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Perspective

G-Protein Coupled Receptors: Models, Mutagenesis, and Drug Design

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1.0. Introduction

During a typical career in medicinal chemistry, most chemists will run across a G-protein coupled receptor (GPCR) as a drug target. Almost invariably, the interest in the receptor will lead to the construction of a three-dimensional model as an aid in interpreting ligand binding and molecular biological data. The quality and usefulness of this model will depend on the assumptions made in its construction and by its supporting biophysical and molecular biological data. These models are typically used for three functions: to visualize the protein interior and to propose modes of ligand binding, to plan mutagenesis experiments, and to support ligand design. This perspective will critically assess the recent trends in model construction, the reliability of models themselves, and their impact on the drug discovery process.

1.1. Biological Background. The guanine nucleotide coupled receptors constitute a superfamily of proteins whose function is to transduce a chemical signal across a cell membrane.[1] This mechanism provides communication between the exterior and the interior of the cell. Such a process requires that the signal transduction be specific to the initiating impulse, and have well-defined intracellular sequelae. Since the impulse may be a very weak signal, amplification of the initial signal may be necessary.[2] In the GPCR, binding of the chemical messenger leads to the association of an intracellular G-protein, which in turn is linked to a second messenger pathway. Typically, the G-proteins either stimulate or inhibit the production of the second messenger.[3] The changing concentration of second messenger can generate an action potential if it leads to the opening of ion channels. Taken together, the GPCRs and second messenger systems form a regulatory loop, with provisions for feedback, signal modulation, and signal amplification.

Individual GPCRs have evolved to recognize a bewildering array of incoming signals. Sequence variation in the interior of the protein and the extracellular loops ensure that the protein activates only in response to a unique signal. The incoming signal may arrive in the form of a photon, a monoamine neurotransmitter, a peptide hormone, a glycoprotein, or gustatory or olfactory functional groups.[4] The receptors themselves can even act as the agonist; the thrombin receptor requires the enzymatic cleavage of its N-terminus, which in turn activates the receptor.[5] Despite the variety of incoming signals, the transmembrane structure and a number of interior residues appear common to all members of the family.

The arrangement of seven transmembrane helices (7TM) into a membrane-spanning bundle was an early evolutionary innovation, as evidenced by its existence in archaeabacteria, in viruses, in yeast, and in eukaryotic organisms. It has since been extensively adapted in almost all forms of life on earth. The ring topology is favorable for transmembrane signal transduction, either by ion transport as in the archaeabacteria,[6] or some form of conformational switching, as has been proposed for the GPCRs.[7] Alignment of the GPCR sequences reveals very few conserved residues; this

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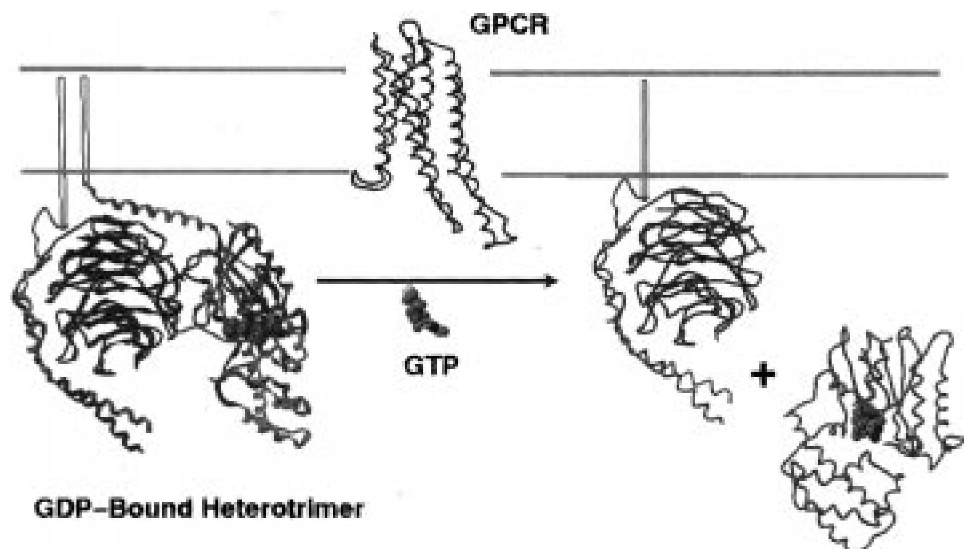

Figure 1. Figure 1 shows a G-protein in its inactivated form. The protein has three subunits: the α (in red) with GDP bound, the β (green), and the γ (magenta) subunits. Upon activation, the GPCR (blue) binds, thus catalyzing the exchange of GTP for GDP. The bound GTP causes the G-protein to dissociate by destabilizing the alpha/beta interface. The α subunit effects a change in the concentration of second messenger, by either activating or inhibiting a second enzyme protein (e.g., adenylate cyclase, guanine cyclase, phospholipase C). The β-gamma subunit has also been shown to be linked to a feedback pathway. In the figure above, the structures of the heterotrimer (1gg2[154]) and activated α subunit (1gia[155]) were obtained from the Brookhaven protein databank.

suggests that the transduction mechanism must either tolerate fairly radical changes in the residues lining the receptor cavity or that different mechanisms exist for individual receptors. A third possibility is that receptor activation is composed of a cascade of events, of which the individual elements may vary, but which collectively produce the required effect.

GPCRs are important both in understanding and in treating disease. Because of their integral role in cellular signaling, GPCR dysfunction can lead to illness. Reversal of these aberrant effects can often contain, if not cure, many forms of disease. The GPCRs are therefore the primary target for a great number of drugs. Recent estimates suggest that up to 60% of the modern pharmacoepia is targeted to GPCRs.[1] Information and models that help us understand how these molecules interact with the receptor can therefore be of great practical interest to the medicinal chemist.

1.2. G-Protein Association and Signal Transduction. A GPCR is the first component of a complex and versatile transduction pathway. Figure 1 shows a general schematic of the activation of a G-protein. Ligand binding is followed by a change in the state of a receptor to one with increased affinity to G-proteins. The receptor binds to the α subunit of the G-protein, which in turn catalyzes the exchange of GTP for GDP. The GTP binding then causes the alpha subunit to separate from the β and γ subunits. The α subunit of the G-protein then couples with an enzyme that catalyzes the production of second messenger molecules, typically cyclic AMP, cyclic GMP, diacyl glycerol, or IP3.[3] The resulting change in concentration of the second messenger causes further downstream effector events—for example, the opening of ion channels—which can ultimately trigger an action potential.[1,7,8]

G-proteins are connected to either excitatory or inhibitory pathways.[9] Thus, the effect caused by one message (e.g., a neurotransmitter) can be modulated by a second message (e.g., a neuropeptide). Complex interrelationships are thereby built up, which can initiate and modulate a variety of incoming signals.

Furthermore, intracellular conditions can affect the sensitivity of the receptors for their ligands. Early on, states were identified in which the receptors had either a high or low affinity for ligands. Both GTP binding and G-protein association affected the equilibrium between these states.[1] In addition to these, cytosolic phosphorylation inactivated the receptors, and consequent protein internalization regulated receptor response and allowed receptor regeneration.

1.3. Scope of Review. As noted above, this review is a critical assessment of the current accuracy and utility of 3D models. It will focus primarily on the role of receptor models and the impact they have had in furthering our knowledge of receptor function and molecular interactions. In this respect, 3D models have had a pivotal role in guiding molecular biological experiments such as site-directed mutagenesis. Since the actual construction of receptor models is covered by a number of excellent and exhaustive papers,[10−20] only recent trends and innovations will be described. Similarly, the amount of mutagenesis data is daunting, both due to the sheer number of experiments and because they are now available across many receptors.[21,22] We have therefore chosen to focus on a limited number of experiments that reveal elements of receptor structure or function, or that demonstrate the complex interplay of model and experiment.

2.0. Biophysical and Structural Foundations of Model Building

2.1. Bacteriorhodopsin. Fortuitously, the proton pump from halobacterium halobium, bacteriorhodopsin, crystallized in a form amenable to electron cryomicroscopy. The resulting 3.5 Å structure is one of the few structures of membrane proteins available. It is also

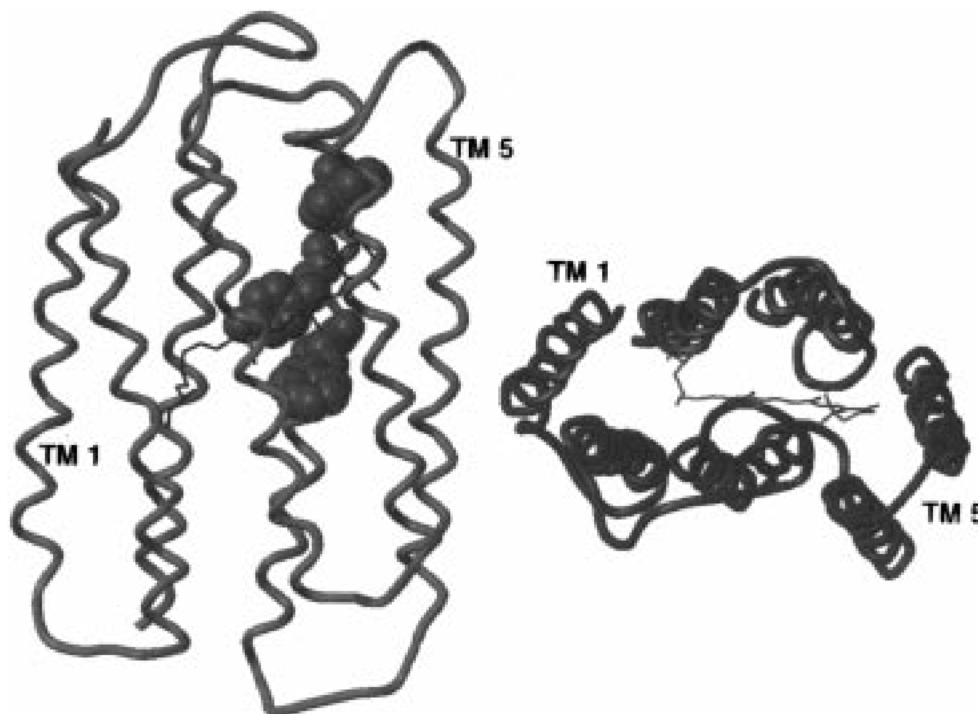

Figure 2. Two views of the most recent structure of the template protein, bacteriorhodopsin (2brd[23]). To the left, the retinal is shown covalently bound to TM7 and a number of the aromatic residues surrounding it are highlighted. To the right, a view of the protein showing the counterclockwise arrangement of helices.

the only 7TM protein with resolution suitable for homology modeling and has therefore been used extensively as a template upon which to develop models of GPCRs. Recently, a revised electron cryo-microscopic structure of this important protein has become available,[23] which differs from the earlier structure by a revision in the location of TM4 and by the addition of extracellular and intracellular loops. A complementary synchrotron structure is now available, which is of the protein crystallized in lipidic cubic phases and has been solved at 2.5 Å resolution.[24] In most essentials, the three structures are in agreement. The following discussion is valid for all three, unless noted.

Figure 2 shows a side and top view of bacteriorhodopsin.[23] The helices pack in an antiparallel fashion that form a counterclockwise ovoid, with TM1 and TM5 occupying opposite ends of the long axis. The retinal molecule is covalently linked by a Schiff base to the epsilon nitrogen of lysine on TM7, and its β-ionone ring projects to a hydrophobic pocket adjacent to TM5. The cavity surrounding the conjugated chain is lined by aromatic residues which are tightly packed around the ligand. The all trans form of retinal is shown bound; this is the light-adapted ground state of the protein.[6] Upon illumination with the appropriate wavelength of light, the retinal undergoes interconversion to its 13-cis form, which initiates proton pumping. Although generally accepted, the details and sequence of this interconversion remain somewhat controversial.[25] A structure of the bound 13-cis form is still unavailable.

The residues lining the cavity of bacteriorhodopsin perform three separable functions; they spectrally shift the retinal's absorbance to 568 nm, they provide the components of the proton pathway for pumping, and they effect the thermal isomerization of the 13-cis form of retinal back to the all-trans form.[26,27] How bacteriorhodopsin accomplishes this is of interest as it affects our understanding of what processes may function within a GPCR.

Side chain polarity is principally responsible for the spectral tuning. Polar and polar aromatic residues which line the cavity adjacent to the conjugated chain of retinal accomplish most of this spectral tuning. This is directly related to the degree of delocalization of the π electrons in the conjugated chain of the retinal. Polar residues adjacent to carbons along the chromophore chain stabilize more of the electron density and increase the delocalization. Increasing delocalization shifts the absorption band into the red; reducing it shifts the absorption to the blue.

The protein interior can also affect the ionization of protein residues. For example, recent IR and NMR assignments suggest that Asp 115 (Figure 3) exists in its neutral form.[28] Similarly, the basis for the proton pumping action is a transfer of a proton from a Schiff base to an acidic aspartate residue.[6,27]

Residues on TM3 and TM7 form the components of a hydrogen bonding network, which provides the pathway along which the proton is transferred.[6] The proton transfer path involves a chain of charged residues. Figure 3 shows the important residues and highlights the discontinuity between Lys 216 and the intracellular Asp 96. Water molecules connect these two residues in the proton pump. Their presence and their importance has been demonstrated by FTIR measurements, but they only appear in the synchrotron structure.[24] Asp 85 performs a critical function as a counterion and proton acceptor. For comparison, Asp 212 appears to have no functional role; it serves to form a hydrogen bonding cage with conserved tyrosines on adjacent helices. Hydrogen bonding involving aromatic residues

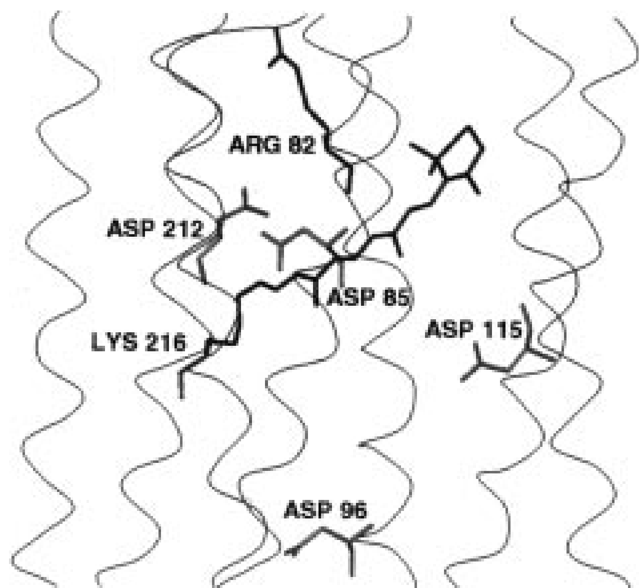

Figure 3. The principal residues responsible for proton pumping in bacteriorhodopsin are shown. The interconversion of *all-trans-* to 13-*cis*-retinal results in a transfer of the Schiff base proton to Asp 85. It is then transferred to solvent, possibly mediated by Arg 82. The reprotonation pathway stretches from Asp 96 to the Schiff base, most probably via a proton wire of water molecules. Asp 115 is not ionized, and Asp 212 has a structural role.

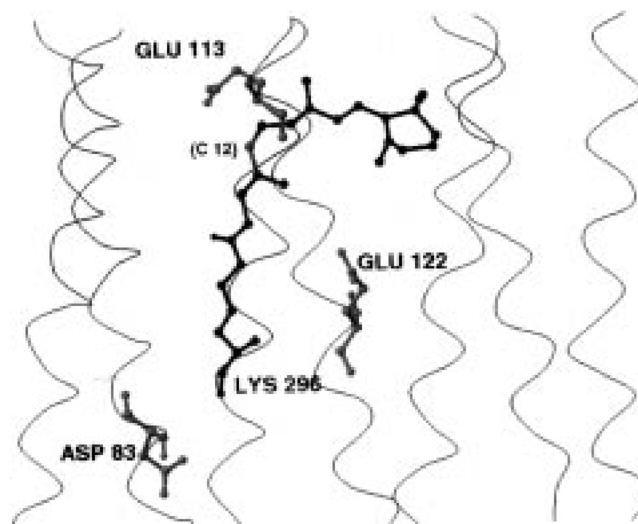

Figure 4. An illustrative model of ionizable residues in the transmembrane portion of rhodopsin. The 11-*cis*-retinal is covalently linked by Schiff base to Lys 296. The counterion to the consequent positive charge is Glu 113, located adjacent to C-12 of retinal (highlighted). Asp 83 on TM3 and Glu 122 on TM3 are not components of the charged system; indeed, both are most likely uncharged (see text).

appears to be an important mechanism by which the protein maintains its structural integrity.[6,24]

The final stage in the cycle is the thermal regeneration of the native protein. Hydrophobic residues such as leucine provide the steric contact responsible for interconversion.

2.2. Rhodopsin. Rhodopsin serves as a link between bacteriorhodopsin and the GPCRs in that, like bacteriorhodopsin it binds an isomer of retinal, but, like GPCRs, it couples to a G-protein. Extensive structural, biophysical, mutagenesis, and spectroscopic studies are available, which are comparable to those performed on bacteriorhodopsin.

Recent electron cryo-microscopic analyses of bovine rhodopsin[29] at 9 Å resolution and of frog rhodopsin[30,31] at 6 Å resolution show a structure that has more of a teardrop or kidney shape than bacteriorhodopsin. The density footprint reveals four helices that are located perpendicular to the cell membrane. An additional arc of density has been recently resolved to three helices located at an angle to the membrane. This arc includes the density now assigned to TM3. The successive contours available show a structure which varies from an arrangement comparable to that of bacteriorhodopsin toward the extracellular surface, to one which changes to a more tightly packed structure near the intracellular surface. It appears that the tilt of TM3 is largely responsible for this transition.[29] Unfortunately, the resolution is still inadequate to determine the location of individual residues.

Some additional structural information has been acquired through NMR experiments. As shown in Figure 4, an oxygen of Glu 113, the Schiff base counterion, has been shown to lie adjacent to C12 of the retinal chain.[32] Furthermore, the Schiff base probably forms a hydrogen bond to a structural water, rather than directly to its counterion residue. Structural studies on a cytosolic loop fragment have also provided a putative helix−turn−helix structure for this element.[33]

Like bacteriorhodopsin, the protein has the ability to produce atypical protonation states for acidic residues. For example, Asp 83, which is highly conserved across all GPCRs, has been shown to exist in its neutral form.[34,35] Similarly, Glu 122, once a candidate Schiff base counterion, has also been shown to be neutral.[35] These residues are included in Figure 4.

The rhodopsin photocycle is substantially different from that of bacteriorhodopsin.[27] The protein binds 11-*cis*-retinal in its ground state, which interconverts to its all-trans form upon exposure to light (498 nm). After a number of transient high-energy intermediates, an equilibrium forms between two states (Meta-I and Meta-II) that bind the all-trans form of retinal. In the Meta-I state a hydrogen bond is retained between the Schiff base and its counterion, and transducin is not bound. Deprotonation of the Schiff base leads to transducin association in the Meta-II state. 11-*cis*-Retinal acts much as an antagonist would, and the *all-trans*-retinal functions much as an agonist.

As in bacteriorhodopsin, receptor activation is accompanied by a domain shift.[36,37] Spin labeling of cysteine residues introduced on the cytoplasmic side of the receptor has shown that formation of the Meta-II state was accompanied by a change in the mobility of residues adjacent to TM3 and TM7.[38,39]

Mutagenesis experiments have also contributed to our understanding. To function correctly, rhodopsin must be able to bind retinal, tune its absorption maximum to the appropriate wavelength, and associate with transducin. Early mutagenesis experiments demonstrated that 11-*cis*-retinal was covalently attached to Lys 296 on TM7. However, additional experiments showed that it was the ion pairing and not the covalent link that was important.[40] This has also been demon-

strated by experiments that moved the counterion, either down one turn on TM3[41] or to TM2.[42] Interestingly, in some opsins, the glutamate residue is replaced by a cage of tyrosine residues.[43,44] These results indicate that the rhodopsins and visual opsins can compensate functionally for significant interior changes.

Residues have also been identified that tune the absorption maximum of the retinal to a wavelength between 420 nm (blue) and 610 nm (red).[45] Mutation of only seven residues changed a red opsin to a green opsin and vice versa.[46] However, it should also be noted that different combinations of residues can tune the retinal to the same wavelength. Rhodopsins which demonstrate poor homology along the binding cavity and even bind modified retinals all have absorption maxima at approximately 500 nm.[43,44]

Additional residues have been identified that affect the Meta-I/Meta-II equilibrium. A disulfide link between the top of TM3 and the TM4−TM5 loop that is conserved in almost all GPCRs is required to stabilize the Meta-II state of the receptor.[47] Furthermore, mutations that disrupt the Schiff base/counterion association (e.g., K296A) produce consitutively active receptors.[48] Recent evidence suggests that even the apoprotein can associate with the G-protein.[49] Thus, in rhodopsin, ligand binding and receptor activation are clearly separable events.

Retinal analogues have also contributed to our understanding of ligand−receptor interactions in the opsins. Removal of the 9-methyl group caused a substantial change in absorption and formed a receptor that activated only weakly. This suggests a close contact at this point. However, removal of the 13-Me yielded a fully functional pigment.[50] The ionone ring was generally tolerant to changes, both by acyclic groups and by bulkier groups, such as adamantyl. This last group also suggests that the ionone ring may lie perpendicular to the plane of the conjugated chain, unlike in bacteriorhodopsin, where it lies parallel.

Analogues in which the 11−12 double bond is constrained in a ring have also shown that the bond photoisomerization is a necessary precursor to activation.[51] Additionally, other studies with *N*-methyl Schiff base analogues have demonstrated that the deprotonation of the Schiff base is required to form the Meta-II state.[52] Thus, photoisomerization and proton transfer are both needed to activate the receptor.

Taken together, the studies on bacteriorhodopsin and rhodopsin provide a suite of information which can be applied to our understanding of GPCRs generally. Bacteriorhodopsin provides a combined view of both structure and function, while rhodopsin provides some indication of the processes initiated by ligand binding. However, the data taken together also point to a marked ability of 7TM structures to compensate for distinct changes within their interior. This is a liability in interpreting mutagenesis experiments and ligand SAR, but probably also confers a powerful evolutionary advantage to these proteins.

2.3. G-Protein Coupled Receptors. There are currently over 700 sequences of G-protein coupled receptors known. These have been subdivided into the rhodopsin-like family, the metabotropic glutamate family, and the calcitonin receptors. The best studied of

```
TM1   xxxxxxxxxxxxxxxxxxxxxxgNxxxxx
                                  18
TM2   xxxxxLaxaDxxxxxxxxxxxxxxxxx
                    10
TM3   CxxxxxxxxxxxxxxxxxxxxxxxxDRY
      1
TM4   xxxxxxWxxxxxxxxxxPxxxxxxxxx
               6
TM5   xxxxxxxxxxxxxFxxPxxxxxxxxxxx
                              16
TM6   xxxxxxWxPxxxxxxxxxxxxxxxxxx
                     15
TM7   xxxxxxxxxxxxxxxxxxxxxxnPxxxx
                                   17
```

Figure 5. Each of the helices in the rhodopsin-like family of GPCRs contains a number of highly conserved residues. These are of considerable use to guide sequence alignments and have been used as a common element to generate generic numbering schemes. The numbering shown in the figure follows Schwartz;[21] the residues used as markers are capitalized.

these families, and the one of most interest to drug design, is the rhodopsin family of receptors. These include the rhodopsins and visual opsins, the monoamine neurotransmitter receptors, the peptide hormone receptors, and various glycoprotein receptors. The majority of these sequences can be aligned simply by matching a number of characteristic, highly conserved residues.[53] As shown in Figure 5, these have been identified for every helix. The characteristic residues have been used as a basis of several generic numbering schemes, which simplify considerably the comparison of residues across GPCRs.[13,17,21,22] Unfortunately, no single scheme has been agreed upon as a common standard, which still complicates comparisons across studies.

There is yet no high-resolution experimental structure of a G-protein coupled receptor. Because of this, common homology methods cannot be directly applied to GPCR modeling. Therefore, two strategies have been followed to construct GPCR models. The most prevalent strategy is to build models using the structure of bacteriorhodopsin as a homology template.[13] A second approach is to build the models de novo, using the 9 Å structure of bovine rhodopsin as a guide by which to orient the helices.[19,54,55] These two approaches have both strengths and limitations.

The key to the relative quality of the bacteriorhodopsin-based models is the way the GPCR sequence is threaded onto the bacteriorhodopsin helices. This determines the interior face of the helix, the relative depth of the helices in the membrane, and the position of interior residues relative to each other. Most alignments to bacteriorhodopsin now differ by at most two helix turns,[18] with common interior faces for all of the helices. As the amount of independent structural information increases, less variation in threading is

tolerated, and these models can be expected to converge further. This development, and the fact that the methodology for receptor construction is now so well-established, has resulted in the model-building process being automated by a number of groups.[56,57]

The recent elucidation of the low resolution electron density map of rhodopsin has forced a reexamination of the suitability of bacteriorhodopsin as a homology template.[58] Consequently, there has been considerable exploration of various methods of generating de novo models. Shortly after the structure of rhodopsin was published, Baldwin reconciled its density footprint and GPCR structure in a study using helical wheels[59] and more recently using molecular modeling combined with refined density contours from frog rhodopsin.[60] In both her models, she incorporated an analysis of interhelical loop lengths and analyses of residue conservation, variability, and polarity. Among her conclusions, she demonstrated that the helices almost certainly had to be arranged adjacent to each other; atypical arrangements such as those by Pardo[61] and Metzger et al.[62] are shown to be fairly unlikely. Furthermore, although her analysis was consistent with either a clockwise or counterclockwise arrangement of helices, she determined that the latter was more likely. Her earlier study forms the basis of many of the "rhodopsin-based" models that have subsequently been published.[19] Given the more refined nature of her subsequent model, this should become a preferred template for model building.[60] Here especially, the additional information about helix tilt and helix orientation will be of considerable interest. The more recent model also has the conserved tryptophans in TMs 4 and 6 shifted toward the extracellular surface.[60] However, the most significant effect of this new information appears to be the change in helix packing at the intracellular surface. This should provide impetus to new investigation of G-protein association.

Interestingly, a similar change in intracellular helix arrangement was predicted by Pogozheva et al.[63] Here, they combined the general distribution of helices as suggested by the density footprint of rhodopsin with the iterative refinement of hydrogen bonds derived from various receptors. The final bundle demonstrates considerable topological similarity to the frog rhodopsin model above. Herzyk and Hubbard[64] have applied a somewhat similar approach to the modeling of rhodopsin, by including constraints derived from biophysical and mutagenesis experiments directly to the development of the 3D model. These constraints were used to restrict a molecular dynamics conformational search. They used bacteriorhodopsin as a verifiable test case and then developed a model of rhodopsin which was consistent with much of the available experimental evidence and the rhodopsin footprint. More generally, Sansom et al.[65] identified "rhodopsin-like" packing as one of three possible arrangements for 7-helix bundles; the other two being "bacteriorhodopsin-like" and "endotoxin-like".

Considering the extensive prior art that has been generated by model building, much information can now be elicited from a simple sequence alignment to a protein that has already been modeled. Such an alignment will suggest the location and possible importance of individual residues. On a larger scale, mutations that affect ligand binding tend to occur at the same locations in many proteins. Analyses of sequence alignments such as that provided by the GRAP database,[22] or the helical wheel analyses of Schwartz[21] demonstrate this clearly. Given an alignment of related receptors, conserved and variable residues can also be determined. The location and amount of sequence variation provides a way of estimating how difficult it will be to obtain drug selectivity. If there is little interior variability, the proteins can be expected to recognize similar ligands. This similarity can also provide a starting point from which to search for lead compounds.

The extracellular and cytosolic loops pose a problem when building a GPCR model. These domains play an important role in both ligand recognition and G-protein association. However, early bacteriorhodopsin-based models tended to ignore the extracellular loops. They were considered to be the most speculative, and therefore the most scientifically dubious regions to add. However, this approach has had its perils, especially in the tendency to ignore even the loop residues which may form part of the ligand binding cavity. This is especially true for peptide receptors, which recognize and bind a portion of their endogenous ligands in the extracellular loop region.[66,67] An increasing number of published models now include the loops, with the caveat that their secondary structure is rather speculative.[68−71] For a number of smaller loops (e.g., between TM1 and TM2, TM2 and TM3, TM6 and TM7, and the part of the TM4−TM5 loop past the disulfide link), the uncertainty of the loops is somewhat higher than the rest of the receptor model. The longer stretches become far more speculative, with the inclusion of 50-mer loops becoming nonsensical. Often, only a single conformation for these regions is provided, even though in reality they are expected to be rather mobile portions of the receptor. However, they do serve to provide some insight into the pattern and approximate distribution of residues which may contribute to binding.

For some receptors, the N-terminus can be modeled using another protein as a template. The N-terminal domain of the LH receptor contains regions that have distinct homology to serine protease inhibitors. The N-terminus can then be modeled by homology to these inhibitor peptides.[72] The receptor models generated for these structures are therefore unique in that the N-terminal domain can be obtained directly using homology modeling methods.

Given the labile nature of the G-protein coupled receptors, a single conformation, although descriptive, is unable to describe important features of receptor function. Both ligand docking and receptor activation are dynamic events, which are poorly described by a static model. The marriage of GPCR structure and molecular dynamics (MD) would therefore appear to be both natural and obvious. Unfortunately, rigorous protein MD simulations require a high-quality starting structure, which has been unobtainable for the GPCRs. However careful the simulation protocol used, the results cannot be expected to overcome this initial liability.

Despite this limitation, MD simulations have been used in conjunction with receptor models fairly fre-

quently. They are useful for exploring the local geometry of a receptor model, and for removing poor contacts from the model. Furthermore, they can be used to perform conformational searches on the labile portions of the receptor, especially the interhelical loops.[71] Simulations have also explored the geometry, location, and orientation of ligands docked into the receptor.[73] In addition to these uses, MD simulations have been performed to explore more complex domain movements, such as the helix motion induced by docking agonist and antagonist molecules.

Unfortunately, the computational power available does not allow simulations of sufficient length to describe protein folding. This limitation precludes MD simulations of being of much use in altering or correcting helical packing. This is demonstrated by considering two early models. One of the first models to be published was of the serotonin 5-HT1a receptor.[74] In this study MD simulations were used to refine the helix packing and the docking of serotonin. As the counterion for the ammonium group in serotonin, the model identified Asp 93 on TM2. This suggestion was disproved by later mutagenesis. In 1993, Zhang and Weinstein published a model of the 5-HT2 receptor.[75] This model was based on the bacteriorhodopsin template, although with an atypical matching of GPCR helices to template helices.[61] Again, residues on TM5 that have been shown to be important to serotonin binding (see section 3.2 below) were not identified by this modeling study. Given the uncertainties inherent in the models and the natural time scale of these events, the use of molecular dynamics alone to explore helix association and receptor activation is inappropriate. As with protein folding, the application of molecular dynamics to these events is a problem awaiting a clever solution, rather than a clever solution to the problem.

3.0. Impact of Receptor Models

Receptor modeling and site-directed mutagenesis have almost become inseparable companions in the quest to determine how a ligand might bind its receptor. This combination has yielded a fairly good general description of how a molecule may bind to its receptor. However, this description is limited both by the inadequacies of the template protein (be it bacteriorhodopsin or low-resolution rhodopsin) and by the uncertainties associated with the mutagenesis experiments.

The limitations of the receptor models have been reviewed in the previous section; the limitations of the mutagenesis data will also be discussed briefly before assessing the impact of the combined techniques.

A substitution of one amino acid by another in the protein may have an effect which can range from negligible to dramatic, either locally or distantly. Unfortunately, the biological readout only provides a global assessment of how a ligand now binds in a new, re-engineered environment. Oftentimes, the observed binding affinity variation is simply attributed to the changes made to the mutated residue, and their immediate impact on ligand binding, leading to a reduced or increased ability of the protein to recognize that particular ligand. However, such interpretation depends on a series of assumptions, which may not necessarily hold up. The ligand may bind differently to the native and mutated protein, which confounds a simple mapping of chemical interactions. The mutation may also act indirectly, altering a residue which is not adjacent to the ligand, but affects the conformation and position of adjacent residues. More subtly, in GPCRs, the residue may affect the ability of the protein to interconvert between high-affinity and low-affinity states, which results in so-called “pseudo-hits”.[112] It is becoming clear that the most informative studies reconcile extensive mutagenesis within the receptor and considerable structure−activity data drawn about a chemical class. Where these form a consistent picture, receptor modeling is at its most convincing.

3.1. Binding Grid Analysis. It is often difficult to reduce the rich description of the protein interior provided by three-dimensional models to an informative two-dimensional picture. This is often done by schematic, helical wheels, or flat helix diagrams of the entire protein. With the information that has emerged from both mutagenesis and modeling, it is now possible to define the residues most important to ligand binding. This subset of residues can be described by a “binding grid”, which focuses attention on the residues lining the binding cavity in the upper half of the receptor. A selection of these grids is shown in Figure 6. Such a description also maintains much of the relative depth and adjacency information of a receptor model. The characteristic conserved residues within the receptors were identified and used to align the sequence to the grid (thus, an inward facing residue in, say, a muscarinic M3 receptor will be in the same location as its aligned residue in the rhodopsin receptor). The grid was constructed so that adjacent helices were also adjacent in the grid. This resulted in the helices being represented in descending order from left to right. The grids are coded by residue property: yellow, hydrophobic (Met, Leu, Ile, Ala, Cys, Val); cyan, aromatic hydrophobic (Phe, Trp, Tyr); magenta, hydrogen bonding (Ser, Thr, Asn, Gln); red, positive ionizable (Lys, Arg, His); blue, negative ionizable (Asp, Glu); green, helix kinking or breaking residues (Pro, Gly).

This formalism will be used to describe the receptors in the following discussion of ligand binding.

3.2. Biogenic Amine Neurotransmitter Receptors. Receptor models provide context for both mutagenesis experiments and for ligand SAR. This ability was first demonstrated in studies of the hamster β-adrenergic receptor.

Exhaustive mutagenesis experiments on the transmembrane domain of the hamster β-2 adrenergic receptor identified a number of residues that affected the binding of agonists.[76] These included Asp 113 on TM3,[77,78] Ser 203, 204, and 207 on TM5,[79] and Phe 289 and 290 on TM6.[80] Experiments with analogues of adrenaline containing only one hydroxyl group on the catechol ring suggested that the meta OH interacted with Ser 204, and the para OH with Ser 207.[79] These are outlined in red in Figure 6a.

The first detailed receptor models based on the refined structure of bacteriorhodopsin were published shortly thereafter by Hibert et al.[11] and Trumpp-Kallmeyer et al.[13] On the basis of the mutagenesis, they located the agonist binding pocket in the extracellular part of the transmembrane domain between TM3, TM4, TM5,

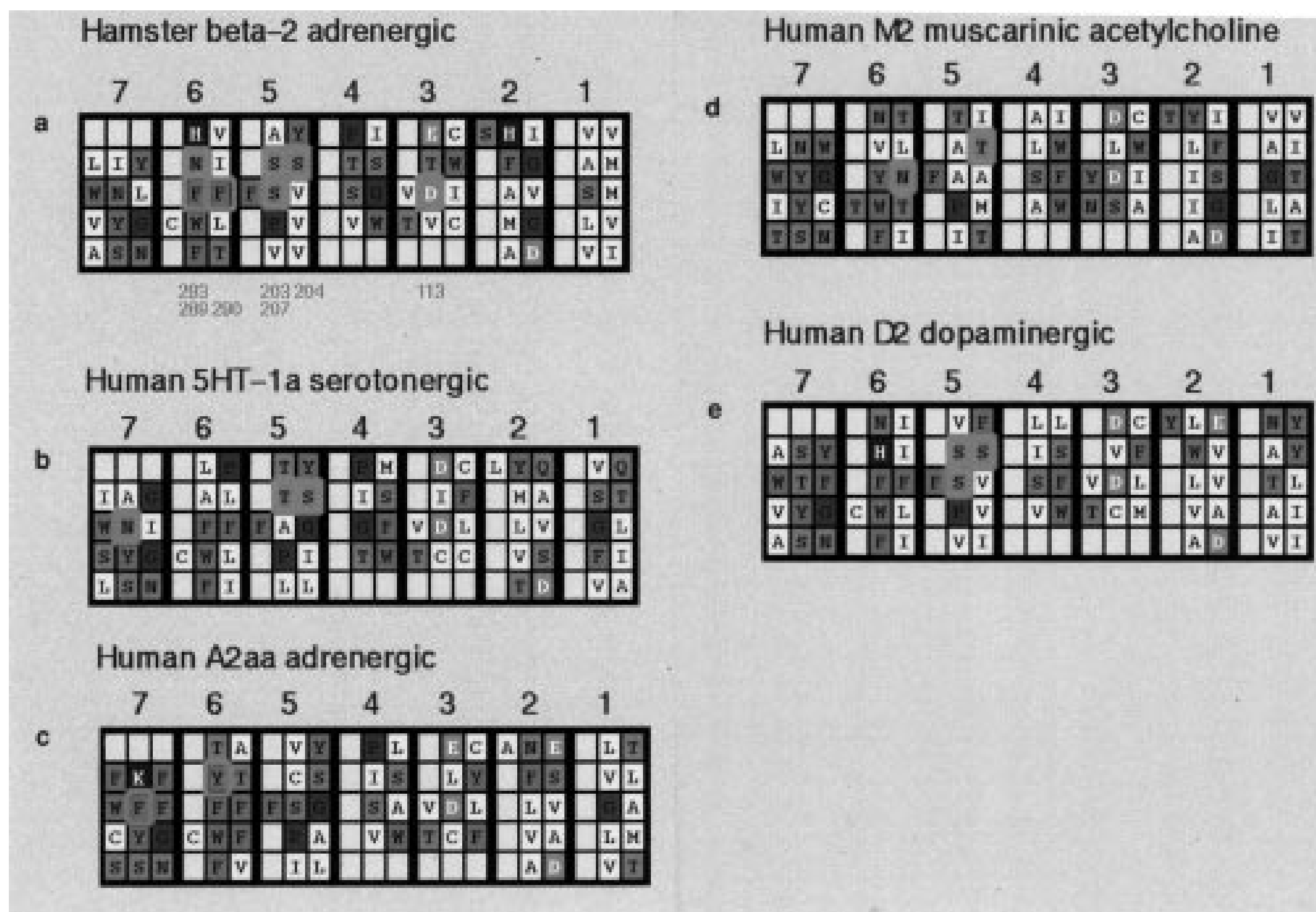

Figure 6. Binding grids are shown for the biogenic amine receptors described in the text. Residues which have been mutated and are discussed in the text are highlighted in red. The helices are numbered from the left in descending order, and residues in the same location in the grids are directly comparable across receptors: (a) the hamster β-2 adrenergic receptor, showing the principal residues involved in agonist binding; (b) the human serotonin 5-HT1a receptor; agonist binding is affected when either the threonine and serine on TM5 are mutated (note also the asparagine which mediates beta blocker binding at the top of TM 7); (c) the human α-2 adrenergic receptor; (d) the human muscarinic acetylcholine receptor (note the absence of serine residues on TM5 and the proximity of the threonine on TM5 and the asparagine on TM6); (e) the human dopamine D2 receptor.

TM6, and TM7. They demonstrated that the mutagenesis data could be reconciled to form a plausible description of ligand binding.

In their model of the β-2 adrenergic receptor, adrenaline was positioned parallel to the membrane so that Asp 113 acted as a counterion to its protonated amine, and the two catechol hydroxyl groups interacted with two serines on TM5 as suggested by mutagenesis (*m*-OH to Ser 204, *p*-OH to Ser 207). The β-hydroxy group of adrenaline was proposed to interact with Ser 165 on TM4. They also suggested that a number of aromatic residues on TM3, TM6, and TM7 formed cation$-\pi$ interactions with the positively charged ammonium group of the ligand. Additionally, Phe 290 was positioned to stabilize the catechol ring. Studies by Lewell,[81] by Weiland et al.,[82] and by Donnelly et al.,[19] the latter using a "rhodopsin-based" model, all used the same anchoring information to dock adrenaline into their models and provide an essentially similar view of ligand binding.

Binding studies have shown that (*R*)-adrenaline binds to the β-2 adrenergic receptor with 100-fold higher affinity and efficacy than does (*S*)-adrenaline.[84] Various modeling studies have identified numerous residues which may be responsible for this stereoselectivity: Ser 165,[13] Ser 161,[81] or Thr 164[84] on TM4, Asn 293[19,82] on TM6 or either a serine or tyrosine on TM7.[83,84] Unfortunately, the postulated interaction between the β-hydroxyl group of adrenaline and Ser 165 in TM4 could not be tested because receptors mutated at this position were not expressed at the cell surface. When Asn 293 on TM6 was mutated to a leucine, the stereoselectivity of the resulting mutant receptor was greatly reduced, but without any decrease in the binding affinity of partial agonist that did not have a β-OH group.[82] This strongly suggests that it is Asn 293 in the β-2 adrenergic receptor which forms a hydrogen bond to the β-OH group of adrenaline. However, this cannot be generalized across all adrenergic receptors. In the alpha adrenergic receptors, a leucine normally occurs at the homologous position (Figure 6c).

On the basis of their modeling, Hibert et al.[11] and Trumpp-Kallmeyer et al.[13] extended the definition of a common agonist binding pocket for cationic ligand GPCRs and other small ligand GPCRs. In particular they predicted a number of amino acids to be located in the agonist binding site, which had not been identified previously by site directed mutagenesis studies. Moreover, they proposed that in cationic neurotransmitter receptors the electrostatic interaction between the conserved Asp on TM 3 and the cationic head of the neurotransmitter is shielded by a number of highly

conserved aromatic residues located on TM3, TM6, and TM7. In subsequent mutagenesis studies, ligand binding was significantly affected when these aromatic residues in the D2,[85] 5HT2a,[86,87] muscarinic M1,[88,89] and the M3 receptors[90] were mutated, supporting these proposals from the modeling studies.

The existence of a general binding pocket for cationic amines has been repeatedly demonstrated across many receptors. Noradrenaline was shown docked in this location in the α adrenergic receptors.[13,91,92] This assumption has been supported by recent mutagenesis.[93] In models of the muscarinic acetylcholine receptors[13,19,73,94] the acetyl part of the molecule was predicted to bind an asparagine on TM6[13,19,94] and later incorporated binding to a threonine on TM5.[19] These interactions replaced the role of the catechol ring binding to the serines on TM5 (Figure 6d). Indeed, ligand binding has been affected when these residues were mutated.[95−97] Similarly, in serotonin receptor models[13,19,98,99] the aromatic hydroxy group of serotonin was predicted to bind to the single serine on TM5 in the serotonin 5-HT1a receptors.[13,19,98] A later study also predicted an interaction to its adjacent threonine.[99] In the latter case, the aromatic NH is shown binding to the serine, thereby explaining the loss of binding upon mutation of either residue.[100] Dopamine has also been shown docked to its receptors in a manner analogous to the *β*-2 adrenoceptor receptors.[13,19,101−103] Studies have postulated contacts of the catechol hydroxy groups to one,[104] two[13,19,101,105] or three[103] of the serines on TM5 (Figure 6e). Mutagenesis experiments on the dopamine receptors suggest that the binding is indeed more complex to interpret than that of the *β*-2 adrenergic receptor.[106−108]

Fewer modeling and mutagenesis studies have addressed the binding location of antagonists. This is due in part to their greater structural diversity and to the strong possibility that these bind in a variety of binding sites and binding orientations. However, a number of studies have demonstrated that modeling studies can have a positive impact on our understanding of antagonist binding. Lewell[81] docked propranalol and predicted that Asn 312 formed a hydrogen bond to the *β*-OH. This observation has since been confirmed by mutagenesis of the *β*-2 adrenergic receptor.[109] It has also been linked to the ability of the 5-HT1a receptor (Figure 6b) to recognize these antagonists, principally due to the existence of an asparagine in an analogous position in these receptors.[110,111]

From recent site directed mutagenesis and modeling studies, a relatively precise binding mode for pirenzepine, a tricyclic antagonist to the M1 receptor, has emerged.[89] Pirenzepine binds parallel to the plane of the membrane with its cationic piperazine ring interacting with Asp 105 on TM3 and its endocyclic amide forming hydrogen bonds with a conserved Asn 386 on TM6. The tricyclic ring is located between TM4, TM5, and TM6 and forms strong aromatic interactions with tryptophans on TM4 and TM6 and a phenylalanine on TM5. Furthermore, the cationic headgroup of pirenzepine is located close to a tryptophan on TM3 and a tyrosine on TM6. The mutagenesis reported in this study did not support an alternative binding site between TM2, TM3, and TM7, a proposal based on molecular dynamics simulations and QSAR, but without the benefit of additional mutagenesis.[73] However, this location proposed by Bourdon et al. is similar to that proposed for tricyclic antagonists to the dopamine D2 receptor.[101] Taking into account the structural homology in the cationic neurotransmitter receptors and the crossreactivity of a number of tricyclic antagonists, the possibility that a tricyclic binding pocket between TM4, TM5, and TM6 is common to many cationic neurotransmitter receptors appears likely.

It is now almost inconceivable to attempt mutagenesis on a novel receptor without modeling support, either by building and inspecting a new model or by using the description of ligand binding built up by previous modeling studies. In many cases, predictions have been confirmed by subsequent mutagenesis. Where they have not, these results serve to refine existing models. For example, any *β*-2 adrenergic model consistent with available mutagenesis must have a plausible relationship between TM3, TM5, TM6, and TM7. Such tests can also be transferred to models of other receptors using sequence homology.

3.3. Peptide Receptors. Over the past decade, an increasing range of peptides have been shown to play an essential role in neurotransmission and endocrine modulation.[112] In most cases, research has followed the path from isolation of a peptide, through synthesis of potent and selective antagonists, to characterization of the peptide's receptor. Only recently and serendipitously have non-peptide agonists been identified for the CCK and angiotensin AT1 receptors.[113,114] The notable exception has been that of the opioid receptors, for which many selective classes of non-peptide agonists and antagonists have been synthesized over the last century.[115] Ironically, it has only been comparatively recently that their endogenous agonists were discovered to be linear pentapeptides,[116] and their receptors have been among the last to be sequenced.

Peptide receptors are now known to be associated with important neuronal and endocrine pathways which modulate effects such as pain (tachykinin NK1), and appetite (neuropeptide Y). However, except that they all act through GPCRs, there is little immediate similarity to these peptides. Unlike the biogenic amines, there is a great range in sizes, from large pituitary hormones which could almost be considered proteins to small hormones such as thyrotropin releasing hormone (TRH), which is a modified tripeptide.

Given this disparity in size, the immediate question arises as to how the peptides interact with their receptors. Synthesis and binding of peptide fragments has demonstrated that it is usually the C-terminus, as a free carboxylic acid or as an amide, or the N-terminus of the peptide that interacts with the receptor.[117] The minimum active fragment of the larger peptides appears to be a hexapeptide, suggesting that only a small amount of the peptide necessarily inserts into the receptor. Compared to the biogenic amines, the interactions with the loop regions of the receptors appear to play a more important role. In receptors such as the tachykinin NK1, mutations that presumably affect peptide binding directly occur exclusively in the extracellular loops.[112] For most, however, the principal binding site appears to be within the transmembrane region, approximately

congruent to the binding cavity of the biogenic amines and opsins. Within the receptors for peptides with free carboxyl groups, there is often a counterion residue, such as LYS 199 on TM5 for angiotensin[120] and LYS 166 on TM3 for endothelin.[118] For amide terminated peptides, the components of the binding site are far more difficult to identify.

The elucidation of the binding sites for peptide hormones can be addressed by a combination of mutagenesis experiments and modeling studies. An example of such an approach is the identification of the binding location of arginine vasopressin (AVP) in the V1a receptor[55] and the identification of the locus that determines its selectivity between the V1a and V2 receptors.[71] AVP is an amide-terminated nonapeptide characterized by the presence of a cyclic backbone formed by the first six amino acids. Primary structure comparison and three-dimensional modeling identified a number of conserved polar residues on TM2, TM3, and TM4 which could form hydrogen bonds to AVP. Subsequent mutation of these residues to alanine reduced the binding of AVP and related agonists by 10–300-fold. Taking into account structure–function studies of AVP, the authors concluded that the cyclic part of the hormone is embedded in the extracellular half of the transmembrane domain with the exocyclic C-terminus forming hydrogen bonds to two glutamine residues at the beginning of TM2. More importantly, they identified a tyrosine in the first extracellular loop which was responsible for the selectivity of AVP to the V1a receptor.[71] Mutation of this residue to the amino acid present at the corresponding position of the V2 or the related oxytocin receptor subtypes switched the pharmacological profile toward the respective receptors.

Studies of peptide binding and receptor activation are also providing useful insight into the fundamental functioning of GPCRs. The vasopressin and oxytocin receptors also provide an example of such a study.[119] In their first paper, the authors proposed that aromatic residues on TM4, TM5, TM6, and TM7 which are conserved in most GPCRs might be involved in transduction of the agonist signal to the intracellular loops.[13] In a later site-directed mutagenesis study, two aromatic residues which line the bottom of the putative binding pocket on TM5 and TM6 in the oxytocin receptor were mutated to their corresponding residues in the V1a receptor. Interestingly, in the oxytocin receptor, the residues are Tyr 509 and Phe 609 (using the author's numbering scheme), but the corresponding residues in the V1a receptor are Phe 509 and Tyr 609. AVP, which is normally a partial agonist to the oxytocin receptor, acts as a full agonist when either residue is mutated to the corresponding residue of the V1a receptor. The authors concluded that these residues are therefore involved in signal transduction and the modulation of the receptor response in the oxytocin receptor. Since aromatic residues are conserved in homologous positions in most of the GPCRs, a similar role in signal transduction can be assumed.

For modeling studies, mutagenesis also plays an important role by identifying residues in contact with the ligand. The structure–function relationships developed for the peptide can then be reconciled to this information, allowing the peptide to be oriented within its binding site. An example of such a combined study is afforded by the docking of the octapeptide angiotensin II to the angiotensin AT1 receptor.[120] Structure–function studies of the peptide have identified Arg 2, Tyr 4, and His-6 as well as the negatively charged carboxy terminus as being important for receptor binding. Subsequent site directed mutagenesis studies support a model in which the negative C-terminal carboxyl group forms a salt bridge to Lys 199 on TM5, which is stabilized by a tryptophan on TM6. A phenylalanine and an aspartate on TM6 provide a docking site for His 6, and guanidinium side chain of Arg 2 forms an electrostatic interaction with an aspartate in the third extracellular loop. To support all these interactions, a somewhat extended conformation of the peptide was chosen. This contrasts with a model of the docked peptide in a much more folded conformation which was proposed without the benefit of the additional mutations afforded by this study.[121] Such studies highlight the dangers inherent in the prediction of peptide binding

Similar studies have been published for the SSTR2 somatostatin receptor,[122] the TRH receptor,[123,124] and the bradykinin receptor[125] and have helped to identify receptor areas interacting with specific parts of the peptide hormones. Recent investigations in the opioid receptor are also instructive. Mutagenesis experiments in the μ-opioid receptor identified His 297 in TM6 as a key residue binding agonists, accompanied by Asn 150 in TM3 (agonists) and Tyr 327 in TM7(both agonists and antagonists).[126] These mutations were suggested on the basis of receptor modeling, with the binding modes subsequently corrected to fit to the mutagenesis results.

The information afforded by studies of peptidomimetic agonists and antagonists further complicates matters. The introduction of an atypical or D-amino acid can often change an agonist into a highly potent peptide antagonist. Although these are generally presumed to bind with considerable overlap between agonist and antagonist binding sites, there is often poor support for this view from mutagenesis experiments. It appears that the shift from agonist to antagonist involves a change in binding mode,[71] and possibly a change in receptor conformation. The tendency of these modifications to produce antagonists, rather than agonists, is possibly due to the unnatural conformations into which the side chains are locked by most "peptidomimetic" groups.

The discovery of non-peptide antagonists to peptide receptors has been the result of high volume screening of small molecule libraries, followed by analogue synthesis. Subsequent mutagenesis experiments suggest that these molecules often bind in a manner that differs from both peptide agonists and antagonists, although there is typically some overlap among them.[112,127] In many cases, discrepancies reflect an inadequate exploration of the residues in the protein interior, but in cases such as the NK1, it does appear that the peptide and non-peptide binding sites are completely separate.[128]

Modeling has played a role in designing mutagenesis experiments to identify the binding location of non-peptide antagonists. An example of such a study is the modeling of devazepide binding to the CCK-A receptor.[129] CCK-A and CCK-B/gastrin receptors are responsible for the respective gastrointestinal and CNS regu-

latory events of cholecystokinin. Devazepide is a benzodiazepine based antagonist which is selective for CCK-A receptors. This ligand was docked into a bacteriorhodopsin-based receptor model, and polar residues on TM3, TM6, and TM7 were identified. Subsequent mutation of these residues to alanine indicated that they did indeed form part of the devazepide binding site. An independent mutagenesis study showed the involvement of additional residues on TM3, TM5, and TM6.[130] However, even with these mutagenesis data in hand, it is still impossible to identify a distinct binding mode of devazepide in the transmembrane pocket of the CCK-A receptor.

As befits such a disparate group of molecules, the receptor−ligand interactions of the peptide hormone receptors defy sweeping generalization. This has been reflected in the receptor modeling. Unlike the biogenic amine receptors, which contain the aspartate in TM3 as a firm anchoring point, many of these receptors offer a range of plausible polar and hydrophobic residues to the modeler. This is further complicated by the size of the ligands, the uncertainty of the binding conformation, and the involvement of the (poorly modeled) extracellular loops. Despite this, modeling has had an important role in planning mutagenesis experiments and in providing inspiration to drug design. The more successful modeling results generally combine an appreciation of existing ligand SAR with an iterative cycle of modeling and mutagenesis. In this way, similarities among peptides and non-peptides and their interaction with complementary receptor features can be tested. This ultimately bases the receptor models on a firm experimental foundation.

3.4. Proposed Mechanisms of Receptor Activation. The description of the receptor interior provided by molecular models allows some discussion of plausible mechanisms of receptor activation. These range from suggestions of individual residues that can act as switches, to more complex movements of entire domains. Although the precise mechanisms have not been elucidated, these suggestions provide a context within which further experimentation can proceed.

A number of residues have been identified which may have some form of switching function within the receptor. One of the earliest suggestions arose from model building in the biogenic amine receptors. Hibert et al. suggested that a tyrosine in TM7 may provide the switch connecting the conserved Asp of TM3 and the conserved Asp of TM2.[11] However, since two of the three residues are conserved only in the biogenic amine neurotransmitters, this cannot be a universal mechanism. In a subsequent study, they proposed that a network of conserved aromatic residues lining the bottom of the pocket might undergo a conformational rearrangement upon agonist binding which is transferred down to charged residues on the intracellular side.[13] This proposal received strong support from the recently published mutagenesis study of the 5HT2a receptor.[87]

More recently, much has been made of the importance of the arginine which is part of the DRY sequence at the cytosolic end of TM3.[131] This has been suggested as a key switching residue, which moves from an inactive position in the receptor interior to an active state in the cytosol, which mediates G-protein coupling. This proposal accords well with the conservation of this residue across all receptors, and its demonstrated importance in G-protein coupling in a number of systems.

A third residue that has been proposed as a switch is a tyrosine one turn down from the conserved NP sequence of TM7. Joseph et al.[121] have proposed a model for the activation of the angiotensin receptor which includes this residue as a component of a proton wire. More radically, other researchers have suggested that the proton pumping function of bacteriorhodopsin is also common to the GPCRs.[132]

Other researchers have proposed models of receptor activation based on entire domain movements. Zhang and Weinstein[75,133] suggested that the introduction of an agonist or antagonist caused a domain shift in the intracellular region of TM 1−3. Underwood and coworkers, working with the angiotensin system, suggest that an agonist serves to shift TM6 and change the interactions between this helix and the third intracellular loop.[68] Despite such efforts, the first reasonable elucidation of the mechanism of receptor activation is dependent on a correct description of the relationships between the key residues. Until this is available, models of the activation mechanism will remain controversial.

3.5. Receptor Function. Until recently, ligands that act on GPCRs have been separated into one of three categories depending on their effect in cellular assays. An agonist bound to the receptor and led to its activation, whereas a partial agonist bound to the same receptor but only produced partial activation, no matter how large its concentration. Typically, full agonism was defined by the native ligand, or a close analogue which was used as a standard. Ligands that competed with the agonist but did not evoke a second messenger response upon binding were classified as antagonists.[134]

The kinetic models which were developed to quantitate competitive binding could not distinguish whether the agonists and antagonists competed directly for the same (isosteric) sites on the receptor, or whether they competed for mutually exclusive (allosteric) sites. With the advent of point mutagenesis experiments, this question has been probed. Much of the early mutagenesis data pointed to isosteric competition. The agonists and antagonists of the biogenic amine receptors appeared to share much the same binding.[76] Data arising from many peptide receptors, including the opioid, the vasopressin, and the CCK are also consistent with this interpretation.[112] However, point mutants of the tachykinin NK1 and angiotensin AT1 receptors is more consistent with allosteric antagonism. In the NK1 receptor, mutants that affect peptide agonists do not affect non-peptide antagonists and vice versa.

To complicate the picture still further, the intracellular milieu also affects receptor activation and ligand binding. Binding studies in the presence and absence of nucleotides defined populations of receptors in “high affinity” and “low affinity” states.[7] G-protein binding shifted the equilibrium between these states to favor the “high-affinity” state. The concentration of guanine nucleotides, which blocked the G-protein binding, therefore favored the “low affinity” state. These observations

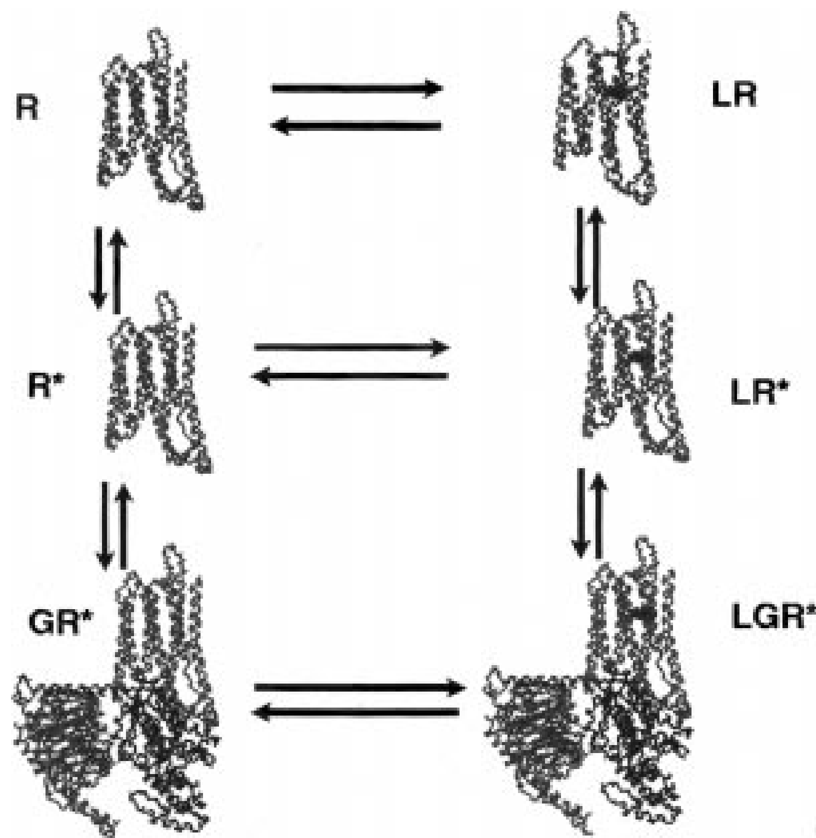

Figure 7. This figure shows the extended ternary complex model, showing G-protein association and ligand binding.[7,136] The ground state of the receptor (R) can be activated either via ligand binding, or spontaneously. Ligand binding can favor either the LR state (antagonist, inverse agonist) or the activated LR* state (agonist). Receptor activation is an explicit stage however, and can also be selected by certain point mutants. The activated, ligand bound state then associates with a G-protein. However, the G-protein itself can increase the concentration of GR*, which has a higher affinity for the agonist.

were integrated into a model whereby receptor function required a ternary complex of agonist, receptor, and G-protein to produce activation. Activation of the receptor was a concerted effort of all three parts, which was modulated by both extracellular and cytosolic conditions.

Two recent linked developments have substantially changed our understanding of receptor activation. The first development was that mutations were described that result in constitutively active receptors (receptors active in the absence of agonists). A variety of mutations in rhodopsin,[41,135] the beta adrenergic receptors,[136] and the muscarinic receptors[137] have all led to receptors showing constitutive activity. This led to the second discovery that a number of antagonists were capable of reversing this consitutive activity. Rather than passively blocking the action of agonists, this subset of antagonists, the "inverse agonists",[112] were capable of shifting the equilibrium of the receptors from an active to inactive form. These developments separated receptor activation from the action of agonists, and changed the role of a class of antagonists.

To better reflect this situation, a revised model of receptor activation, the allosteric ternary complex model, has recently been proposed.[136,138] In this, the description of the receptor has been broadened to include two states, one an inactivated ground state, and the other an "active" form. A schematic of this model is shown in Figure 7. Agonists favor the latter, and inverse agonists favor the former state. Partial agonists would be expected to have affinity for both states, but still lead to receptor activation. Because the ligands would favor both states, some proportion of the receptors would be held inactive, leading to less than full efficacy. True antagonists would be neutral in that they would block the interconversion of states rather than favor one state or another.

A number of experiments appear to support this interpretation. One consequence of this model is a predicted resting population of active receptors. It has been recently shown that this is indeed the case for the opsin apoprotein, which can activate transducin in the absence of retinal. A second consequence of the model is that the interconversion of the receptor from the resting to active state is an identifiable step. A number of point mutations have been identified which appear to interfere with this step—the so-called "pseudohits". Finally, Bond et al.[139] have shown that transgenic mice which overexpress B2-adrenergic receptors are spontaneously activated. In their studies, they showed that a 200-fold overexpression of normal adrenergic receptors leads to a state which is physiologically similar to normal receptors in the presence of agonist. This constitutive activity could be reversed by inverse agonists. This experiment supports a two-state model of receptor activation.

The introduction of the allosteric ternary complex model of receptor activation has also forced a reexamination of the role of the agonist. The agonist has traditionally been considered to induce the receptor

activation, by providing the necessary energy from that liberated upon binding. The alternate interpretation which is also consistent with the allosteric model is that the agonist simply selects the active form of the receptor and shifts the equilibrium by forming an activated, agonist bound pool. The energy barrier to interconversion is much lower in the latter interpretation.

These developments pose both a challenge and an opportunity to receptor modeling. The challenge results from increased complexity of the model, which allows at least two states for the receptor model, and a greater variety of binding modes to the ligand. The opportunity arises from the improvement in receptor pharmacology. With better classification of ligands, SAR relationships may become more clearly defined, which will simplify their reconciliation to binding modes within the receptor models. Paradoxically then, increasing fragmentation in the description of ligands may lead to better focus.

4.0. Perspective

Seven years have elapsed since the publication of the 3.5 Å structure of bacteriorhodopsin.[6] During the intervening years, the number of available GPCR sequences has increased from tens to hundreds, and published modeling and mutagenesis studies have proliferated exponentially. This information has deepened our understanding of both receptor structure and function. During this period, many researchers have built receptor models, initially using bacteriorhodopsin as a template, but more recently also incorporating information from the low resolution electron density footprint of bovine rhodopsin.[29] It is difficult to overstate the impact of such models on our understanding of GPCR structure, ligand binding, and function. Although there is still considerable debate about many of the details, a fairly comprehensive picture of the structure, ligand binding, and functioning of the GPCRs has emerged. In developing this picture, molecular models have been an invaluable tool in generating ideas, rationalizing experimental results, and providing a structural framework upon which to assess sometimes contradictory results.

However, this effort has been hindered by the inherent "fuzzy" nature of the receptor interior. Residues that are adjacent in one plausible model may be separated by considerable distance in another model. Models retain an uncertainty of approximately 100 degrees in the inward directed face of each helix, and an uncertainty of 1−2 turns in the relative depth of each helix in the receptor. For comparison, an uncertainty of two turns (6.8 Å) falls within the uncertainty of 10 Å perpendicular to the membrane that was suggested for bacteriorhodopsin.[6] This uncertainty is further compounded by the effect of different helix tilt angles and packing. All in all, this has been a boon to the proliferation and a bane to the credibility of GPCR models.

A minor revolution may shortly occur as new experimental data is introduced into the modeling process. A number of recent experiments have identified interhelical contacts that substantially constrain the relative position of the helices. Figure 8 shows the approximate location of engineered residues that now constrain interhelical packing. Zinc binding sites introduced into

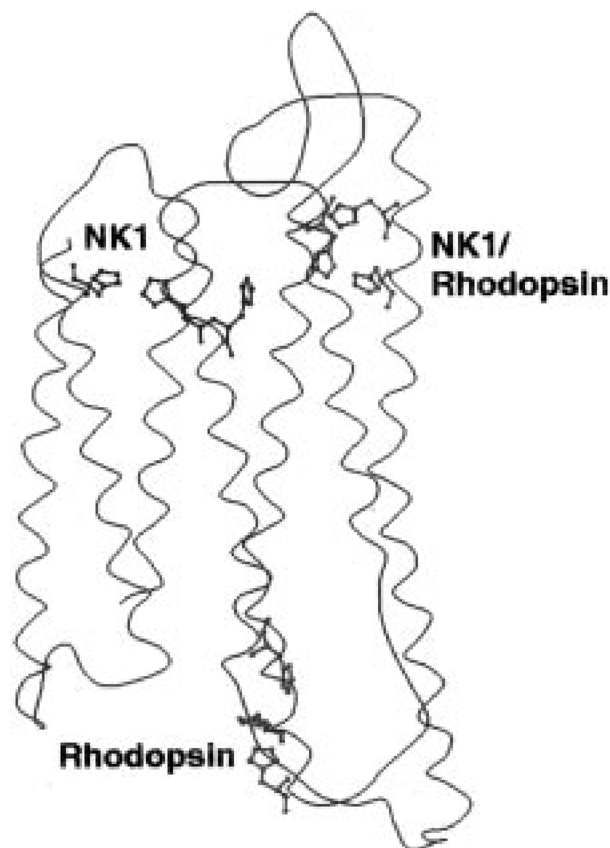

Figure 8. A model of a GPCR showing the locations of the histidines that have been introduced into the NK1 receptor and rhodopsin. These sites constrain the disposition of helices in the receptors. The sites at the intracellular face constrain TM3 and TM6 with respect to each other (red). The residues at the top of TM5 and TM6 fix their relative depth, both by engineered zinc sites in the tachykinin NK1 and opioid receptors, and by disulfide bridging in rhodopsin (cyan and magenta). Finally, the disposition of TM2, TM3, and TM5 have also been constrained by engineered zinc sites (blue and cyan). The receptor model is show with the helices of TM5 and TM6 extended, as suggested by random mutagenesis experiments.[156]

the NK1[140] and κ opioid[141] receptor now serve to position TM5 with respect to TM6. These data are bolstered by the insertion of a disulfide bridge between two of the homologous residues in rhodopsin.[142] More recently, the relative disposition of TM3 and TM6 has been constrained by a further intracellular zinc binding site in rhodopsin.[143] Additional zinc binding sites constrain TM2 with respect to TM3 and TM3 with respect to TM5.[144] These results strongly support a counterclockwise orientation of helices, a conclusion that is also supported by chimeric studies of the adrenergic receptor.[145] One datum which is inconsistent with this view is a mutagenesis experiment in which a pair of potentially complementary residues on TM2 and TM7 in the gonadotropin receptor were exchanged. Individual mutations reduced receptor function, but the exchange mutation retained function. This result is somewhat controversial, as it appears to favor a clockwise orientation of helices.[146] However, the results from this experiment may be unique to the gonadotropin receptor, as analogous experiments in the serotonin 5HT2a receptor,[147] β-adrenergic receptor,[148] and CCKB[149] receptors were less conclusive. The balance of the experiments

appear to favor a counterclockwise arrangement of helices. Taken together, these data, combined with a judicious choice of ligand binding information (e.g., from the β-adrenergic receptor or rhodopsin) should serve to progressively constrain future models. In doing so, the models will converge to structures that are presumably not dissimilar to the native structures themselves.

The receptor models have favorably impacted drug design although it remains difficult to point at highly visible examples since they more intangibly influenced thought processes or creativity in molecular design.[20,150−153] Clearly, they have been most effective when used in conjunction with ligand structure−activity relationships or pharmacophore hypotheses. Receptor models also had a role in the development of random screening libraries. The principal limitation of the current generation of models when used for rational drug design is that the resolution of the binding cavity is too low to predict specific ligand−receptor interactions. Attempts to dock ligands into various GPCR models are further complicated by difficulty in identifying unique, sensible modes of binding, especially when dealing with molecules of the size of the neurotransmitter ligands. Best results are still obtained when pharmacophore models or ligand SAR is used to direct the orientation of the ligand within the putative binding cavity. Unfortunately, this often is unavailable until a research project is well underway.

In random design, the nature of the questions has changed. Here, arrays of molecules are designed to test a series of hypotheses simultaneously. Rather than requiring a clear picture of ligand binding to assist in design, the design of random libraries needs an assessment of the nature of the residues lining the ligand binding cavity, which can then be used to identify complementary functional groups to include in the designed arrays of molecules. In this case, precise distances and angles are less important than some plausible distribution of functional groups. Such information is immediately available from the current generation of receptor models.

What will change when the first high resolution structure of a GPCR is solved? First and foremost, it will satisfy the natural curiosity of every researcher in the field as to what the interior of the protein actually looks like. It will also provide a much more rigorous template upon which to build homology models and provide the dynamics community with a reliable starting structure for their simulations. Furthermore, it will reduce the complexity of the current problem, which is that of two unknowns. The first unknown is the location and conformation of the bound ligand, and the second is the uncertainty associated with the receptor model itself. These will be reduced to a more tractable problem which is comparable to that presented by a homology model of a globular protein. Finally, with a proper disposition of interacting residues within the protein, better postulates can also be made regarding receptor activation.

Biography

Jack A. Bikker received a B.S. in Engineering Chemistry from Queen's University, followed by an M.S. and Ph.D. in Medicinal Chemistry from the same university. In 1993, he joined Parke-Davis, Ann Arbor, MI, as a Postdoctoral Research Fellow in the Molecular Modeling and Drug Design Section. This was followed in 1996 by a postdoctoral position in the Department of Chemistry, University of Cambridge, U.K. Since 1997, he has held a Group Leader position in the Molecular Modeling Group at Parke-Davis, Cambridge, U.K.

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Christine Humblet received a Ph.D. in Chemical Sciences from the Facultés Universitaires de Namur, Namur, Belgium, in 1978. Following a Postdoctoral Fellowship at Washington University, St. Louis, MO, she joined the Centre National de la Recherche Scientifique at the Centre de Neurochimie in Strasbourg, France, in 1981. Since 1984, she has been leading the Biomolecular Structures and Drug Design Section at Parke-Davis, Ann Arbor, MI.

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