receptor. (A, B) Contact probabilities for agonist-free (A) and agonist-bound (B) receptor. Clusters are indicated, as described in the text and Table 1. C and D show cluster C1 in the agonist-free state in the crystal structure (C) and in the simulations (D). Residues are coloured: Phe44, light green; Phe93, blue; Cys128, green; Cys185. Orange. In (C) the side chain –SH groups of Cys128 and Cys185 are shown in yellow and the backbone oxygen of Phe93 in red, all with VDW radii scaled by a factor of 1.1. A fatty acyl chain observed in the crystal structure close to cluster C1 is shown in ball-and-stick representation. (D) Five overlaid snapshots from the simulations with cholesterol

molecules shown as surface meshes; in this view Cys128 is buried under the bound cholesterol molecules.

Fig. 7. Interaction clusters for deep cholesterol molecules on the agonist-bound A_{2A} receptor. (A) The crystal structure with cluster D1 residues coloured: Leu131, pink; Ser132, purple; Ile135, yellow; Phe180, red; Asn181, pale green; Ala184, cyan; Cys185, orange, and cluster D2 residues coloured: Phe93, blue; Cys128, green; Ile124, olive. (B, C) show five overlaid snapshots from the simulations, for cluster D1 (B) and cluster D2 (C); residues are coloured as in (A) and cholesterol molecules are shown as surface meshes. The views are tilted with respect to that in (A) for clarity.

Fig. 8. Cholesterol and POPC molecules binding to β_2-adrenergic receptor. (A, B) show snapshots of cholesterol and POPC molecules on the surface of agonist-free β_2-adrenergic receptor in the regions of clusters A1 (A) and A2 (B), with residues coloured as in Fig. 4. Cholesterol molecules are shown in space-fill representation with –OH groups in red. Annular phospholipid molecules are shown in ball-and-stick format, with chains in salmon and the phosphate group in purple. (C, D) illustrate the changes at cluster A1 of the agonist-free receptor that occur on agonist binding. (C) The locations of Ile43, Gly320, and Phe321are shown in the crystal structure of the agonist-bound form with the same colour coding as in Fig. 4A. (D) An overlay of snapshots from the simulations for the agonist-free and agonist-bound structures. The surface of the agonist-free structure is shown as a solid surface (green) and that of the agonist-bound structure is shown as a mesh (brown), so that the agonist-bound surface

is only visible when it lies above the surface of the agonist-free structure. The yellow mesh shows a cholesterol molecule bound in the agonist-free structure. Gly320 is shown in space fill format (dark blue, agonist-free; light-blue, agonist-bound).

Fig. 9. Comparison of the binding clusters. Crystal structure surfaces of the agonist-free (A) and agonist-bound (B) β_2-adrenergic receptor and the agonist-free (C) and agonist-bound (D) A_{2A} receptor. All views are in the same orientation with the N-most residue resolved in the crystal structure shown in green. The binding clusters visible in these views are labelled as in Table 1. The clusters are coloured on the basis of similar residue numbers in the Ballesteros-Weinstein numbering system for some of the residues in the cluster: 7.48 (A1, D4, yellow). The horizontal lines indicate the approximate positions of the glycerol backbone regions of the lipid bilayer, taken from the OPM database [36]. C and D have been tilted by 18^o relative to the crystallographic z axis to account for the tilted orientation of the protein in the bilayer reported in the OPM data base.

Fig. 10. Comparison of the binding clusters. Details as in Fig. legend 9, the views shown being related to those in Fig. 9 by a 180^o rotation around the bilayer normal. The clusters are coloured on the basis of similar residue numbers in the Ballesteros-Weinstein numbering system for some of the residues in the cluster: 4.52, 4.53, 5.45 (A2, D1, sky blue); 3.41, 4.49 (B1, C1, D2, pink); 5.43-5.48 (B2, C3, cyan); 4.50, 4.54 (C2, D3, orange).

Table 1
Interaction clusters.

Receptor	Cluster[a]	Residues in cluster[b]	Occupancy[c]	K_d[d]	ΔG^{o} [e]
Agonist-free β_2	A1	$I43^{1.42}$, $G320^{7.47}$, $F321^{7.48}$	0.72 ± 0.02	0.0027	14.6
	A2	$T118^{3.37}$, $V157^{4.49}$, $V160^{4.52}$, $S161^{4.53}$, $V206^{5.45}$	0.69 ± 0.01	0.0031	14.3
Agonist-bound β_2	B1	$F71^{2.42}$, $E122^{3.41}$, $V126^{3.45}$, $I153^{4.45}$, $V157^{4.49}$	0.60 ± 0.03	0.0046	13.3
	B2	$F208^{5.47}$, $Y209^{5.48}$	0.27 ± 0.04	0.0189	9.8
Agonist-free A_{2A}	C1	$F44^{2.42}$, $F93^{3.41}$, $C128^{4.49}$, $C185^{5.46}$	0.49 ± 0.03	0.0088	11.7
	C2	$W129^{4.50}$, $F133^{4.54}$	0.29 ± 0.02	0.0208	9.6
	C3	$F182^{5.43}$, $F183^{5.44}$	0.24 ± 0.02	0.0269	9.4
Agonist-bound A_{2A}	D1	$L131^{4.52}$, $S132^{4.53}$, $I135^{4.56}$, $F180^{5.41}$, $N181^{5.42}$, $A184^{5.45}$, $C185^{5.46}$	0.74 ± 0.06	0.0030	14.4
	D2	$F93^{3.41}$, $I124^{4.45}$, $C128^{4.49}$	0.38 ± 0.13	0.0139	10.6
	D3	$W129^{4.50}$, $F133^{4.54}$	0.32 ± 0.02	0.0181	9.9
	D4	$V283^{7.48}$, $F286^{7.51}$	0.27 ± 0.03	0.0230	9.3

[a] Clusters are defined in Figs. 3 and 6.

[b] Ballesteros-Weinstein numbers are given as superscripts.

[c] Fractional occupancies, where a cluster is defined as occupied if a cholesterol –OH group is within 5 Å of at least one residue in the cluster. The uncertainty is the standard error over five repeat simulations.

[d] In units of mole fraction.

[e] Units of kJ mol^{-1}.

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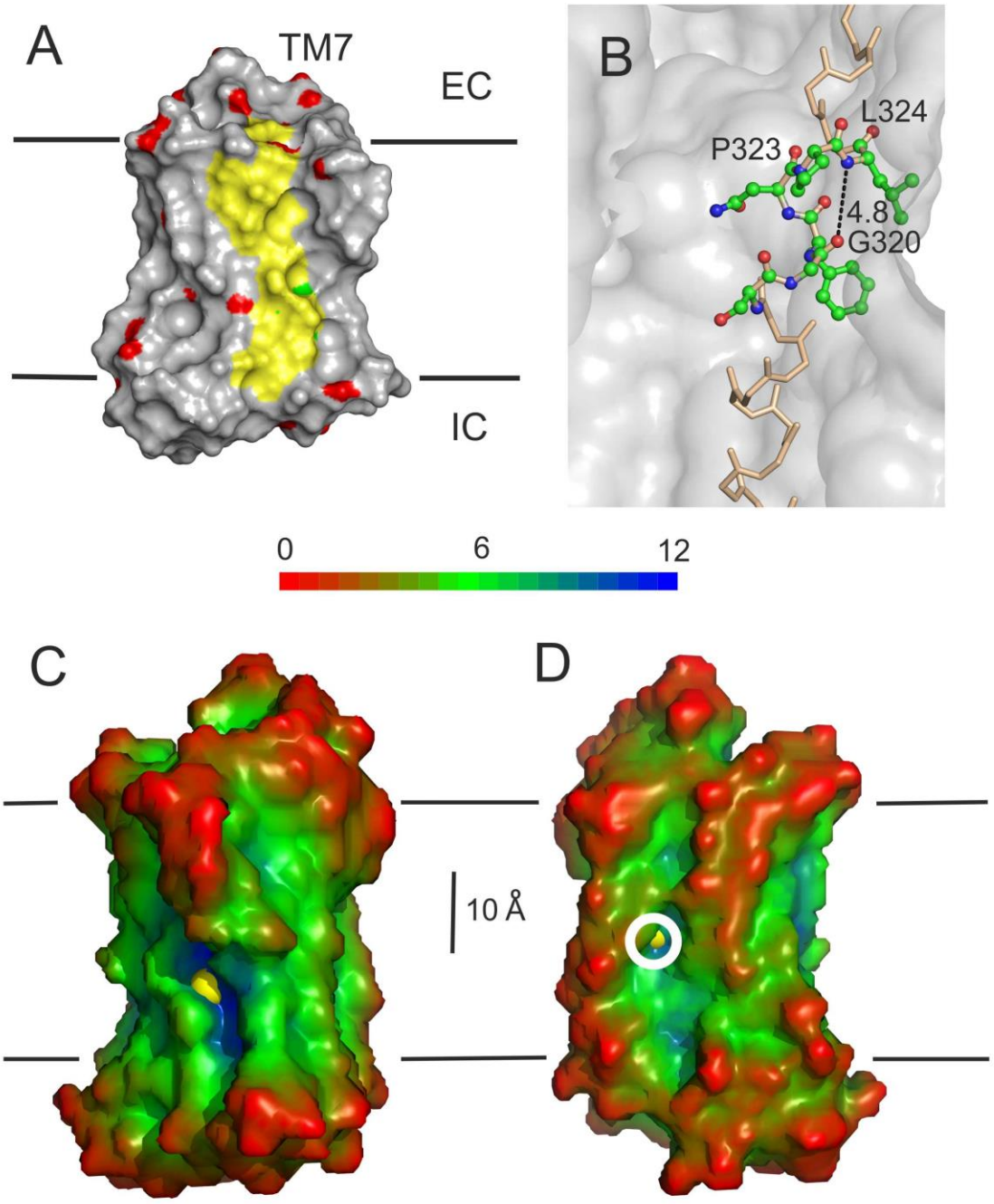


Fig. 1

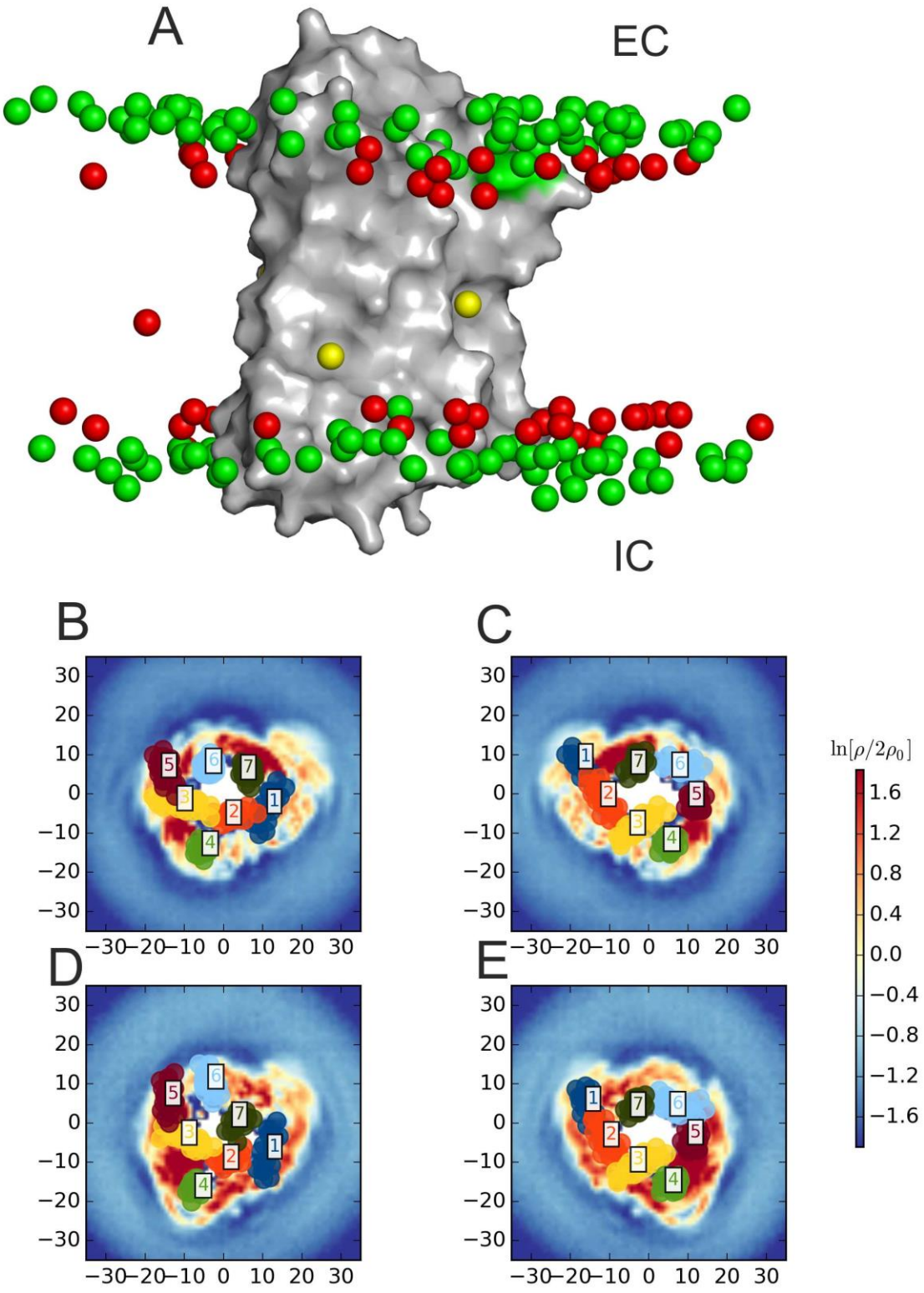


Fig. 2

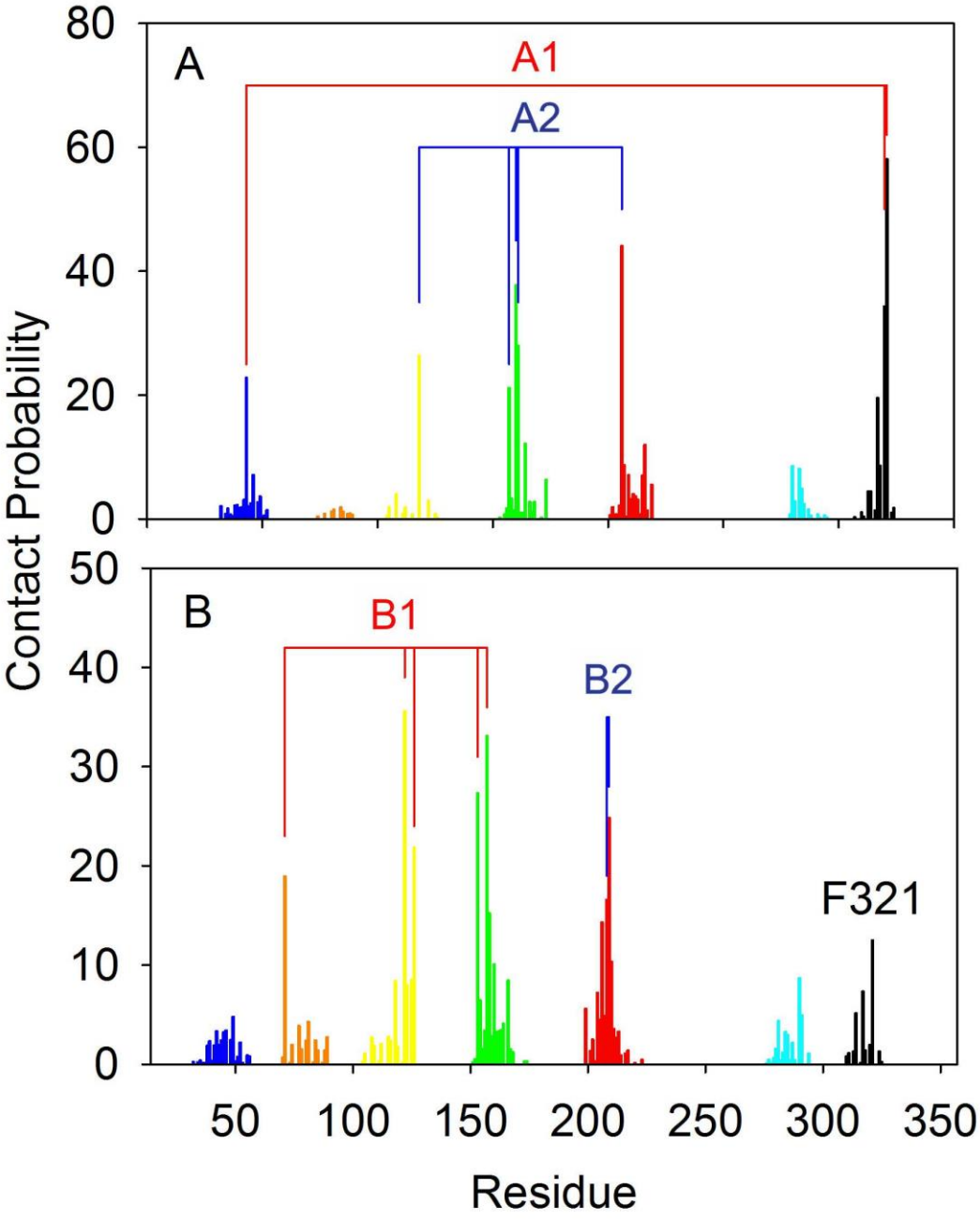


Fig. 3

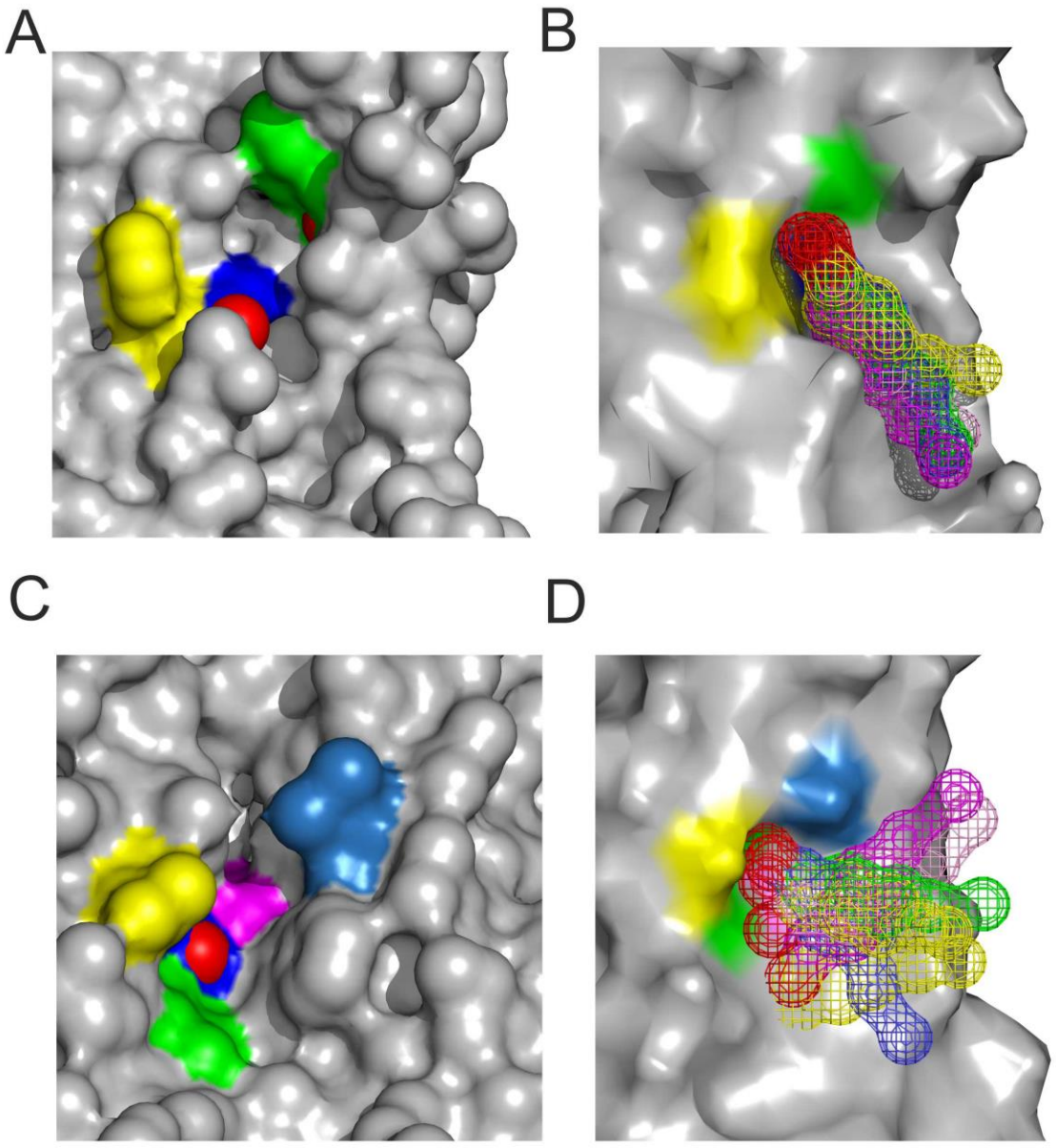
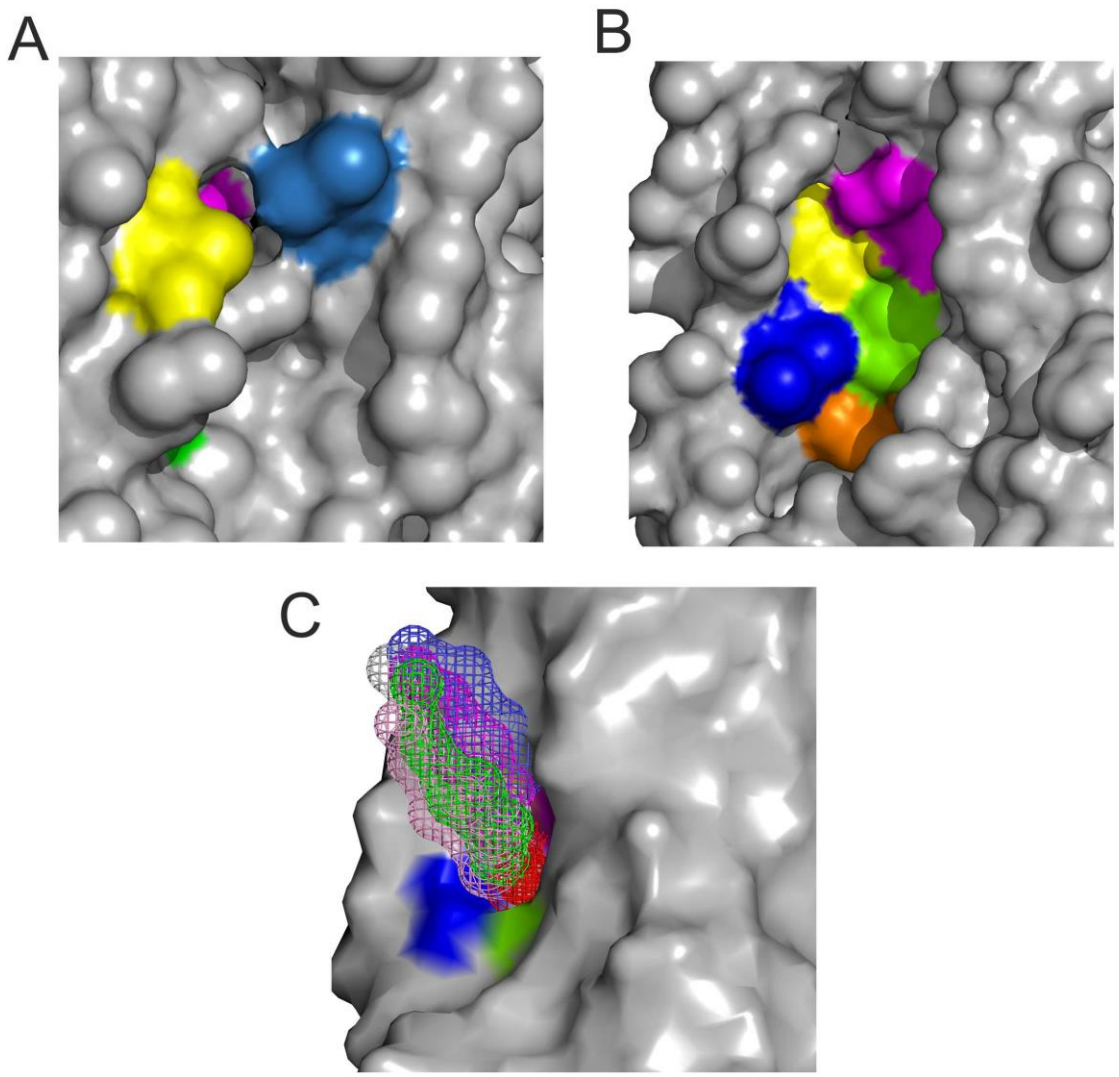


Fig. 4



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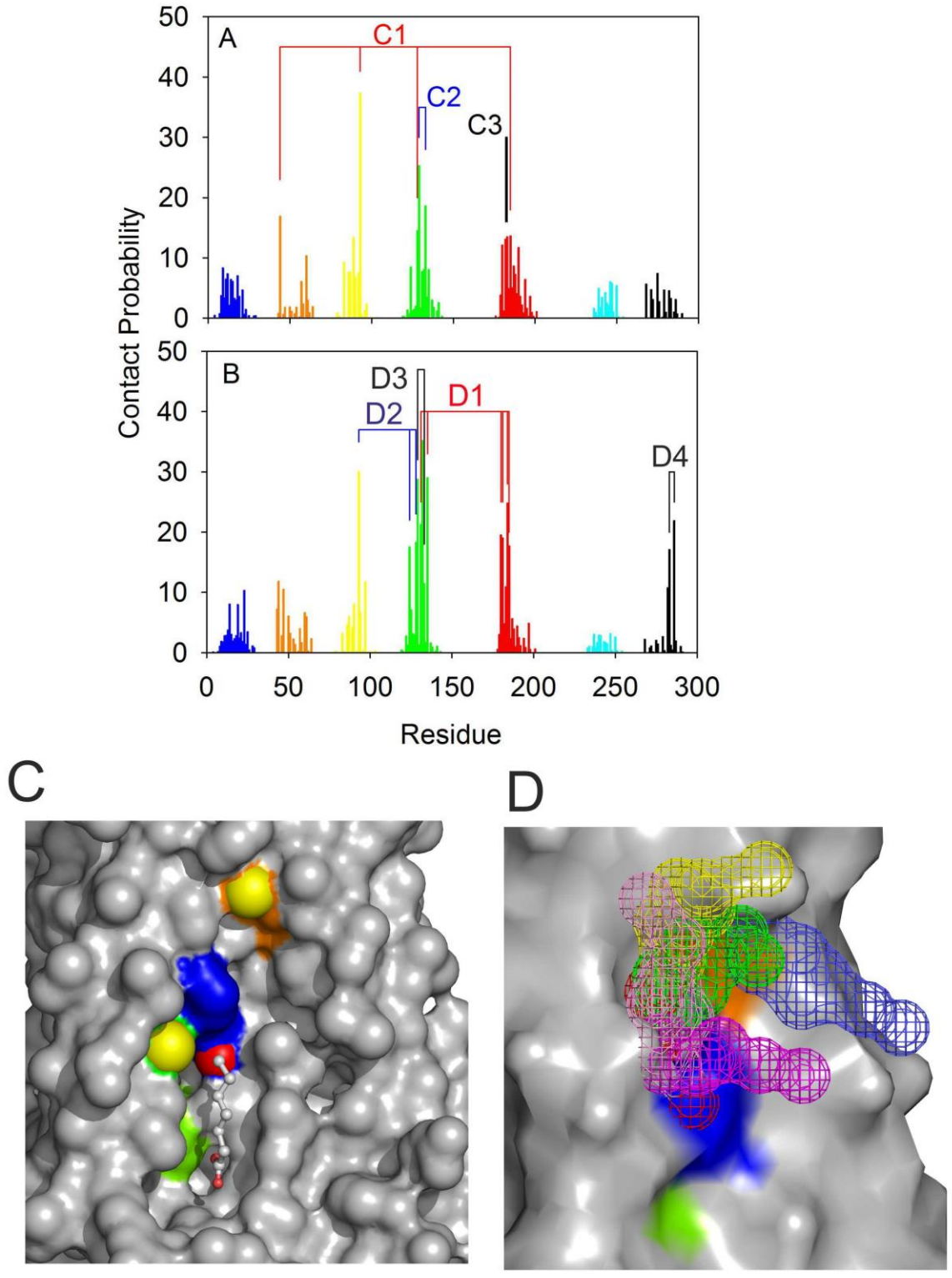

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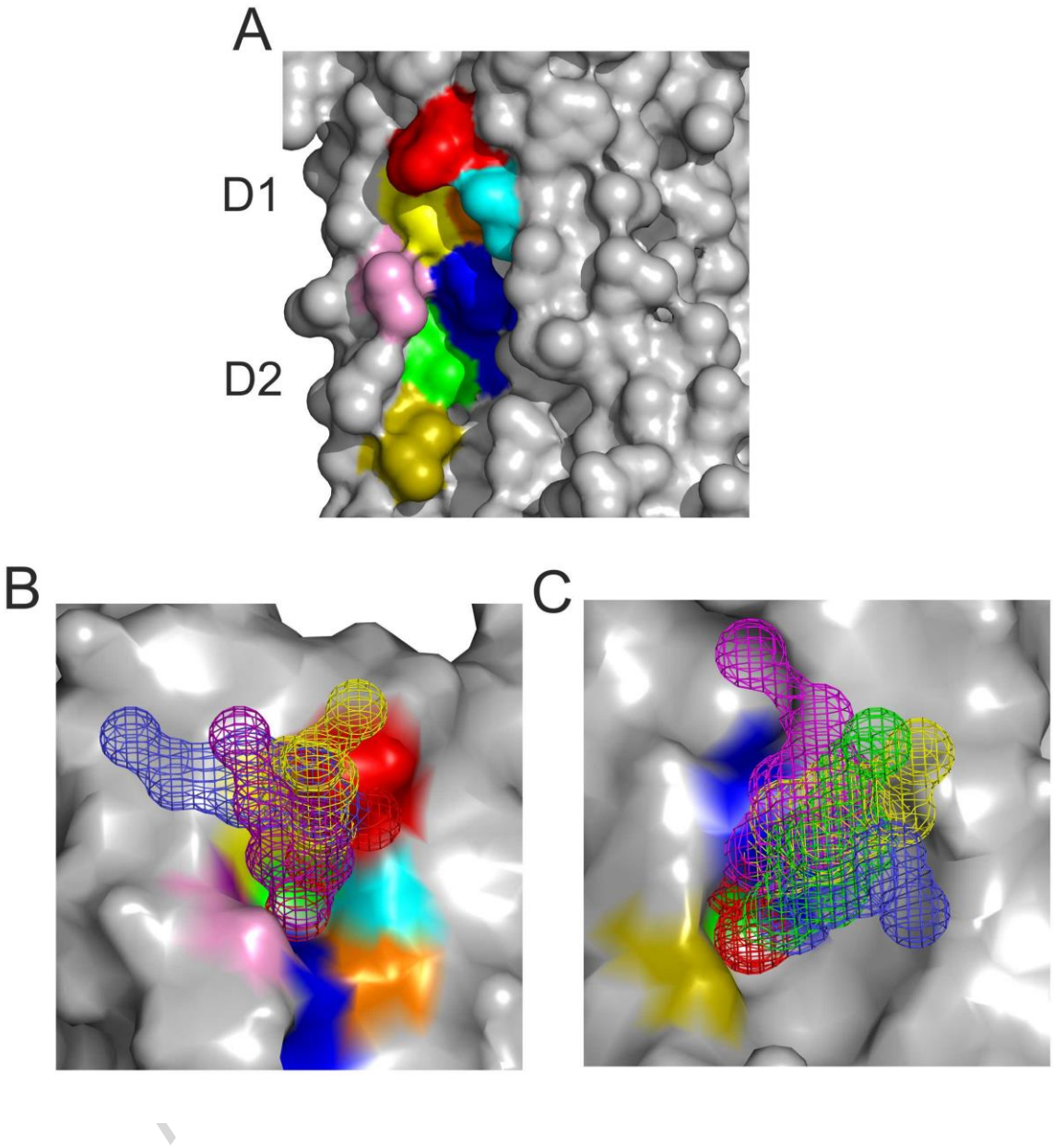

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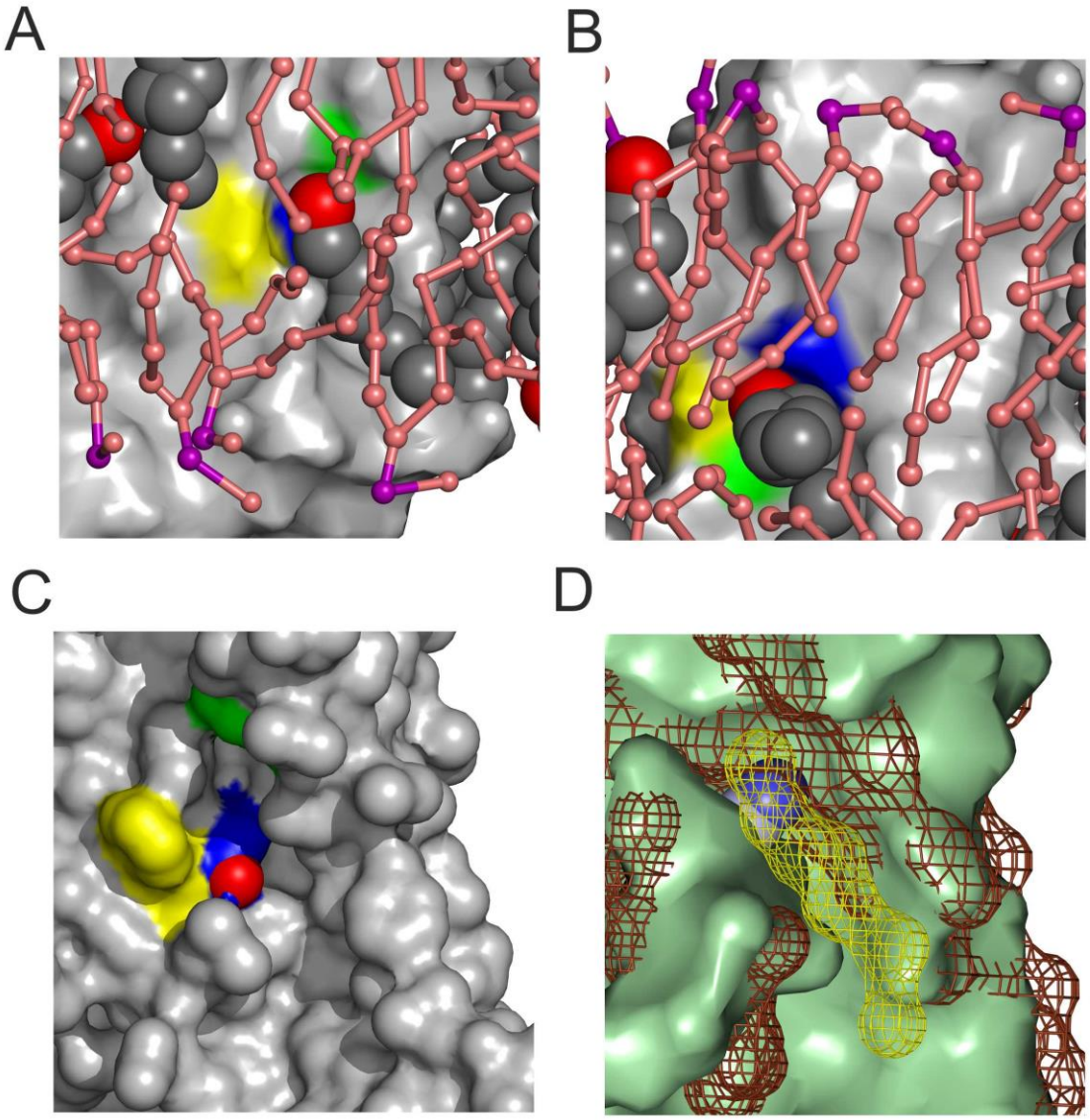


Fig. 8

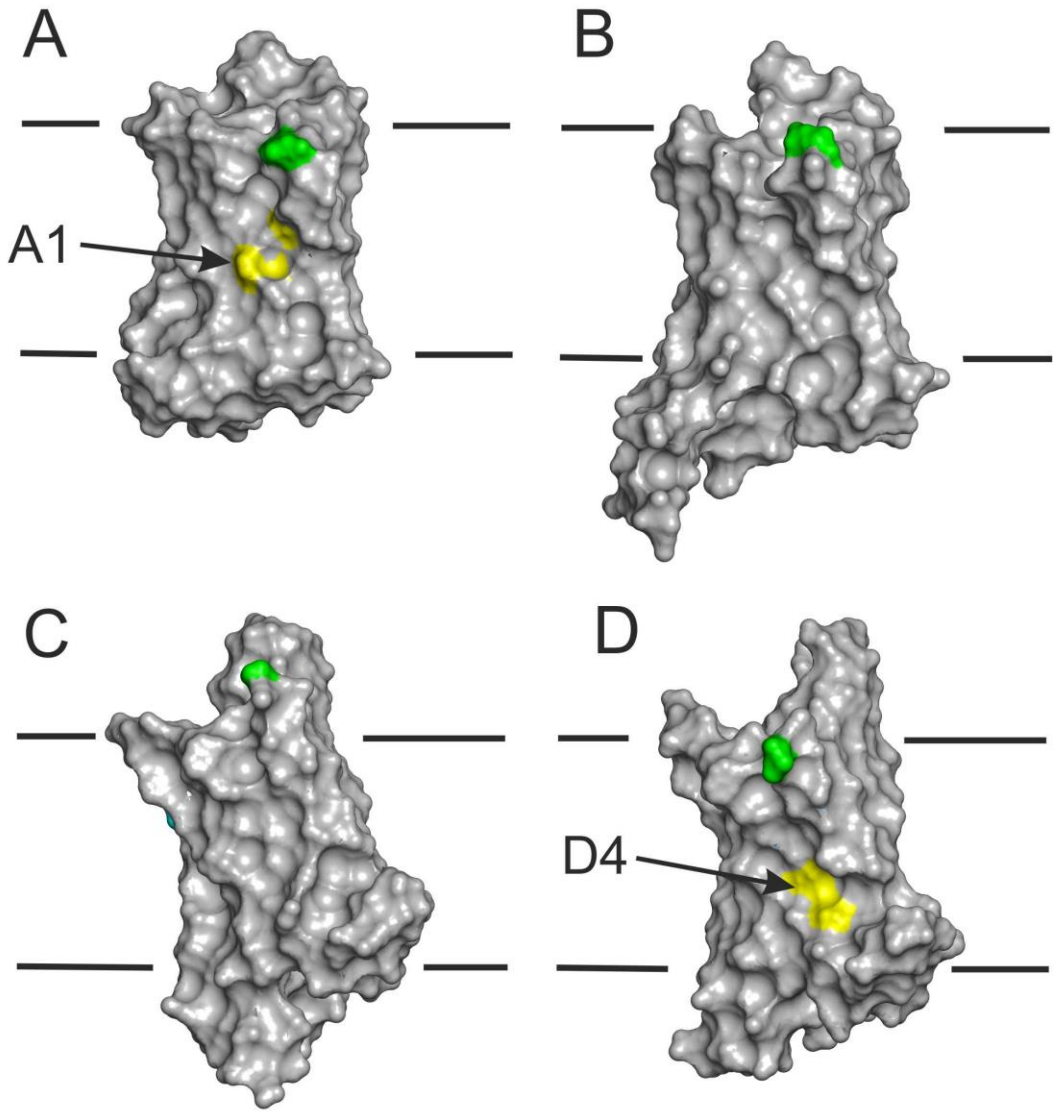


Fig. 9

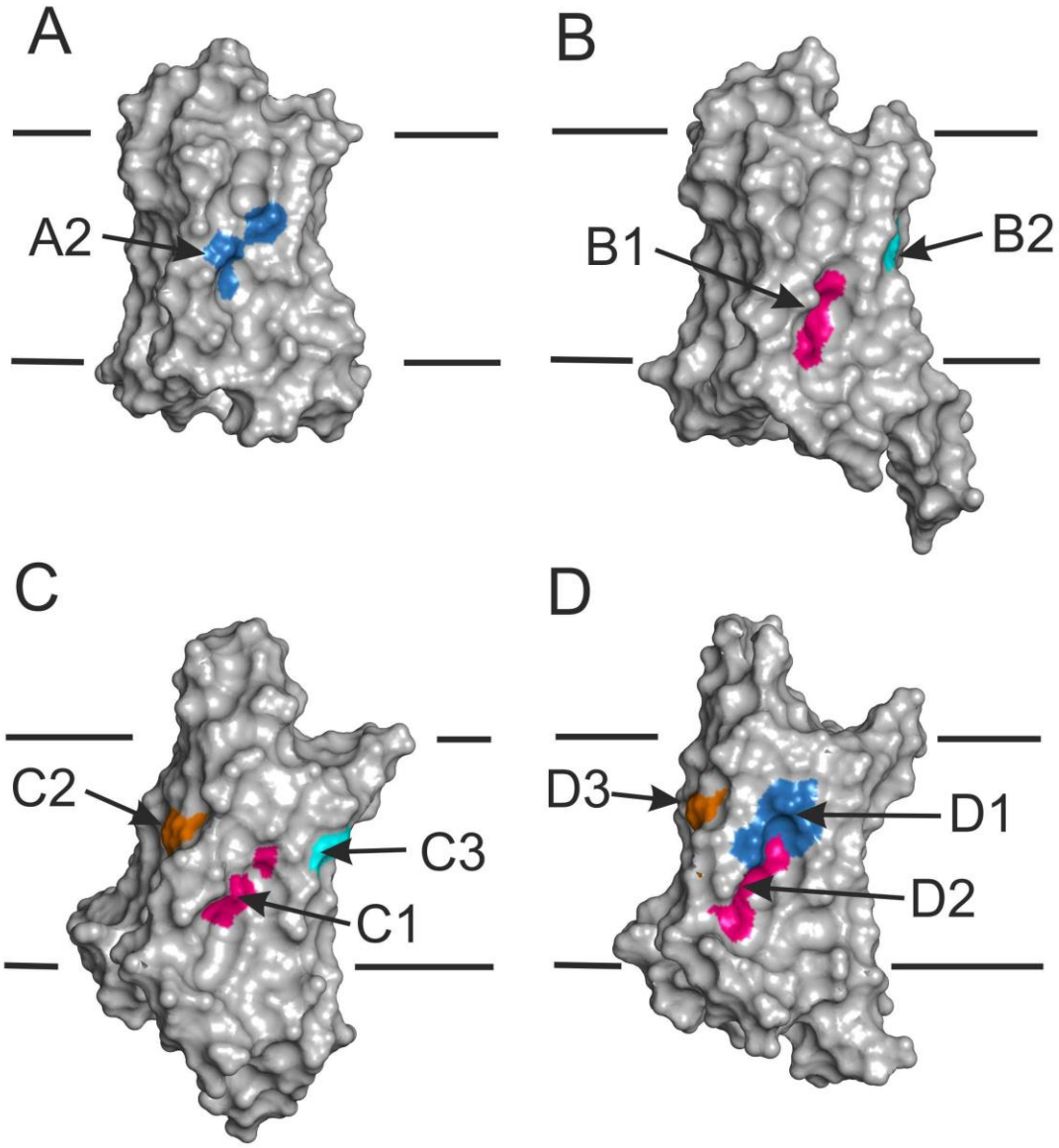
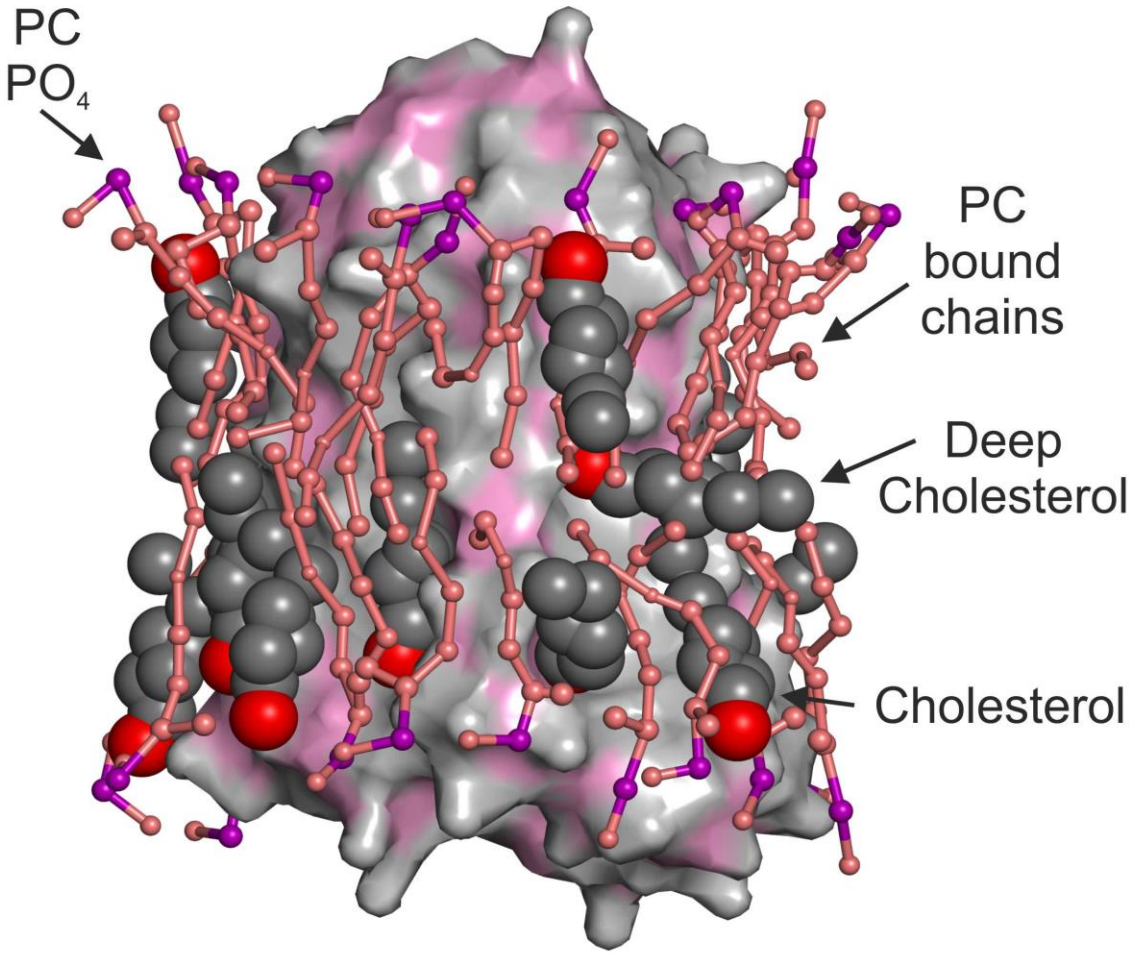


Fig. 10



Graphical abstract