Introduction

GPCRs are the largest family of membrane proteins, serving as key components of signal transduction pathways across cell membranes and as important drug targets. GPCRs are activated by diverse ligands, including odorants, fatty acids, peptides and neurotransmitters. GPCRs contain seven membrane-spanning $\alpha$-helical segments separated by alternating intracellular and extracellular loop regions, and are commonly divided into five families based on their sequence and structural similarity: (i) rhodopsin (family A, the largest and most diverse family of GPCRs); (ii) secretin (family B); (iii) glutamate (family C); (iv) adhesion; and (v) Frizzled/Taste2 [1].

Computational models of the 3D structures of GPCRs have become important tools for studying these receptors and for drug discovery [2-4]. The available crystallographic structures of family A GPCRs have recently been expanded to include the $\beta_1$-adrenergic receptor ($\beta_1$AR), the $\beta_2$-adrenergic receptor ($\beta_2$AR), the $\alpha_2$-adrenergic receptor (A$\alpha_2$R), squid rhodopsin and bovine opsin (reviewed in references [2,5-7]), in addition to the pioneering structures of rhodopsin [8,9]. The new structural data has broadened the knowledge of the conserved and variable structural features and dynamic properties of GPCRs, providing additional templates for homology modeling. Traditionally, GPCR models were validated by indirect mutational data analyses or measures of successful docking, estimated by improvement in enrichment factor [3,4]. More recently, modeling protocols have been tested and refined via direct comparison with experimental structures. The existence of multiple templates, as well as the apparent variability in loops, ligand-binding modes and activity states, create a situation in which many choices must be made to obtain optimal modeling. This review focuses on four, somewhat interconnected topics: (i) choosing and combining templates; (ii) using knowledge-based data; (iii) applying molecular dynamics simulations; and (iv) modeling loops.

Are more templates always better?

The availability of several different GPCR templates provides three main options in GPCR modeling: (i) the use of one template; (ii) the use of multiple templates; and (iii) fragment-based modeling (Figure 1). The selection of a single template may be based on the choice of a template with high sequence similarity, usually in the transmembrane (TM) helices [10], similarity in binding-pocket regions [11] or similar structural motifs [12]. For example, although A$\alpha_2$R has the highest sequence
similarity of any known GPCR to A₃AR, A₂AR has distinct structural features (e.g., three disulfide bonds in the second extracellular loop [ECL2]) that do not occur in A₃AR [13].

In an elegant study of β₂AR models, the effect of template choice was evaluated by examining whether docking poses from the largest cluster of flexibly docked ligands had a root mean-square deviation (RMSD) from the crystal structure of < 2 Å [10]. If all templates shared low sequence identity with the target, a multiple-template-based model obtained in the Modeller program [14] performed slightly better than single-template models [10]. The percentage of docking solutions with < 2 Å RMSD from the crystal structure obtained from multiple template-based models was slightly higher than the percentage obtained from single template-based models.

The benefits of using multiple templates for modeling the neurokinin (NK)1 receptor have been analyzed in terms of enrichment in virtual ligand screening (VLS) [15]. Models based on a single template were incompatible with mutagenesis data; however, the use of multiple-sequence alignment of the NK1, NK2 and NK3 receptors and bovine rhodopsin improved the results. A model based on two templates, bovine rhodopsin and β₂AR, provided similar or even better results [15].

The Critical Assessment of Techniques for Protein Structure Prediction (CASP) is an important community-wide effort to assess structure prediction, refinement and evaluation methods [16]. A study of CASP targets and additional reference sets demonstrated that the use of multiple templates can improve the quality of models, mostly via extension of the target-template alignment, as a result of the different sequence lengths of the templates [17]. Only one of three commercial modeling methods demonstrated some improvement in model quality beyond this alignment effect. Because poorer quality models are also created using the multiple-template method, the average model quality does not improve with increasing number of templates unless the data are ranked by a quality-assessment program [17].

Automated fragment-based methods have been demonstrated to be successful in CASP. One such fragment-based method is I-TASSER, a hierarchical approach to protein structure modeling in which fragments are excised from template structures and reassembled based on threading alignments [18]. The I-TASSER server performed well in predicting the structure of A₃₄R.
Knowledge is power: Data-driven homology construction

The incorporation of knowledge-based constraints has been successful in ab initio folding of modeled membrane proteins [20] and in various docking applications [21,22]. This section focuses on the incorporation of constraints in the context of the application of resulting models to the docking or virtual screening of family A GPCRs, which share features within their TM-bundle binding pockets [11]. Other characteristics of computational models, such as description of the intracellular regions, require further investigation and are thus not reviewed.

One particularly important initiative involved a community-wide modeling and docking experiment prior to the release of the structure of A2AR [19]. The most accurate model in terms of ligand RMSD and correct contacts was submitted by Stefano Costanzi (National Institute of Diabetes & Digestive & Kidney Diseases). The poses were selected and ranked on the basis of their docking scores and agreement with available mutational data, interpreted on the basis of previous modeling studies by the author (for additional details, see the supplementary information of reference [19]).

Additional successes, in terms of the percentage of correctly predicted native contacts, were also obtained by researchers who used ligand-based information [23]. Two of the research groups used LiBERO (ligand guided backbone ensemble receptor optimization). In the LiBERO method, known ligands of the receptor are docked into multiple conformations of the initial homology model. The resulting ligand-receptor models with acceptable conformational energy are clustered according to the conformation of the binding-pocket residues and are assessed by VLS. The best models performed as well as crystal structure data in selecting antagonists over decoys, antagonists of other adenosine subtypes and A2A agonists [23]. The quality of the ligand-receptor complexes predicted from ligand-docking into β2AR homology models was improved by applying a knowledge-based filtering procedure on docked poses [Levit A, Niv MY: unpublished data].

Ligand-supported homology modeling was applied to the construction of a model of metabotropic glutamate receptor 5 (mGluR5), which was then used in a VLS test [24]. The mGluR5 is a member of the C family of GPCRs, for which no example of an experimental structure has been published. The available mutational data for mGluR5 were ambiguous and not sufficient to determine a binding pose with the predicted interactions; therefore, ligand-binding mode information from the whole GPCR family, obtained from publications from 2005 and 2006 [25,26], was added. Renner and colleagues identified a possible binding mode that correlated with the observed SARs and mutational data, and used these data to create and refine a model for docking. A database of drug-like molecules was then docked to this model by a VLS protocol. Finally, information on the patterns of interactions between known ligands of the receptor and the binding-site residues was used to rank the docking solutions (by interaction fingerprint similarity scoring approach). This approach was able to successfully discriminate between known active and inactive ligands of the receptor [24].

Ensembles of models of melanin-concentrating hormone receptor 1 (MCHR1) were constructed based on bovine rhodopsin [27]. The positions and orientations of the ligands were randomized and minimized using knowledge-based distance constraints between atoms in the ligand and receptor. A model that recognized diverse antagonists was used in virtual screening, resulting in enrichment, with more than a 10-fold improvement over high-throughput screening [27].

A systematic rotation of the TM helices of a bovine rhodopsin-based model of β2AR (constructed prior to the publication of the structure of β2AR) was combined with energy minimization to predict the conformational changes that occur upon ligand binding [28]. The structures predicted for models using full agonist, partial agonist and an inverse agonist correlated with other published data [29,30]. Because the X-ray structure of β2AR has since been published [31], a retrospective analysis of these models would be of interest.

The examples highlighted in this section illustrate the potential benefits of incorporating ligand information, site-directed mutagenesis and other experimental data as constraints in modeling and docking protocols. However, experimentally derived constraints should be applied with care; for example, when cocrystallized with A2A, R, the high-affinity A2A antagonist ZM-241385 is closer to the interface of TM6 and TM7, and higher in the TM bundle compared with the location of several ligands when cocrystallized with other GPCRs [6]. The effects of mutagenesis can also be ligand-dependent, providing different results for agonists and antagonists and generating a high-resolution, multiple-template model with a Cα TM RMSD of 2 Å, but was unsuccessful in predicting the ligand-binding mode [19]. I-TASSER has been used recently to model a bitter taste receptor in order to predict ligand-binding residues, which were confirmed experimentally [Brockhoff A, Behrens M, Niv MY, Meyerhof W: unpublished data]. In summary, the use of multiple templates may improve models, and automated fragment-based methods may be a good and practical choice because the GPCR structures and fragments from additional templates will be selected automatically. Additional testing and GPCR-focused optimization of quality-assessment programs for such models is warranted.
potentially even for different chemotypes exhibiting the same pharmacological effects (see reference [11] and references therein). Furthermore, allosteric binding sites exist for some receptors [32]. In a study that did not relate to GPCRs that attempted to account for variations in the ligand-binding pocket on binding to different ligands from a compound library [33], 'consensus' enrichment was calculated by combining results from docking to multiple structures of the same target, and ranking each compound using the best docking score across all docked structures. The use of this strategy for multiple models was either comparable to or better than the use of single holo and apo X-ray structures. Thus, a combination of individual knowledge-supported models was proposed to provide a good platform for alleviating the bias that could result from using a single model in a virtual screening application.

Applying molecular dynamics
An important tool for exploring the conformational space of proteins and other large atomic-scale structures is molecular dynamics (MD) simulations. With the improvements in the speed and accessible timescales of MD simulations that have occurred in the past few years [34], long time-scale MD simulations of GPCRs in a membrane environment have become feasible. MD simulations of X-ray structures have provided important insights into the structural details of GPCRs [29,30,35]. For example, MD simulations were able to provide additional understanding of the GPCR 'ionic lock' feature, which is a salt bridge between two highly conserved residues: Arg3.50 of TM3 and Asp/Glu 6.30 of TM6. The salt bridge interaction holds the cytoplasmic ends of TM3 and TM6 in close proximity and stabilizes the inactive state; disruption of the ionic lock is an established marker of GPCR activation [36]. Surprisingly, the ionic lock is not present in the inactive crystal structures of turkey β2AR [37], human β2AR [31] and human A2aR [38]. This absence could be an artifact resulting from the crystallization procedure, but could also suggest that the ionic lock is not a general constitutive activity-reducing interaction for family A GPCRs. Importantly, MD simulations of wild-type β2AR conducted in the presence or absence of a bound antagonist demonstrated predominant formation of the ionic lock [29,30]; however, in constitutively active mutant forms of the protein, the equilibrium shifted toward conformations containing a broken lock [29]. These results support the hypothesis that the ionic lock stabilizes the inactive conformation of the receptors.

Are molecular dynamics simulations also helpful for models?
There are several methods that address issues relating to model refinement or the 'last mile of protein folding' [39]. MD-based methods performed well in the refinement category in CASP8 [39] and have been used in other refinement protocols [40]. In some cases, the use of MD with periodic boxes containing explicit water molecules was not as effective as models that employed implicit solvent minimization [41]. For small- to medium-sized proteins, implicit solvent replica-exchange refinement MD simulations provided reasonable convergence when reliable structural information of the initial models was incorporated using restraints [42]. For example, symmetry-restrained MD simulations were reported to improve homology models of potassium channels [43].

Several recent MD-based simulations relating to GPCR modeling are highlighted in the following paragraphs. Docking performed with the short ghrelin derivatives Gly-Ser-Ser Octa-Phe-NH₂ and Gly-Ser-Ser Octa-Phe-Leu-NH₂, enabled the identification of a well-defined position of these peptides in the active site of the receptor, sharing common interactions with active-site residues [44]. The model that was obtained in this manner was further refined by MD simulation and validated by docking experiments performed on a set of 55 ligands of the ghrelin receptor [44]. In addition, an enhanced sampling of collective MD simulation coordinates has been used to study possible pathways for entry of the opioid antagonist naloxone into the accepted binding pocket of a δ-opioid receptor [45].

A homology model of the human histamine receptor hH4R was constructed based on bovine rhodopsin complexed with an agonist (histamine) or a selective antagonist (JNJ-7777120; Abbott Laboratories/Johnson & Johnson Pharmaceutical Research & Development LLC), and the model was subsequently subjected to MD simulations in a membrane-embedded environment [46]. These simulations enabled the elucidation of important interactions and helix motions, which were in agreement with mutational and other experimental data. Novel interactions between amino acid residues could be predicted by subjecting a bovine rhodopsin-based homology model of gonadotropin-releasing hormone receptor to long time-scale (160 to 200 ns) MD simulations that included explicit lipid and water molecules [47].

Pressure-guided MD simulations, termed 'balloon potential', have recently been described [48]. The application of increasing pressure to the binding site causes the site to expand in a manner similar to inflating a balloon. For example, in a relaxed structure of bovine rhodopsin, from which retinal had been detached, the binding pocket was occluded; when pressure was applied to the binding site, the side chains moved and allowed retinal to dock in an approximately correct pose. Moreover, a model of the chemokine receptor CCR2 that could not accommodate known ligands when initially refined with retinal was able to dock three known ligands in orientations consistent with published mutagenesis data following a pressure-guided simulation [48]. Although some caveats are associated with this method, such as the difficulty in deciding what pressure to apply and which constraints to use, the strategy is
Homology modeling of GPCRs

Yarnitzky et al

promising and should be tested further with available GPCR complexes.

GPCR oligomerization is an important feature in GPCR signaling and has begun to emerge as a novel target for selective drug design [49,50]. Various computational approaches (eg, MD [51], normal mode analysis [52] and Brownian dynamics [53]) have been applied to study oligomerization. In a recent study, 40-ns MD trajectories of the dimeric assembly between the TM regions of a heterodimeric mGluR2/5-HT$_{2A}$ complex (TM4/TM5 interface) were compared with that of a 5-HT$_{2A}$ monomer [54]. An analysis of the binding site demonstrated that the formation of the interface between the two protomers in the dimer allosterically affected the shape of the binding pockets of the individual protomers. Lysergic acid diethylamide (LSD; a hallucinogen) and serotonin (a non-hallucinogenic natural neurotransmitter), both of which are 5-HT$_{2A}$ agonists, were docked into the 5-HT$_{2A}$ binding pocket in both the dimeric and the monomeric models. LSD did not dock well in the 5-HT$_{2A}$ monomer, but could be docked into the 5-HT$_{2A}$ binding pocket of the heterodimer. In contrast, serotonin, could be properly docked to the monomer, but not into the heterodimer as a result of the reshaping of the binding pocket [54].

In summary, MD simulations (illustrated in Figure 2) have been used to investigate GPCR functional dynamics, to improve models in the context of experiment-derived constraints and to provide new insights into interactions with ligands and allosteric influences on binding sites. Although these observations are promising, possible difficulties associated with convergence should be addressed. Further validation and an improved understanding of the limitations of the methods are needed. Additional validation studies are now possible as a result of the recently expanded availability of experimental GPCR structures.

Modeling of extracellular loops

The role of GPCR extracellular loops in binding high-molecular-weight peptidic ligands is well established [55]. Recent studies have demonstrated that the extracellular loops, specifically ECL2, also interact with low-molecular-weight ligands, such as biogenic amines or adenosines [56]. Furthermore, several site-directed mutagenesis studies have highlighted the effects of mutations in ECL2 on agonist and antagonist binding, reinforcing the importance of this region for ligand binding and structural integrity [56]. The recently reported X-ray structures of various GPCRs demonstrate that these extracellular loops also differ greatly in their structural features [5]. Furthermore, NMR studies have revealed the dynamic and ligand-dependent character of ECL2 in β$_{2}$AR [57], and the activation-induced and TM5-coupled changes in the rhodopsin ECL2 [58]. The accurate modeling of these loops is therefore non-trivial and important for comprehending ligand recognition and functional attributes of the structure, as well as for docking quality and VLS.

If the structures of membrane proteins are similar, comparative loop modeling is a potential strategy. The SuperLooper server provides a database containing 180,000 loops in membrane proteins (LIMPs) [59], enabling the prediction of multiple knowledge-based conformations of the loops. Another strategy for modeling loops that is being actively pursued is the de novo restoration of loops with the aim of improving the

Figure 2. Reshaping of the receptor binding site during molecular dynamics simulations.
description of longer loops [60-63]. Loops that are > 13 amino acids in length, such as ECL2, present a formidable challenge. The results from several research groups that have evaluated the effect of ECL2 modeling on the quality of GPCR homology models are reviewed in the following paragraphs.

Two models of β2AR based on the structure of bovine rhodopsin were created, in which ECL2 was structured either according to rhodopsin homology modeling or de novo, and known restrictions on ECL2, such as the occurrence of a disulfide bridge, were enforced [64]. The two models demonstrated high overall similarity with the X-ray structure of β2AR. Loops without gaps in alignment (first intracellular and extracellular loops) were well modeled, but longer loops that contain such gaps were less suitably modeled. The de novo modeled ECL2 loop performed better in predicting solvent exposure, having a smaller RMSD of the binding-pocket-facing residues and docking carazolol in a similar manner to the experimental structure [64]. If de novo modeling is problematic, as a result of loop length or incompatibility with or lack of experimental data, the use of models without ECL2 is suggested by the author.

Another study evaluating β1AR models suggested that loopless models represent a better alternative for flexible-ligand/rigid-protein docking, because loop configurations, particularly ECL2, are highly variable, as illustrated in the existing GPCR crystal structures [10]. The impact of ECL2 conformation on VLS has been assessed by docking studies with rhodopsin-based β1AR homology models; VLS in the absence of ECL2 provided high enrichment factors and hit rates [65].

Modeling the A2AR ECL2 based on bovine rhodopsin is also problematic, as a result of the low similarity between the two structures [66]. A2AR has been modeled based on either rhodopsin [8] or β2AR [31] crystal structures, with the β2AR-based model producing better docking modes, consistent with the higher similarity of A2AR ECL2 to the β2AR compared with the rhodopsin crystal structures [66].

A recent de novo method progressively constructed loops by geometrical sampling of dihedral angles, starting from stem residues in the TM helices, to select possible loop-closing backbone conformations [67]. The energy was calculated for all the selected conformations of the individual extracellular loops and then for the selected ECL1-2-3 combination of loops. The docking of ligands to structures with predicted loops was successful and compared well with docking to loopless X-ray structures of β1AR and β2AR, although less satisfactory results were obtained for A2AR [67].

The effect of ECL2-modeling strategies on VLS has been evaluated for three targets: (i) the dopamine D2 receptor; (ii) the A2AR; and (iii) the thromboxane A2 receptor [55]. These test cases were selected based on experimental evidence for the involvement of ECL2 in antagonist binding, and because they represent different GPCR subfamilies and vary in their ECL2 lengths. Based on the VLS performance of the models, the researchers recommended using loopless models of GPCRs for virtual screening, unless high homology to existing structures or receptor-specific experimental data were available to enable the construction of a complete high-quality model [55].

The available approaches to loop treatment, namely homology modeling, the use of loopless models and de novo prediction, are summarized in Figure 3. The emerging consensus is that models perform better without modeled loops at all than with badly modeled loops, and that de novo modeling approaches are sufficiently promising in certain cases to warrant further development.

**Conclusion**

The research field of GPCR modeling has been separated to some extent from the general protein structure prediction field as a result of the lack of experimental GPCR structures, and the strong focus on drug design and screening applications. With the increase in available experimental structural information in recent years, novel strategies that are being continuously developed via the CASP community-wide experiments are being adopted, and are being combined with the practical and powerful knowledge-based methods that are well established in the GPCR field.

The studies described in this review suggest multi-template or fragment-based modeling as a promising direction that requires careful model assessment and will benefit from additional GPCR templates. The informed and cautious incorporation of mutational and other knowledge-based constraints is advantageous for docking and VLS quality. Progress in MD simulations has made these a useful tool for GPCR studies, and the applicability of MD simulations to homology models warrants further exploration. Finally, long-loop modeling...
remains a significant challenge. Although de novo loop modeling techniques are improving, loopless models provide a practical alternative in certain cases.

Among the exciting topics that will become increasingly accessible via a synergistic combination of experimental data and computation are ligand-specific interactions, activated receptor conformations, allosteric binding sites and oligomerization. The available and anticipated GPCR structures are creating exciting possibilities for advancements in modeling and for the increasingly rigorous validation of modeling, refinement and assessment methods. It is hoped that community-wide modeling experiments for any new GPCR structure will be routinely conducted prior to the release of the full data, as these experiments are likely to advance the field of GPCR modeling.

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References


- Protein Structure Prediction Center: University of California, Davis, CA, USA (2010). predictioncenter.org


• A thorough molecular dynamics study of wild-type and mutated β2AR based on the recently published X-ray structure. The study reconciled the discrepancy between biochemical and structural data regarding the role of the TM3-TM6 ionic lock in the stabilization of the inactive state of GPCRs.


• Presents a novel method for binding pocket expansion using a pressure-guided MD simulation.


- An interesting study that proposes a de novo method for modeling extracellular loops using an unsophisticated force-field and a coarse sampling grid to provide energetically reasonable conformers of the loops.