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Approaches to Molecular Modeling Studies and Specific Application to Serotonin Ligands and Receptors

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WESTKAEMPER, R. B. AND R. A. GLENNON. *Approaches to molecular modeling studies and specific application to serotonin ligands and receptors.* PHARMACOL BIOCHEM BEHAV **40**(4) 1019–1031, 1991.—Molecular modeling studies are useful in as much as they may allow us to understand the activity and selectivity of currently existing agents, and, furthermore, may aid in the design of completely novel therapeutic agents. There are two basic modeling strategies: the ligand-ligand approach and the ligand-receptor approach. Both approaches possess certain inherent advantages and disadvantages and, in addition, make certain assumptions about the agents and/or receptors being investigated. Keeping with the spirit of this minisymposium, we describe these two approaches, their general usefulness, and their limitations. Using serotonin (5-HT) receptors as a focal point, we review and provide novel examples of molecular modeling studies involving both strategies. Presented for the first time are examples of ligand-receptor models to account for the binding of serotonergic agents at 5-HT_2 and 5-HT_{1C} receptors.

5-HT_{1A} 5-HT_{1C} 5-HT_2 Molecular modeling Receptor Receptor models Serotonin

MOLECULAR modeling studies can be approached from two perspectives: manipulations involving only the ligands (ligand-ligand approach; receptor mapping), modeling interactions between a ligand and receptor macromolecule (ligand-receptor approach). The ligand-ligand approach is by far the most often employed and is an attempt to infer information about the macromolecular binding site, and/or modes of binding interactions, from the structural features and experimentally determined biological activities of a series of small molecules without direct consideration of the structure of the binding site. Although closer to the event of interest, binding of the ligand to the receptor, the ligand-receptor approach is less common because it requires a working knowledge of receptor structure. The latter approach has been applied successfully to model the interactions between small molecules and soluble proteins for which detailed structures are available from x-ray crystallographic studies. Quantitatively accurate calculation of binding energies has been accomplished using dynamics simulations in favorable cases (6). However, there are very few examples of the ligand-receptor approach for neurotransmitter receptor systems. In the serotonin field there have been studies that utilize the ligand-ligand approach for 5-HT_{1A} (31, 38, 81), 5-HT_2 (31), and 5-HT_3 receptors (31, 39, 61, 70). With the exception of the work of one group whose receptor model is not based on known properties of the serotonin receptors (78), no studies using the ligand-receptor approach have appeared for serotonin systems; however, the ligand-receptor approach has been applied to nicotinic (17), muscarinic (44,74), and adrenergic receptors (41, 56, 74, 75) with varying levels of sophistication and degrees of success.

Ligand-Ligand Approach or Receptor Mapping

This approach involves the comparison of properties within a series of molecules. The derivable properties of small molecules that can be compared include steric attributes (Van der Waals surface, solvent accessible surface, hydrophobicity/hydrophilicity), flexibility (from dynamics), electronic properties including potential fields (electrostatic potentials, probe atom affinities), properties from quantum mechanical calculations (semiempirical, ab initio), point atomic charges, shape and energies of molecular orbitals (LUMO, HOMO). In order to carry out a three-dimensional comparison for a series of ligands, it is necessary to select an alignment rule, i.e., to decide which structural features are of common function and are likely to occupy comparable regions when bound to the receptor. The mode of superimposition of a series of ligands may be obvious if a common structural feature exists. However, for structurally diverse ligands, the alignment rule may be quite obscure. Even an obvious superimposition mode may not reflect the true mode of orientation or conformation of the bound ligands.

Superimposition of a series of ligands requires that the bioactive conformation of each is known. Once this information regarding the three-dimensional shape of active compounds is reasonably certain, rigid superimposition is accomplished by least squares fitting of the coordinates of selected pharmacophoric features. Superimposition can be carried out in one of several ways. Manual, rigid superimposition requires the user to select atoms or structural features (such as a dummy atom placed at the center of mass of a phenyl ring or a best plane defined by

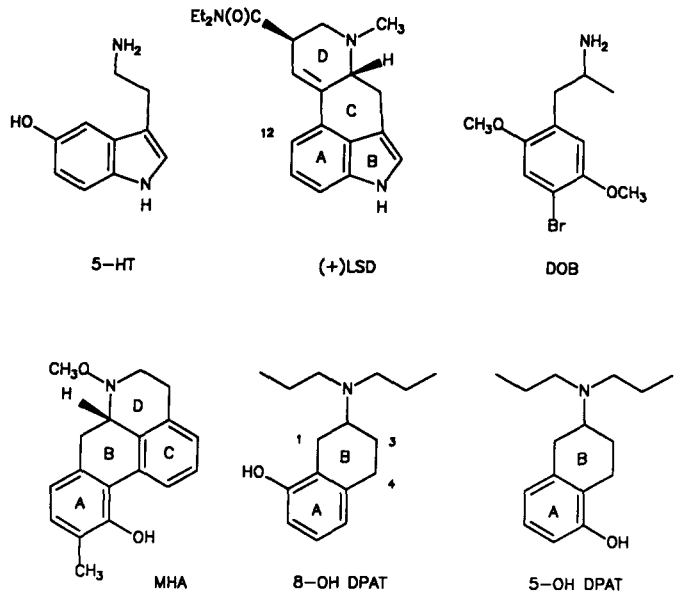

FIG. 1. Structures of serotonin receptor ligands: serotonin (5-HT), (+)lysergic acid diethylamide (LSD), 1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane (DOB), R(−)10-methyl-11-hydroxyaporphine (MHA), 8-hydroxy-2-(dipropylamino)tetralin (8-OH DPAT), 5-hydroxy-2-(dipropylamino)tetralin (5-OH DPAT).

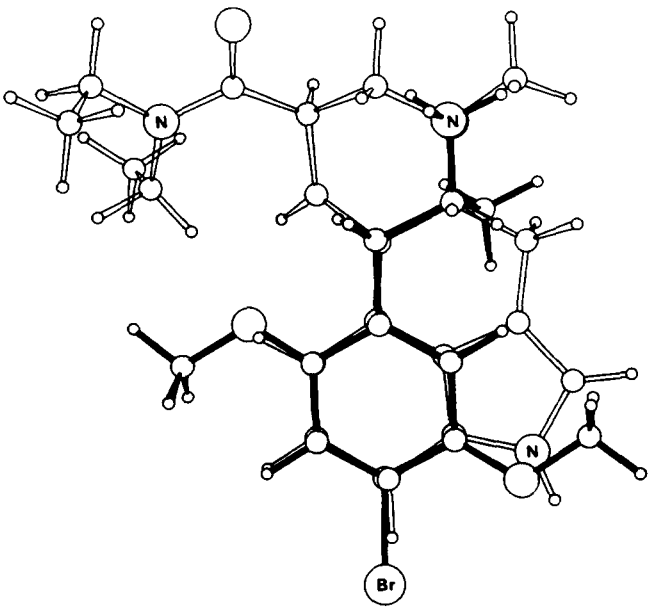

FIG. 2. Superimposition of (+)LSD (═) and DOB (—).

three or more atoms) that are thought to coincide. Qualitative evaluation of graphic displays of regions of similarities and differences can lead to the formulation of a hypothesis about structural features responsible for interaction of the ligand and receptor. Quantitation of three-dimensional molecular fields (steric and electronic) can be accomplished using programs such as CoMFA (18) that allow the prediction of the potency of newly proposed structures based on the initial alignment rule, and correlations between potency and three-dimensional properties.

As an alternative to manual rigid fitting, distance geometry methodology (19,29) can be applied (e.g., the program DGEOM) to the problem of exploring superimposition modes independent of the starting geometry of the ligands. The program DGEOM (7) allows random, unbiased generation of sterically feasible fits of a pharmacophoric ensemble. DGEOM describes molecular structure not as a set of cartesian coordinates, but as a matrix of maximal and minimal interatomic distances between all pairs of atoms. All sterically accessible conformations lie between the upper and lower bounds of these interatomic distances. Randomly selected trial interatomic distances are generated, refined, and three-dimensional coordinates are generated subject to the constraints inherent in generating a realistic structure, as well as any supplied by the user. In the ensemble distance geometry approach (62) several molecules are combined into a single distance matrix. Randomly generated sterically allowed conformers are generated subject to the additional constraint that selected groups superimpose. An advantage of this approach is that fitting constraints can be minimal, and potential solutions are generated without operator bias.

Modeling assumptions. All superimposition methods are based on the assumption that correspondence exists amongst the selected pharmacophore structural features when the ligand is bound to the receptor; that is, the ligands are assumed to bind in the same way to the receptor. A further assumption is that neither ligand nor receptor binding site conformation changes upon complex formation. The major assumption of receptor mapping methods that rely on establishment of a superimposition or alignment rule is that the ligands bind in such a manner that common structural features of small molecules occupy common positions in space when bound to the receptor, and that structurally similar functionalities serve comparable roles. This is not necessarily true even for very close structural analogs.

Experimental structures of dihydrofolate reductase complexed with trimethoprim or a trimethoprim analog support the notion of common modes of binding for structurally related compounds; when an aromatic methoxy substituent of trimethoprim is replaced with an extended carboethoxy group, the analog is bound in the anticipated fashion with superimposition of the structurally common components of the two ligands (52). The structures of complexes of the peptidase thermolysin with two peptide analog inhibitors have been determined. The peptide analogs are identical except that in one case the peptide amide bond is replaced with a phosphonate ester moiety and in the other with a phosphoramidate. The phosphoramidate is bound in a fashion consistent with the orientation of peptide substrates, and the ligand NH functions as a hydrogen bond donor to a peptide backbone amide carbonyl. The phosphonate, the ester oxygen of which cannot function as a hydrogen bond donor, is bound at the active site in a fashion that is superimposable to that observed for the phosphoramidate. In this case there is a good atom-by-atom correspondence, and the difference in affinities of the two compounds is attributable to the differences in a specific inhibitor functional group and its interaction with the macromolecule (72). However, there are also cases that support noncommonality amongst modes of binding. For example, the x-ray crystal structure shows that two equivalents of acetyl-Ala-Pro-Ala, the tripeptide product of the enzymatic hydrolysis of acetyl-Ala-Pro-Ala-p-nitroanilide, are bound to the peptide binding site of elastase, and both are bound backwards relative to the productive mode of binding of other peptide substrates (55). Ketone analogs of normal peptide and ester substrates for

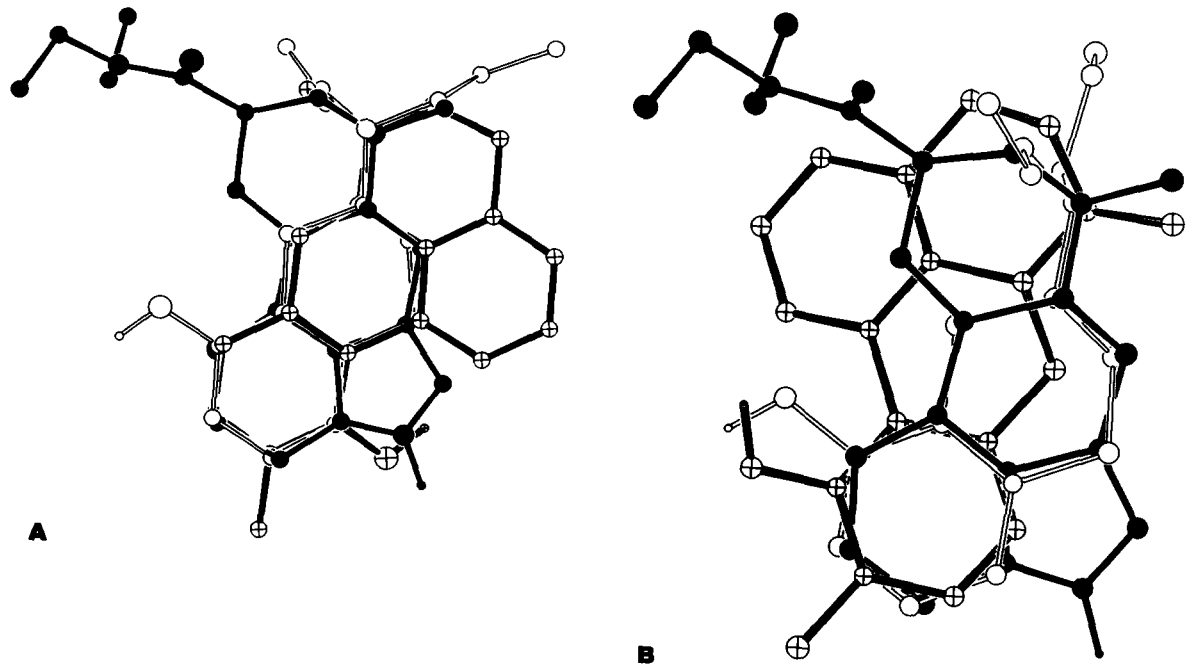

FIG. 3. (A) Superimposition of (+)LSD (bonds —), R(−)10-methyl-11-hydroxyaporphine (≡), and 8-OH DPAT (═) in the intuitive mode. (B) Superimposition of (+)LSD (bonds —), R(−)10-methyl-11- hydroxyaporphine (≡), in the counter-intuitive mode, and 8-OH DPAT (═) in the intuitive mode.

carboxypeptidase (a methylene replaces the amide N or ester O and the remainder of the structures are identical) are tightly bound inhibitors and very close structural analogs of substrates that are known to bind with the labile carbonyl coordinated to the active site zinc atom. The structure of the enzyme-inhibitor complexes shows the ketone carbonyl is not coordinated to the metal, but accepts a hydrogen bond from a nearby guanidinium side chain of an arginine (15). The next example is, perhaps, the most compelling illustration of the fact that obvious modes of superimposition may not reflect the way in which a compound is oriented at the binding site. The crystal structure of a complex of a transition state analog, 2-phenylethane boronic acid, and the α-chymotrypsin dimer has been reported (73). Main chain folding of the two halves of the dimer is nearly identical, but there are small differences in positions of some amino acid side chains, principally at the dimer interface. There are only slight differences in the orientation of the active site side chain residues. The bound inhibitor molecules differ significantly in conformation and orientation within the site. The aromatic rings are in approximately the same region of the site, but one is rotated by approximately 30° along the phenyl C1–C4 axis. The conformations of the ligand side chain are completely different; in one case the ligand is bound in its extended or trans conformation, a prediction one might have made under the assumption that the more stable conformer will be the "bioactive" conformer; in the other subunit of the protein dimer, the phenylethane boronic acid is bound as the gauche conformer with the boronic acid moiety in an entirely different area of the binding site. In as much as that we are dealing with two molecules of the same ligand, this is a situation in which one would expect an atom-by-atom correspondence of functional groups and common mode of orientation of bound ligands; however, the expected results are inconsistent with the experimental findings.

Establishment of an alignment rule provides useful hypotheses, but there is really no reason to assume it is correct, even when there is an obvious structural similarity between the superimposed structures. Varying degrees of difference in binding modes are possible, ranging from near perfect atom-by-atom overlap of the functionally important structural features of different compounds, to very dissimilar modes of binding. Since there is no way to predict whether or not a proposed alignment rule actually reflects the manner in which compounds occupy a binding site, such superimpositions must be regarded as hypotheses to be verified rather than as solutions to the problems themselves.

Application. Molecular superimposition of structurally different ligands that contain some common structural feature allows qualitative assessment of three-dimensional similarity and dissimilarity. For example LSD and DOB (see Fig. 1 for structures), both of which bind with high affinity at 5-HT_2 receptors, contain in common an aryl ethylamine fragment. Superimposition of the aryl ethylamine portions of the two compounds (Fig. 2) suggests other structural features that may interact with the receptor in an analogous fashion. For example, the depiction suggests that the oxygen atom of the 5-methoxy substituent of DOB may interact with the portion of the binding site occupied by the indole nitrogen atom of LSD (31). The underlying assumption implicit in such analysis is that the two ligands share a common mode of interaction with the receptor and, when bound, the aryl ethylamine functionalities of each occupy the same space. Identical binding modes for two different compounds is likely to be the exception rather than the rule, but such analysis provides a useful starting point for further analysis [see (31) for additional examples of the ligand-ligand approach as it applies to serotonergic agents].

Often, attempts to rationalize activity with the obvious alignment rule fail to explain the facts. For example, certain aporphines bind at 5-HT_{1A} serotonin receptors. In particular, R(−)

TABLE 1

SEQUENCES OF THE ASPARTATE-CONTAINING SEGMENT OF TRANSMEMBRANE HELIX 2 AND 3 FOR 5-HT RECEPTORS*

Helix 3															
5-HT_2	148 –	C	A	I	W	I	Y	L	D	V	L	F	S	T	A	S
5-HT_{1C}	128 –	C	P	V	W	I	S	L	D	V	L	F	S	T	A	S
5-HT_{1A}	109 –	C	D	L	F	I	A	L	D	V	L	C	C	T	S	S
Helix 2																
5-HT_2	113 –	L	M	S	L	A	I	A	D	M	L	L	G	F	L	V
5-HT_{1C}	93 –	L	M	S	L	A	I	A	D	M	L	V	G	L	L	V
5-HT_{1A}	75 –	I	G	S	L	A	V	T	D	L	M	V	S	V	L	V

*Sequence data from (26, 42, 43, 58).

10-methyl-11-hydroxyaporphine (MHA; Fig. 1) is a high-affinity and potent 5-HT_{1A} agonist (13,14). Although the aporphines and the standard 5-HT_{1A} agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH DPAT; Fig. 1) share a common aminotetralin structure (compare rings A and B; Fig. 1), interaction of these agents at 5-HT_{1A} receptors in an orientation with the aminotetralin rings superimposed fails to satisfactorily account for the pharmacological activity of the aporphines. That is, the 8-hydroxyl group of 8-OH DPAT is important for selective 5-HT receptor stimulation (3), and yet MHA, which apparently lacks the appropriate hydroxyl group, is a potent agonist (13). In addition, the aminotetralin derivative that possesses a hydroxyl group corresponding to the hydroxyl group of MHA (i.e., 5-OH DPAT; Fig. 1) lacks activity as a serotonergic agonist (4). Thus it is rather difficult to reconcile the agonist potency of MHA; that is, the hydroxyl group of the aporphine appears to be on the "wrong" side of the molecule. Furthermore, substitution at the 3-position of 8-OH DPAT is not well tolerated; this substitution reduces 5-HT_{1A} receptor affinity by more than 100 fold and essentially abolishes serotonergic agonist activity (54). When one considers that MHA is a 3-substituted aminotetralin derivative, this too is difficult to reconcile. Thus the most intuitive mode of orienting the MHA molecule with that of 8-OH DPAT (i.e., an atom-by-atom superimposition of the aminotetralin moieties) does not account for the high affinity and agonist activity of the aporphine.

(+)LSD, although nonselective, also binds at 5-HT_{1A} receptors with high affinity. The distance geometry approach (29) was used to explore the 3-dimensional relationships between R(−) 8-OH DPAT, (+)LSD, and MHA while imposing as few geometric constraints as possible in arriving at superimpositions. The goal was to randomly explore all geometrically and sterically feasible relationships that fit geometric constraints without bias. In this manner, the DGEOM (7) procedure was used to generate 100 superimpositions that met the constraints. Hierarchical cluster analysis (60) was used to sort the superimpositions into conformationally similar families. One family of 20 individual superimpositions consisted of slight variations on the "intuitive" model (see Fig. 3A for a representative example) in which the hydroxyl group of 8-OH DPAT corresponds to the C12 hydrogen atom of (+)LSD, with the hydroxyl group of MHA being in the vicinity of the indole nitrogen of (+)LSD. A second family of 28 superimpositions (see Fig. 3B for a representative example) retained the intuitive orientation of 8-OH DPAT with (+)LSD, but produced acceptable amine and aromatic-ring superimpositions for MHA in what we have termed a "counter-intuitive" fashion; as shown in Fig. 3B, the 11-position hydroxyl group of the aporphine is in the vicinity of the C12 hydrogen atom of (+)LSD and the hydroxyl group of 8-OH DPAT. The only major differences between superimpositions within these two families were in the conformations of the aliphatic ring of 8-OH DPAT, in the conformations of the 8-OH DPAT *n*-propyl groups, and in the conformations of the B and D ring of the aporphine. In at least one of the superimpositions in each family, the geometry of 8-OH DPAT and MHA closely resembled the minimized starting geometry. MM2 (11) minimization of each produced geometries indistinguishable from the global-minimum starting geometries. Even when the DGEOM-generated structures were different from a global minimum, they usually produced the global minima after MM2 minimization. The distance between the aromatic centroids and the amine nitrogen atoms were comparable for the three structures: 5.17, 5.13, and 5.22 Å for (+)LSD, MHA and 8-OH DPAT, respectively. The two superimposition modes are essentially equivalent with respect to goodness of fit (RMS deviations with respect to the superimposition criteria were 0.18, 0.20, and 0.16 Å for (+)LSD, MHA and 8-OH DPAT, respectively, in the intuitive mode, and 0.17, 0.16, 0.19 Å in the counterintuitive mode). Thus, on the basis of the hypothetical minimal pharmacophore model imposed during fitting (i.e., correspondence of the aromatic rings and the amine nitrogens), each model is a satisfactory theoretical representation of the manner in which the agents may interact with the receptor. However, on the basis of radioligand binding and other pharmacological data, only one of the orientations (i.e., that shown in Fig. 3B) accounts for the biological activity of R(−)-10-methyl-11-hydroxyaporphine. The results of this investigation reveal that in addition to the intuitive or obvious atom-by-atom superimposition of the aminotetralin moieties of 8-OH DPAT and MHA, there is also a less obvious superimposition that better accounts for the biological data.

A problem that may be encountered when considering non atom-by-atom superimpositions is the positioning of structural features of a drug molecule into regions of receptor space that have not been considered previously and about which nothing is known. For example, in the case of the aporphine MHA, the most obvious question is whether or not the added bulk associated with the aromatic C-ring of the aporphines can be tolerated by a region of the receptor as shown in Fig. 3B. We have already proposed that there exists a area of bulk tolerance associated with this region of the receptor (31). Further support for this concept comes from the recent finding that dihydroergotamine (Ki = 1.2 nM) binds with high affinity at 5-HT_{1A} receptors (53). This and related ergolines possess extraordinarily large substituents that are readily accommodated by the receptor.

Ligand-Receptor Interactions

Since the molecular event of interest is the association of a

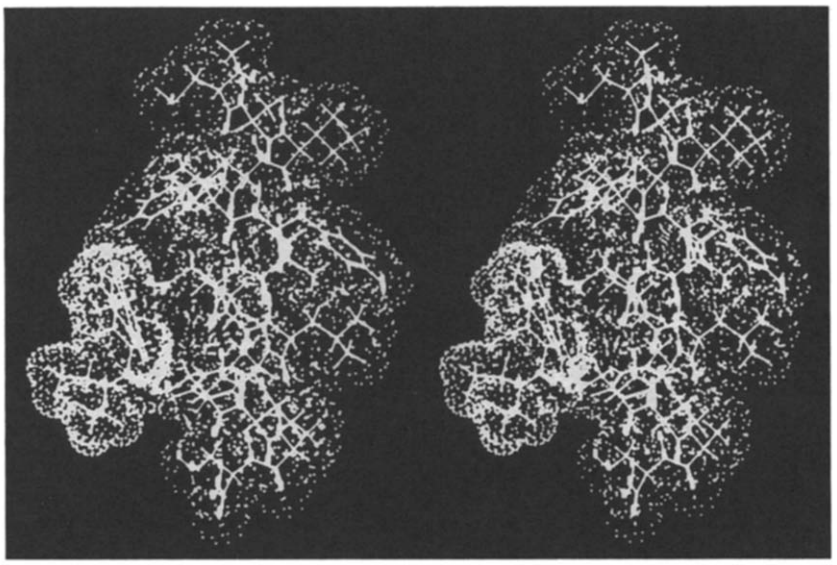

FIG. 4. Stereoscopic representation (dot surface at van der Waals radius) of (+)LSD bound to the helix 3 model of the 5-HT_2 receptor.

ligand with a protein receptor, it is most desirable to directly model that process using the conformation and location of a small molecule bound to an experimentally determined structure. Unfortunately, such empirical data (e.g., an x-ray crystal structure) for a neurotransmitter-receptor complex are presently unavailable.

Computational assumptions. Computational methods used to evaluate ligand-receptor association range in sophistication from (a) simple manual docking accompanied by qualitative evaluation of goodness of fit based solely on steric grounds, (b) systematic or automated docking coupled with molecular mechanics energy minimization, to (c) dynamic simulation of interactions of ligand and receptor with explicit inclusion of water. Only the computationally intensive dynamics simulations allow estimation of a parameter proportional to entropy. Force field methods used dynamically require supercomputer power for large systems. In addition to the limitations inherent in the rigor of the energy calculation method itself (molecular mechanics, semiempirical molecular orbital methods, ab initio methods), there are other approximations attendant to the completeness of the receptor ligand model. Complete evaluation requires explicit consideration of water coupled with dynamics. Prudent use of less complete systems requires careful evaluation of the inherent assumptions. For example, if solvent is not considered explicitly in a dynamic calculation, the inherent assumption is not that solvent is unimportant, but that the effect of solvation (free ligand and macromolecule, and bound ligand macromolecule-ligand complexes) is of the same magnitude and sign for the series of ligands evaluated. For a static minimization, entropic factors are not considered. The assumption is not that entropy is negligible (in fact it is probably of enormous significance), but that entropic changes associated with binding are of comparable sign and magnitude for a series of ligands.

Receptor model assumptions. Superimposed on many of the problems and assumptions made in the ligand-ligand approach is an entirely new layer of complications associated with the construction of a receptor model. All known neurotransmitter receptors are membrane-bound. Obtaining experimental structures of membrane-bound proteins at atomic resolution is difficult simply because, unlike soluble proteins, membrane-bound proteins do not typically yield crystals suitable for x-ray diffraction analysis. Generation of a receptor model for proteins of unknown structure is not a trivial task. In theory, it should be possible to predict the three-dimensional structure of a protein receptor from the amino acid sequence. However, in spite of intensive effort, such an undertaking is impractical at this time. If the protein of interest has a high degree of sequence homology with a protein of known structure, it may be possible to use this information as the starting point for building a three-dimensional model. To date, only one integral membrane protein structure has been obtained at atomic resolution using x-ray crystallographic techniques; the photosynthetic reaction center from three related photosynthetic bacteria: *Rhodopseudomonas viridis*, *Rhodobacter sphaeroides* and the mutant *Rhodobacter sphaeroides* R-26 (1). None of these is functionally or genetically similar to membrane-bound neurotransmitter receptors (with the exception that both classes contain several membrane spanning α-helices).

With the advent of techniques of molecular genetics, many protein sequences are becoming available, including those of membrane-bound proteins and receptors such as the photosynthetic reaction center, bacteriorhodopsin, and mammalian visual pigments (rhodopsin). Bacteriorhodopsin is a membrane-bound, retinal-dependent, light-driven proton pump from microorganisms. Its three-dimensional structure has been determined to near-atomic resolution (3.5 Å parallel to, and 10 Å perpendicular to, the membrane) using high-resolution cryo-electron microscopy (36,37). Bacteriorhodopsin consists of a bundle of seven membrane spanning amphiphilic α-helices linked by extra- and intracellular loops. The helices are thought to form a pore in the membrane with lipophilic sides oriented toward the lipid membrane and polar sides facing inward. The small molecule ligand, retinal, binds in the pore and forms a covalent imine with a ε-amino group of a lysine residue. Ten amino acid residues near the middle of the channel contribute to the retinal binding site. Bacteriorhodopsin, a proton pump, is functionally distinct from the mammalian visual pigment rhodopsin, which is G-protein coupled, but both proteins are light and retinal-dependent. There is no sequence homology between the mammalian

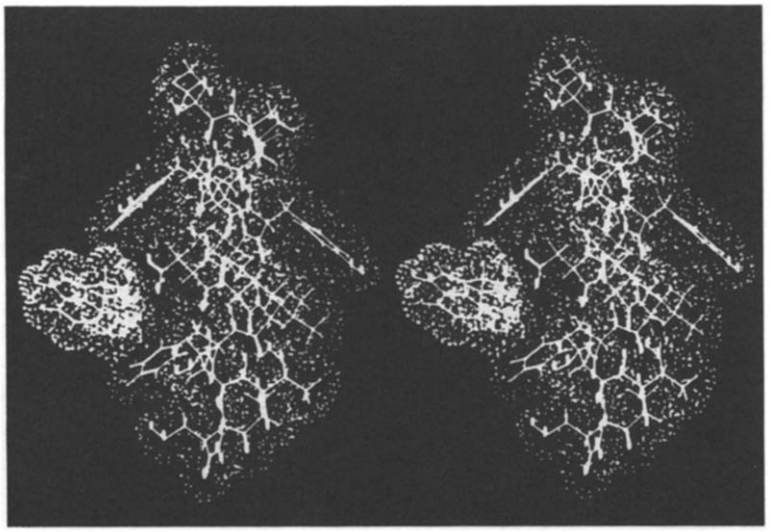

FIG. 5. Stereoscopic representation (dot surface at van der Waals radius) of 5-HT bound to the helix 3 model of the 5-HT_2 receptor.

protein and bacteriorhodopsin (22). However, there is significant homology between the mammalian visual pigments and G-protein neurotransmitter receptors, and even greater homology amongst the neurotransmitter receptors themselves. The notion that rhodopsin consists of seven membrane spanning helices is supported by the distribution of polar and nonpolar amino acids. Within the rhodopsin sequence, seven segments of approximately 24–28 nonpolar residues are linked by more hydrophilic sequences, providing indirect support for a bacteriorhodopsin-like topology. More direct evidence for the existence of the membrane spanning α-helices has been obtained from chemical modification experiments using hydrophobic nitrenes, which selectively react with portions of the protein thought to be contained within the lipid bilayer. Proteolytic susceptibility studies provide data similarly consistent with the existence of membrane spanning segments. The α-helical nature of the membrane spanning segments is consistent with CD spectra that indicate 50–60% α-helical content, and low-angle x-ray and neutron diffraction determinations indicate approximately 50% of the protein is located within the lipid bilayer. Infrared dichroism studies provide evidence that the α-helical segments are perpendicular to the membrane. Thus, analogy to bacteriorhodopsin, sequence analysis, and experimental evidence support a structural model for rhodopsin consisting of a bundle of seven membrane spanning α-helices; but, the precise relationship between the seven helices has yet to be verified by experimental observation (2).

G-protein coupled neurotransmitter receptors that have been cloned and sequenced include human and rat m1, m2, m3, m4, m5 subtypes as well as porcine m1 and m2 subtypes of the muscarinic cholinergic receptors; the α-adrenergic receptor from human kidney, human and avian β1-adrenergic, human and hamster β2-adrenergic receptors, the human 5-HT_{1A}, rat 5-HT_{1C}, and rat 5-HT_2 serotonin receptors (8–10, 20, 26, 28, 42, 43, 45, 47–51, 57, 58, 80). Studies utilizing secondary structure-predicting methods, analysis of hydrophobicity indexes, and hydrophobic moment plots support the notion that the gross architecture of the neurotransmitter receptors is similar to that of bacteriorhodopsin and to that presumed for rhodopsin; they may all contain seven amphiphilic α-helices (presumed to consist of a pore-forming aggregate similar to bacteriorhodopsin) connected to each other by extra- and intracellular peptide sequences of varying length. The degree of sequence homology varies, but is significant with the greatest degree of similarity within the putative membrane spanning α-helices, and between subtypes (22). There is no direct structural data, and very little indirect experimental evidence, to support this notion. Proteolytic susceptibility experiments, similar to those carried out earlier for mammalian rhodopsin, have been reported for β-adrenergic receptors (59). These results support suggested similarities between the membrane spanning topology of rhodopsin and the neurotransmitter receptor proteins (24). While this view of receptor structure is on the verge of becoming dogma, and should provide a valuable starting point for the construction of atomic models of receptors, it should be remembered that uncertainties still exist in building a model of even bacteriorhodopsin from the experimental data: 1) which helix in the diffraction pattern corresponds to which in the sequence (connectivity of the observed helical regions), 2) the precise length of each transmembrane helix, 3) the size and structures of the interconnecting extra- and intracellular loops, 4) specific interactions between helices (both adjacent helix rotation along the helical axis as well as translational orientation of interacting helices), 5) the positions of the amino acid side chains (36, 37, 46). Obviously, models built for mammalian visual pigments and neurotransmitter receptors by analogy to bacteriorhodopsin are subject to these same and additional ambiguities.

Site-directed mutagenesis studies of specific transmembrane residues and deletion mutation of extra- and intracellular loops have provided valuable clues as to the location of the ligand binding site for several of the receptors. The general conclusion has been that the ligand binding site is contained within the membrane spanning α-helices. In the case of the β-adrenergic receptor, most of the extra- and intracellular loops can be deleted without affecting ligand binding, indicating that the hydrophobic core most likely contains the ligand binding site (21). In all, there are only four acidic aspartate (Asp) and glutamate (Glu) residues in the membrane domain of the β-adrenergic receptors. These are all highly conserved within the G-protein neurotransmitter family. All neurotransmitter receptors have conserved aspartate residues near the middle of the second and third transmembrane helices. These absolutely conserved anionic amino acids are logical candidates for the ligand ammonium ion binding site (69). Early site-directed mutagenesis studies of the ham-

TABLE 2

CALCULATED BINDING ENERGIES FOR SELECTED AGENTS USING THE 5-HT_2 (HELIX 3) RECEPTOR MODEL

	ΔE (kJ/mol)	ΔΔE (kJ/mol)
High-Affinity Ligands (K_i <10 nM)		
5-Hydroxytryptamine	−239	0
Ketanserin	−226	13
(+)LSD	−224	15
R(−)DOB	−220	19
S(+)DOB	−220	19
N-Methyl DOB	−220	19
2-Methoxy-4-bromoamphetamine	−219	20
Low-Affinity Ligands (K_i >100 nM)		
Amphetamine	−207	32
8-OH-DPAT	−195	44
N,N-dimethyl DOB	−185	54
(−)LSD	−126	113
N,N,N-trimethyl DOB	−106	135
Nonselective Ammonium Ion		
Trimethylamine	−195	44

ster β-adrenergic receptor indicated that replacement of Asp-79 (helix 2) with alanine had no effect on antagonist affinity, but reduced agonist affinity slightly (10 fold). Replacement of Glu-107 (helix 3) with alanine affected neither agonist nor antagonist affinities. In contrast, replacement of Asp-113 (helix 3) resulted in a receptor protein that has no detectable affinity for antagonists (68). A more complete evaluation of mutants from the same source revealed that replacement of Asp-113 (helix 3) by asparagine (Asn) resulted in a 10,000-fold increase in dissociation constants for antagonists and between 8,000–40,000 increase in Kd values for agonists without reducing the maximal adenylate stimulation (67). Interestingly, substitution of Asp-113 with a glutamic acid resulted in decreased agonist affinity (100 to 1000 fold), but the relative affinities of various agonists remained the same as for the wild-type receptor. Similar studies have been conducted using mutants of the human β2-adrenergic receptor (16). Mutation of Asp-79 (helix 2) to Asn did not affect the affinity for antagonists. Small increases (40 to 240 fold) in dissociation constants for agonists were observed, but the mutation uncoupled agonist-induced G-protein coupled adenylate cyclase modulation. Replacement of Asp-130 (bottom of helix 3) with asparagine had no effect on antagonist affinity (0.5 to 2 fold) and actually increased agonist affinity slightly (4 to 10 fold), but resulted in a protein no longer coupled to adenylate cyclase (27). Replacement of Asp-79 (helix 2) did not alter antagonist affinity, decreased agonist affinity somewhat (20 to 240 fold), but uncoupled agonist association and G-protein coupled adenylate cyclase activation.

Interpretation of these results varies from investigator to investigator. There are problems in evaluating what constitutes a small change, how a small change in the structure of the protein at a physically remote site can alter binding at the actual binding site, and what constitutes complete loss of the essential structural features at the binding site. These problems are common to all site-directed mutagenesis studies (76). Based on these data, it is plausible that Asp-113 (helix 3) is the most important cation binding site for the adrenergic receptors.

Other residues have been implicated as potential components of the ligand binding site by site-directed mutagenesis. For example, it was reasoned that since adrenergic agonist activity requires a catechol ring, additional receptor features that comprise the ligand binding site may have functional groups capable of forming hydrogen bonds and hydrophobic interactions (69). Site-directed mutagenesis studies have been undertaken to evaluate this possibility (76). Of the seven serine (Ser) and two tyrosine (Tyr) single-site mutant receptors that appeared to fold correctly, substitutions of Ser-161 (helix 4) or Tyr-219 (helix 5) for alanine had little or no effect on ligand binding. Substitution of alanine for Ser-204 (helix 5), Ser-207 (helix 5), and Ser-319 (helix 7) resulted in a 10- to 25-fold decrease in the affinity of an agonist without affecting the affinity of the receptor for an antagonist, consistent with the fact that agonist but not antagonists require aromatic hydroxyl groups for activity. These observations led to the proposal that Ser-204 and Ser-207 constitute a pair of hydrogen bonding residues that interact with the dihydroxy functionalities of catechols (66). It should be noted that the substitution of alanine for Ser-319 (helix 7) caused a 10-fold decrease in ligand affinity, but the authors did not choose to ascribe a specific function to it or identify it as part of binding site (66).

Affinity labeling studies have been undertaken as an independent means of establishing the ligand binding site locus. Unfortunately, the results do not present a consensus. An alkylating analog of the β-receptor antagonist p-aminobenzylcarazolol, p-(bromoacetamido)benzyliodocarazolol, was found to be covalently incorporated into a peptide fragment corresponding to residues 83–96 (helix 2) of the hamster β2-adrenergic receptor. Since the only nucleophilic side chains contained in this sequence are Ser-92 and His-93, both located near the extracellular surface, it is presumed that one of these is the site of alkylation, and thus a portion of helix 3 may form part of the ligand binding site (23). The photoaffinity agent 3-[^{125}I]iodocyanopindolol diaziridine, which bears significant structural similarity to the previous agent and similarly contains the electrophilic/alkylating functionality on the end of a spacer distal from the arylamine end, was found to label two sites of purified turkey erythrocyte β-adrenergic receptor, Trp-330 of the extracellular half of helix 7, and an undetermined site within helices 3–5 (79). These results have been taken to indicate that the ligand binding site may be constructed of multiple helices. However, the possibility remains that alkylation could occur at a site remote from the primary ligand binding site, particularly with a long spacer between the alkylating functionality and the arylamine portion of the structure that is essential of both agonist and antagonist activity, as in this case.

Application. Of the serotonin receptors, three have been cloned and sequenced: 5-HT_2, 5-HT_{1C}, and 5-HT_{1A} (26, 33–35, 40, 42, 43, 58, 63) [see also the subsequent manuscript by Shih and co-workers (64) in this issue]. All are G-protein coupled receptors that are presumed, from hydrophobicity analysis of sequence data and by analogy to bacteriorhodopsin, to consist of seven transmembrane α-helices coupled by six hydrophilic extra- and intracellular peptide loops. There is significant amino acid sequence homology between the serotonin receptors and the other neurotransmitter receptors, with the greatest degree of homology occurring in the putative transmembrane helices. Of the three serotonin receptors, 5-HT_{1C} and 5-HT_2 share the highest homology, 51% overall and 80% for the transmembrane regions. The sequences of the 5-HT_{1A} and the 5-HT_{1C} receptor are only about 39% homologous in the transmembrane regions (34). These observations are consistent with the fact that the ligand binding profiles for the 5-HT_{1C} and 5-HT_2 receptors are similar to each other, but different from the ligand binding profile of the 5-HT_{1A} receptor.

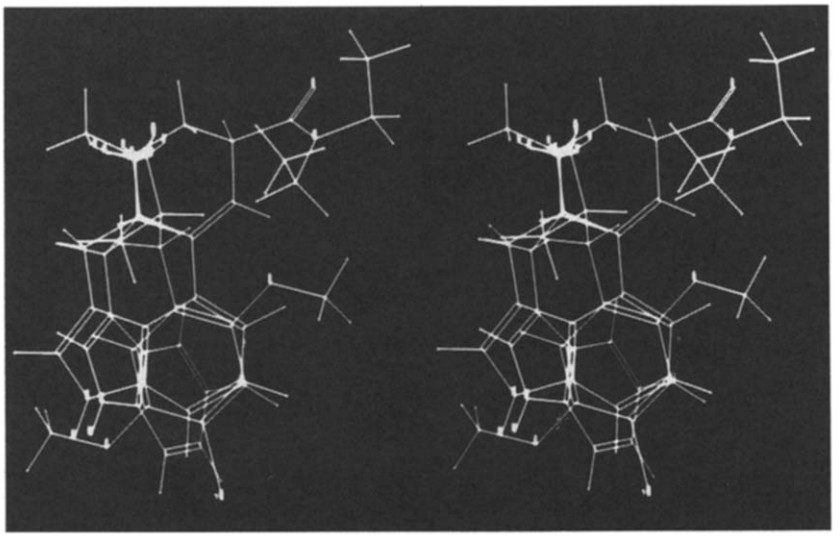

FIG. 6. (+)LSD, 5-HT, and DOB superimposed as bound to the helix 3, 5-HT_2 receptor model.

Unfortunately, site-directed mutagenesis studies have not been undertaken to provide experimental evidence for the location of the serotonin binding sites. There are only two acidic residues in the transmembrane regions of the three serotonin receptors that are uniformly conserved: Asp-120 (helix 2) and Asp-155 (helix 3) for 5-HT_2; Asp-100 (helix 2) and Asp-135 (helix 3) for 5-HT_{1C}; and Asp-82 (helix 2) and Asp-116 (helix 3) for 5-HT_{1A} (40). Both of these aspartate residues occur without variation in the sequences of all known G-protein coupled neurotransmitter receptors. While experimental support is lacking, it has been variously suggested by analogy with results from the adrenergic receptors that the Asp of helix 2 and of helix 3 may be the important ligand cation binding residue of the 5-HT_2 receptors (33). We have used computational methods to investigate potential interactions of a variety of serotonin ligands with models of the two putative binding sites (i.e., helix 2 and helix 3) of 5-HT_2 receptors. Given the uncertainties concerning the complete three-dimensional structure of any of the neurotransmitter receptors, it was desirable to construct receptor models having the least ambiguity. While it has not been proven, the most reliable structural information available is that the transmembrane segments that contain the ligand binding site are α-helices. Although there is evidence to suggest that multiple helices may be involved in the binding of certain adrenergic agents at their receptors (see above discussion), evidence is contradictory. Furthermore, there is no compelling evidence for the precise orientation of the seven transmembrane helices with respect to each other. For these reasons, we have chosen, at least initially, to evaluate potential interactions between ligands and a single helix. In effect, the assumptions in the construction (and evaluation) of these abbreviated receptor models are as follows: 1) the transmembrane helices are, indeed, α-helices, 2) a receptor anion is responsible for the association of the ammonium ions of ligands, 3) the helical segment containing the anionic functionality is responsible for most, if not all, of the ligand-receptor interactions, i.e., the abbreviated receptor model is complete enough to qualitatively account for relative affinities of a series of structurally diverse ligands. Site-directed mutagenesis studies of the adrenergic receptor are quantitatively consistent with the assumption that most of the binding site is contained within a single helix. All mutations of adrenergic receptors other than those that alter the Asp of helix 3, have only small effects on ligand affinity. Similar abbreviated models of adrenergic receptors have been reported (41, 56, 75).

Sequences of the aspartate-containing fragments of the third transmembrane helix of the 5-HT_2, 5-HT_{1C}, and 5-HT_{1A} receptors are shown for comparison in Table 1. The structure of the 5-HT_2 helix 3 receptor model (CH_3-Cys-Ala-Ile-Trp-Ile-Tyr-Leu-Asp-Val-Leu-Phe-Ser-Thr-Ala-Ser-NH_2) was constructed using the MacroModel software system with the peptide backbone constrained to an α-helix. The Φ, Ψ, and Ω torsion angles were set initially to −52, −53, and 180°. Energy minimization was carried out using the AMBER all-atom force field (77) as implemented in MacroModel (65), allowing optimization of all geometries to a gradient of 0.1 kJ/Å-mol. The AMBER force field has explicit energy terms for hydrogen bond formation as well as a distance-dependent dielectric constant intended to at least crudely mimic the effect of solvent. The energy-minimized peptide retained the helical structure and the associated hydrogen bonded network. Optimization resulted in small adjustments to the backbone torsion angles (Φ = −53 to −67° and Ψ = −38 to −50°). We initially chose to evaluate potential interaction modes between the receptor binding site model and LSD, a conformationally rigid, nonselective, high affinity (K_i<1 nM) ligand. Since (+)LSD binds to serotonin receptors without a great degree of selectivity, it should prove useful for performing docking calculations for most serotonin subtypes [see preceding paper by Glennon and Dukat (32), this issue]. The x-ray structure of (+)LSD (5) was flexibly docked to the receptor 5-HT_2 model using the program DGEOM to produce several hundred sterically feasible, randomly generated complexes. Two constraints were imposed. The (+)LSD ammonium ion nitrogen atom was forced to lie (randomly generated) between −1.0 and 3.8 Å from either carboxylate oxygen atom of Asp-155 to maintain the putative ionic interaction between ligand and receptor. The distance range chosen was based on the experimentally determined average of 2.9 Å for O-N hydrogen-bonding distances for ligands bound to protein carboxyl groups (71). Defining the distance to either of the two carboxylate oxygens is based on the fact that ammonium ion-carboxylate complexes show no clear preference

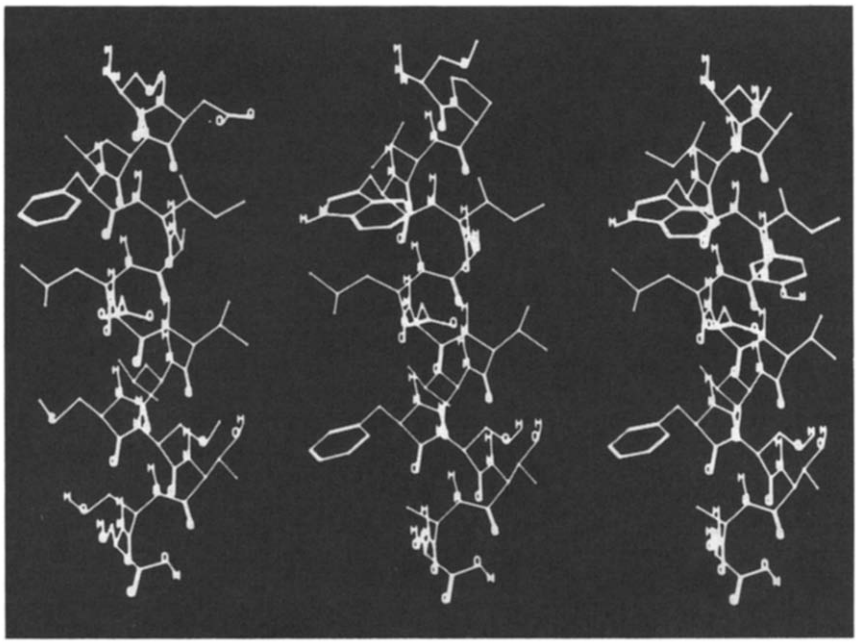

FIG. 7. Models of the helix 3 portions of the 5-HT_{1A}, 5-HT_{1C}, and 5-HT_2 receptors.

for a chelate (both carbonyl oxygens) or lateral geometry of interaction (single carboxylate oxygen) (71). A second set of distance constraints was imposed that, in effect, enforced nonbonded interactions between the remainder of the ligand and receptor. This was accomplished by requiring the indole NH of LSD to lie within −1.0 and 10 Å of the receptor Asp-155 carbonyl carbon. Solutions for which the angle between a plane bisecting the helix and the line defined by the ammonium ion and the indole NH is greater than approximately 60° are rejected automatically. The combined constraints, in effect, define a toroidal region of space within which LSD may lie. Sterically unreasonable solutions were automatically rejected by setting the lower bounds of ligand-receptor approach to van der Waals radii.

The numerous complexes were divided into structurally related families using hierarchical cluster analysis, and representatives of each were fully energy minimized without constraints using the AMBER force field (65,77). Interaction energies were calculated as the difference between the energy of the complex and the sum of the energies of the optimized receptor and optimized ligand alone. In all cases, complex formation was energetically favorable. Of the structures generated, one family of related orientations proved not only to have the highest calculated binding energy, but also showed a 4-point attachment that was compelling from the standpoint of chemical intuition. The complex featured an ionic interaction between the ammonium of (+)LSD and the carboxylate (Asp-155) of the receptor model (mean NH-O distance 1.8 Å), a hydrogen bond between the hydroxyl group of Ser-137 and the amide carbonyl of LSD (C=O-HO distance 1.8 Å, 156°), and two nearly perpendicular edge-to-face hydrophobic interactions between the indole nucleus of LSD and the receptor, one with Trp-129 toward the N-terminal end and one with Phe-136 toward the C-terminal (Fig. 4). The latter edge-to-face geometry for aromatic-aromatic interaction has extensive experimental precedent in crystal structures of amino acids, peptides, proteins, and ligand-protein complexes (12). This type of interaction may be more energetically favorable, in many cases, than the more familiar aromatic ring stacking that occurs in the DNA double helix (12). It should be pointed out that preliminary manual docking experiments failed to produce this complex, even though it was obviously attractive after visualization.

(+)LSD bound to the receptor model in this fashion was used as a template for starting geometries of other serotonergic agents; the structure of (+)LSD was simply "mutated" to another ligand, AMBER minimization was performed, and binding energies were calculated as described. As an example of what was done, the structure of LSD, which contains the basic structure of 5-HT, was "mutated" to 5-HT by deleting bonds and atoms not common to both structures, and adding substituents as required (the 5-OH group in this case). In all cases, the final geometries of the new complexes were different from the starting geometry, but all maintained the following essential features: 1) an ionic bond between the ammonium ion and the receptor carboxylate, 2) aromatic-aromatic interactions between the ligand aromatic ring and one or both flanking receptor aromatic side chains (Phe, Trp), 3) van der Waals interactions between aliphatic features of the ligand and hydrophobically compatible receptor side chains. Figure 5 shows a stereoscopic depiction of the energy minimized complex of the receptor model and 5-HT.

The energy calculations were performed using an approximate empirically-derived force field method in the absence of solvent. In addition, the methods are static (calculated quantities can be proportional only to enthalpy not total Gibbs free energy) and not dynamic, and are based on an abbreviated receptor model. Given these limitations, one should not hope to achieve quantitatively accurate predictions of experimental binding energies. Absolute affinities cannot be accurately calculated using these methods. However, the relative calculated binding energies should be realistic if entropy changes on binding (due to changes in solvation and loss of translational and rotational degrees of freedom) are comparable for a series of compounds. Indeed, qualitatively acceptable predictions would be remarkable and provide evidence for the accuracy of the principle hypothesis: the ligand binding site is comprised primarily of the features

surrounding a carboxylate on a single receptor α-helix. Table 2 provides evidence for the qualitative success of this approach. The principle finding is simply that compounds that are known to bind with high affinity (K_i<10 nM) are tightly associated with the receptor model with calculated binding energies ranging from −239 to −219 kJ/mol, whereas low affinity agents (i.e., K_i>100 nM) produce much lower calculated binding energies (−207 to −106 kJ/mol). Electrostatic interactions will be overestimated by any method that does not include explicit solvent. To help establish what value of calculated binding energy corresponds to low affinity association (i.e., one dominated by electrostatic terms), trimethylamine, which lacks all known structural features required for high affinity association except for the ammonium ion, was evaluated as a control. The calculated binding energy of −195 kJ/mol indicates that association occurs, but that the interaction is much weaker than that for the high affinity agents. It is interesting to note that, although the structural template for the starting geometry was a conformationally restricted compound [(+)LSD], the less conformationally restricted endogenous receptor agonist, serotonin, is calculated to bind with high affinity, even though its final bound orientation is quite different from the template (Fig. 6). The observed qualitative agreement between calculated and experimental affinities holds up well even for structurally diverse compounds such as serotonin, LSD, ketanserin, DOB, and 8-OH DPAT (Fig. 1). In a series of structurally related amphetamine analogs that includes both high and low affinity agents, the agreement between calculated affinities and experimental data (30) is also good, suggesting that the model is appropriately sensitive to aromatic substitution as well as amine alkylation. The model seemingly accounts for stereochemistry; for example, (+)LSD has a much higher calculated binding energy than its inactive enantiomer. The enantiomers of DOB, which have comparable experimental 5-HT_2 binding affinities (30), also have similar calculated binding energies.

Inspection of a similar model of the 5-HT_{1C} receptor, the aspartate containing region of helix 3 (Fig. 7), proves that the proposed binding site is identical to that of the 5-HT_2 receptor. The two structures differ only at sites remote from the binding site or on the opposite helical face, the region presumed to be associated with the membrane. Limited computational exploration of the association of the ligands with the 5-HT_{1C} model has indicated that the qualitatively accurate prediction of ligand affinities produced for the 5-HT_2 receptor also applies to the abbreviated 5-HT_{1C} receptor model (Table 1). In addition, similar modes of binding were observed for a common ligand at both model receptors. This result is consistent with the parallel affinities of a wide variety of ligands for 5-HT_2 and 5-HT_{1C} receptors (30,31).

Since it has not yet been experimentally determined which aspartate-containing helix, helix 2 or helix 3, is the most likely to be the binding site locus, it was of interest to examine the question computationally. An α-helical peptide model of helix 2, (CH_3-Leu-Met-Ser-Leu-Ala-Ile-Ala-Asp-Met-Leu-Leu-Gly-Phe-Leu-Val-NH_2), was constructed as described for the helix 3 model (Table 1). Inspection of the model indicates that the region surrounding the aspartate is nearly featureless with respect to potential sites of ligand-receptor interaction. (+)LSD was randomly docked to the model using DGEOM with the same constraints used previously. AMBER minimization of representative conformational families, performed as described for helix 3, proved that the best orientation produced a calculated binding energy of only −208 kJ/mol (as compared to −224 kJ/mol for helix 3). We suggest on the basis of calculated energetics of association, that helix 3, not helix 2, contains the ammonium ion binding aspartate.

Conclusion

Recent advances in molecular modeling methods allow its application to complex problems of biological interest. Of particular interest to us is the application of molecular modeling to the investigation of 5-HT receptors. There are two basic approaches that can be applied in conducting these studies: the ligand-ligand or receptor mapping approach, that attempts to indirectly gather information about receptors by studying agents that bind at these receptors, and the ligand-receptor approach, that directly examines the interaction of ligands with an experimental or model receptor structure. Very few 5-HT modeling studies using the ligand-ligand approach have been published; we provide several examples and have cited most of the available studies, and Evans and co-workers (25) provide additional insight with regard to 5-HT_3 receptors. Essentially nothing has been published with regard to the ligand-receptor approach.

For both approaches, we have provided a discussion of the various assumptions that are made in conducting modeling studies, and we described the limitations of such studies. Determination of an alignment rule poses a serious dilemma, but serves to provide a first approximation to solving modeling problems. Even greater problems are encountered with the ligand-receptor approach. This approach requires the use of a receptor model; because precise information about receptor structure is lacking (including what portion of the entire receptor protein serves as the active site of the receptor), there is a some uncertainty associated with the receptor model itself.

Nevertheless, by analogy to other receptors, we have constructed a receptor model and have conducted the first ligand-5-HT receptor studies described to date. Our initial studies utilize a single-helix model. Although more than one helix may be involved in the actual binding of (at least some) serotonergic agents, the single-helix model seems to account for differences in ligand affinities. The results of these modeling studies suggest that Asp-155 of helix 3 is an important feature for the binding of serotonergic ligands at 5-HT_2 receptors. Additional structural features were identified as contributing to the binding of (+)LSD, 5-HT, and related agents. The proposed model also accounts for (a) the lack of binding of 8-OH DPAT (a selective 5-HT_{1A} ligand), (b) the stereoselective binding of (+)LSD versus (−)LSD, and (c) the decreased affinity of several DOB analogs. 5-HT_2 receptors also possess an aspartate residue in helix 2. A parallel series of studies was conducted using a helix 2 model, and the results suggest that the binding at helix 3 better accounts for the affinities of the agents investigated. The aspartate region of helix 3 is essentially identical for 5-HT_2 and 5-HT_{1C} receptors (although it is quite different at 5-HT_{1A} receptors); thus many agents found to bind at the helix 3 5-HT_2 receptor model should (and do) bind in a similar fashion with a helix 3 5-HT_{1C} receptor model. These findings are consistent with experimental observations of parallelism in the binding of various ligands at 5-HT_2 and 5-HT_{1C} receptors as demonstrated in radioligand binding studies. The experience gained in the work described here will form the basis for continued studies involving the construction and evaluation of more complete structural models of 5-HT receptor subtypes.

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