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# Molecular Mechanisms of Ligand Interaction with the Gonadotropin-Releasing Hormone Receptor

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## I. Introduction

GONADOTROPIN-RELEASING hormone plays a central role in the biology of reproduction (1), and synthetic GnRH analogs have proven valuable in the treatment of a wide variety of endocrinological and nonendocrinological disorders (2–10). The decapeptide GnRH is generated in neurons of the medial basal hypothalamus through enzymatic processing of a larger precursor. Released in a pulsatile manner into the portal circulation, GnRH interacts with high-affinity receptors on the gonadotropes in the anterior pituitary, leading to the biosynthesis and release of the gonadotropins LH and FSH. The pulse-timing and concentration levels of GnRH are critical for the maintainence of gonadal steroidogenesis and for normal reproductive function.

Chronic, high concentration agonist stimulation of the pituitary GnRH receptors induce regulatory changes that lead to gonadal hypoactivity. This paradoxical suppression of gonadal function in response to pharmacological levels of agonist is the basis for the utility of GnRH analogs in the treatment of gonadal-steroid sensitive tumors, such as prostate cancer.

The GnRH receptor has been an unabatedly intense and productive subject of research for several decades because of its dual significance both for understanding reproductive biology and for developing medical therapies. The landmark elucidation of the primary sequence of GnRH by the laboratories of Schally (11) and Guillemin (12) inaugurated the field. Previous reviews have documented the subsequent evolution of research into GnRH and its receptors over the intervening decades. The complex regulation of the mammalian receptor, which is critical both for normal reproduction and for therapeutic response to analogs, has been studied in many species (reviewed in Refs. 13–15). The various signal transduction pathways used by the receptor have been investigated (reviewed in Refs. 15–20). Several thousand GnRH analogs have been synthesized and characterized (reviewed in Ref. 21) and the amino acid and cDNA sequences for GnRHs have been determined from many vertebrates (reviewed in Refs. 22 and 23).

GnRH receptor clones have recently been isolated. These clones provide the tools and impetus for recent progress in studies of the structure-activity of the receptor-ligand complex (24). The availability of the primary amino acid sequences and cDNAs has made possible the study of the molecular mechanism of action of GnRH and its analogs through receptor mutagenesis and computational modeling of the receptor and peptide (*e.g.* see Refs. 51 and 174). Thus, the structure-activity of GnRH and its analogs can now begin to be placed in the context of the receptor itself. The present review aims to summarize such work on the structure-activity relations and computational modeling of GnRH analogs and of the receptor. Recent developments will be em-

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phasized, with earlier studies presented in illustrative rather than comprehensive fashion.

## II. Amino Acid Sequences of GnRH Receptors

### A. GnRH receptor cDNAs

The cDNA sequence of the mouse GnRH receptor was the first determined. The receptor cDNA was cloned by three groups using the murine gonadotrope cell line, $\alpha$T3–1 cells (25), as a source of RNA. Efficient heterologous expression of the mammalian GnRH receptor in oocytes using $\alpha$T3–1 RNA suggested that this cell line would be a suitable source for cloning the receptor (26). The first clone was isolated using a PCR-based homology cloning strategy (27). Mouse GnRH receptor clones were also identified using *Xenopus* oocyte (28) and mammalian cell line (29) expression cloning. After the elucidation of the mouse receptor sequence, the homologous pituitary cDNAs were identified in five additional mammalian species and one nonmammalian vertebrate: human (30, 31), rat (29, 32, 33), sheep (34, 35), cow (36), pig (37), and catfish (38). An alignment of the cloned GnRH receptor sequences is shown in Fig. 1. The predicted amino acid sequence for the GnRH receptors is more than 85% conserved overall in the six mammalian species reported and is nearly identical within the putative transmembrane domains.

The cow, sheep, and human receptors are 328 amino acids long, while the mouse and rat receptors are 327 amino acids, due to the absence of a residue in the second extracellular domain. According to the consensus-numbering scheme used in this review, this residue is $Lys^{4.77(191)}$ in the human receptor and $Glu^{4.77(191)}$ in the three receptors of ungulates cloned to date. (For a description of the consensus-numbering scheme used in this review, see Fig. 3 or Ref. 39.) The catfish receptor is 370 amino acids in length and is notable for having a 49-amino acid cytoplasmic carboxy terminus domain not present in the mammalian receptors (38). GnRH receptor cDNAs were also isolated from human breast and ovarian tumors (40) and from rat gonads (41). The sequences obtained from these extrapituitary sources were identical to the pituitary GnRH receptor cDNAs of the corresponding species.

### B. General structural features

Analysis of the primary sequence identifies the GnRH receptor as a member of the rhodopsin-like G protein-coupled receptor (GPCR) family. Three distinct families of G protein-coupled receptors have been identified by molecular cloning. The three classes are: the metabotropic glutamate receptors (42, 43), the secretin-calcitonin-PTH class (44–48), and the large rhodopsin-like GPCR superfamily (49, 50), of which the GnRH receptor proved to be a member. Sequences of the members of the three classes of G-protein coupled receptors, when analyzed for hydrophobicity, all contain seven putative transmembrane domains (see Fig. 1). However, the three classes do not share any discernible sequence homology. Included within the rhodopsin-like family of G protein-coupled receptors are the opsins, G protein-coupled neurotransmitter receptors (adrenergic, serotonergic, dopaminergic, muscarinic acetylcholine, etc.), glycoprotein hormone receptors (FSH, LH/CG, and TSH), and a variety of peptide receptors (49, 50).

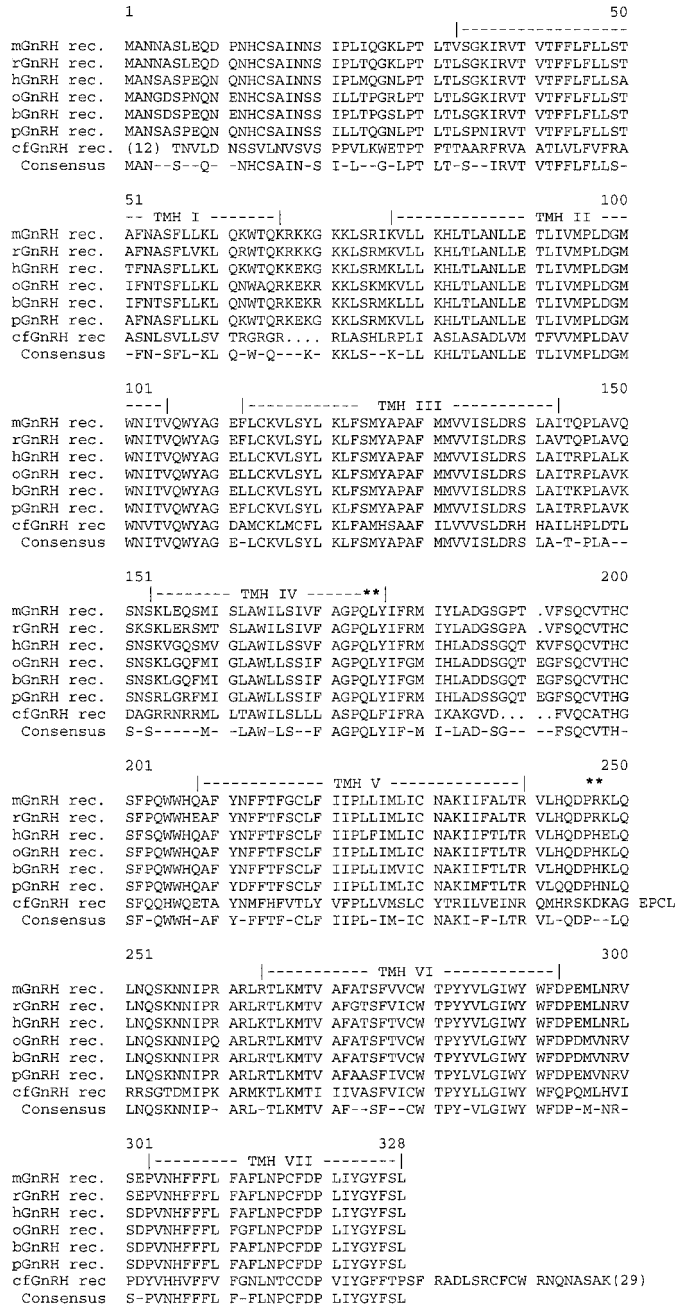


FIG. 1. Sequence alignment of cloned GnRH receptors. The putative transmembrane domains are indicated. m, Murine; r, rat; h, human; o, ovine; b, bovine; P, pig; cf, catfish. The helix boundaries were determined by sequence comparison, using the helix ends proposed by Donnelly *et al.* (233) for the human $\beta_2$-adrenergic receptor. The consensus sequence reflects the mammalian sequences only. The junctions between exons in the murine and human receptors are indicated by **.

A given receptor can be identified as belonging to the rhodopsin-like GPCR family by the presence of certain amino acid motifs conserved within the transmembrane helix domains (TMD), a pattern of conservation that has also facil-

itated the cloning of a large number of these receptors (see Fig. 2). Variations in the pattern of conservation in the GnRH receptor in comparison with other rhodopsin-like GPCRs has proven valuable in elucidating the functional and structural roles of some of these side chains (51) (see Figs. 2, and 3 and below).

```
                    Helix I
                                    |
hGnRH          LSGKIRVTVTFFLFLLSATFNASFLLKLQKWTQK
hNK1           WQIVLWAAAYTVIVVTSVVGNVVVMWIILAHKRM
hß2 adr.       VWVVGMGIVMSLIVLAIVFGNVLVITAIAKFERL
h rhodopsin    WQFSMLAAYMFLLIVLGFPINFLTLYVTVQHKKL

                    Helix II
                        |
hGnRH rec.     KLLLKHLTLANLLETLIVMPLDGMWNITV
hNK1           NYFLVNLAFAEASMAAFNTVVNFTYAVHN
hß2 adr.       NYFITSLACADLVMGLAVVPFGAAHILMK
h rhodopsin    NYILLNLAVADLFMVLGGFTSTLYTSLHG

                    Helix III
                                          |||
hGnRH rec.     LLCKVLSYLKLFSMYAPAFMMVVISLDRSLAI
hNK1           FYCKFHNFFPIAAVFASIYSMTAVAFDRYMAI
hß2 adr.       FWCEFWTSIDVLCVTASIETLCVIAVDRYFAI
h rhodopsin    TGCNLEGFFATLGGEIALWSLVVLAIERYVVV

                    Helix IV
                        |
hGnRH rec.     SKVGQSMVGLAWILSSVFAGPQLY
hNK1           ATATKVVICVIWVLALLLAFPQGY
hß2 adr.       KNKARVIILMVWIVSGLTSFLPIQ
h rhodopsin    ENHAIMGVAFTWVMALACAAPPLA

                    Helix V
                              |
hGnRH rec.     QAFYNFFTFSCLFIIPLFIMLICNAKIIFTLTR
hNK1           IYEKVYHICVTVLIYFLPLLVIGYAYTVVGITL
hß2 adr.       NQAYAIASSIVSFYVPLVIMVFVYSRVFQEAKR
h rhodopsin    NESFVIYMFVVHFTIPMIIIFFCYGQLVFTVKE

                    Helix VI
                               || |
hGnRH rec.     KTLKMTVAFATSFTVCWTPYYVLGIWYWFD
hNK1           KVVKMMIVVVCTFAICWLPFHIFFLLPYIN
hß2 adr.       KALKTLGIIMGTFTLCWLPFFIVNIVHVIQ
h rhodopsin    EVTRMVIIMVIAFLICWVPYASVAFYIFTH

                    Helix VII
                                ||  |
hGnRH rec.     PVNHFFFLFAFLNPCFDPLIYGYFSL
hNK1           QVYLAIMWLAMSSTMYNPIIYCCLND
hß2 adr.       EVYILLNWIGYVNSGFNPLIYCRSPD
h rhodopsin    IFMTIPAFFAKSAAIYNPVIYIMMNK
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FIG. 2. Alignment of the TMD domains of the human GnRH receptor primary sequence with the human NK1 receptor (234), $\beta$2 adrenergic receptor (235), and rhodopsin (236) sequences. Some residues conserved among most GPCRs are marked.

The identification of the GnRH receptor as a member of the GPCR superfamily is consistent with previous studies of guanyl-nucleotide modulation of binding and coupling, which suggested that the GnRH receptor interacts with a G protein (52, 53). Recent studies with $\alpha$T3–1 cells have demonstrated that the G protein involved in coupling to phospholipase C in this cell line is pertussis-toxin insensitive, being $G_q$ and/or $G_{11}$ (54, 55).

A representation of the putative topology of the human GnRH receptor sequence is presented in Fig. 3. The receptor is composed of a single polypeptide chain. Hydrophobicity analysis of the receptor's primary sequence confirms the presence of seven hydrophobic stretches corresponding to putative transmembrane helical domains, with an extracellular amino terminus and an intracellular carboxy terminus. Direct structural information is available for only two heptahelical membrane proteins. The structure of bacteriorhodopsin, which has little if any sequence similarity with the mammalian receptor proteins (see Ref. 56), has been elucidated at 3.5 Å resolution by cryo-electron microscopy of two-dimensional crystals (57). Using the same approach, a 9-Å projection map of bovine rhodopsin, shown in Fig. 4 (58), and a 6-Å projection map of frog rhodopsin (59) have been obtained. The maps of rhodopsin are consistent with the presence of seven transmembrane domains, as had been predicted from primary sequence analysis. The transmembrane domains of all GPCRs are believed to be $\alpha$-helical and arranged around a hydrophilic core in a manner similar to the rhodopsin map (39).

Several features conserved among GPCRs are altered in the mammalian GnRH receptors. The mammalian GnRH receptor is the only rhodpsin-like GPCR identified to date that lacks the entire intracellular C-terminal domain. Also unique in the GnRH receptor is the presences of an Asn at the position of a conserved Asp residue in other GPCRs. The GnRH receptor also has unusual substitutions at loci that are highly conserved, such as the modification of the more common DRY sequence of the proximal second intracellular domain to DRS and the substitution of Asp for Asn in the TMD 7 consensus NPXXY motif. Notably, most of these unique features are not conserved in the nonmammalian catfish GnRH receptor sequence (see Fig. 1). Mutation of DRS to DRY in the mammalian receptor was reported to cause only a small increase in agonist affinity with no discernible change in signal transduction (60). The other unique features of the mammalian receptors seem to have functional significance and will be discussed below in light of recent experimental results.

### *C. Covalent modifications*

*1. Glycosylation.* Most GPCRs have consensus glycosylation sites, and several receptors have been found to be glycosylated at these sites (61–63). Biochemical studies of the GnRH receptor have suggested that it is a sialic acid residue-containing glycoprotein (64, 65). The cow, sheep, pig, and human receptor sequences contain two potential sites for N-linked glycosylation (N-X-S/T), one in the amino terminus and one in the first extracellular domain (see Figs. 1 and 3). The rodent species contain an additional potential glycosylation site in

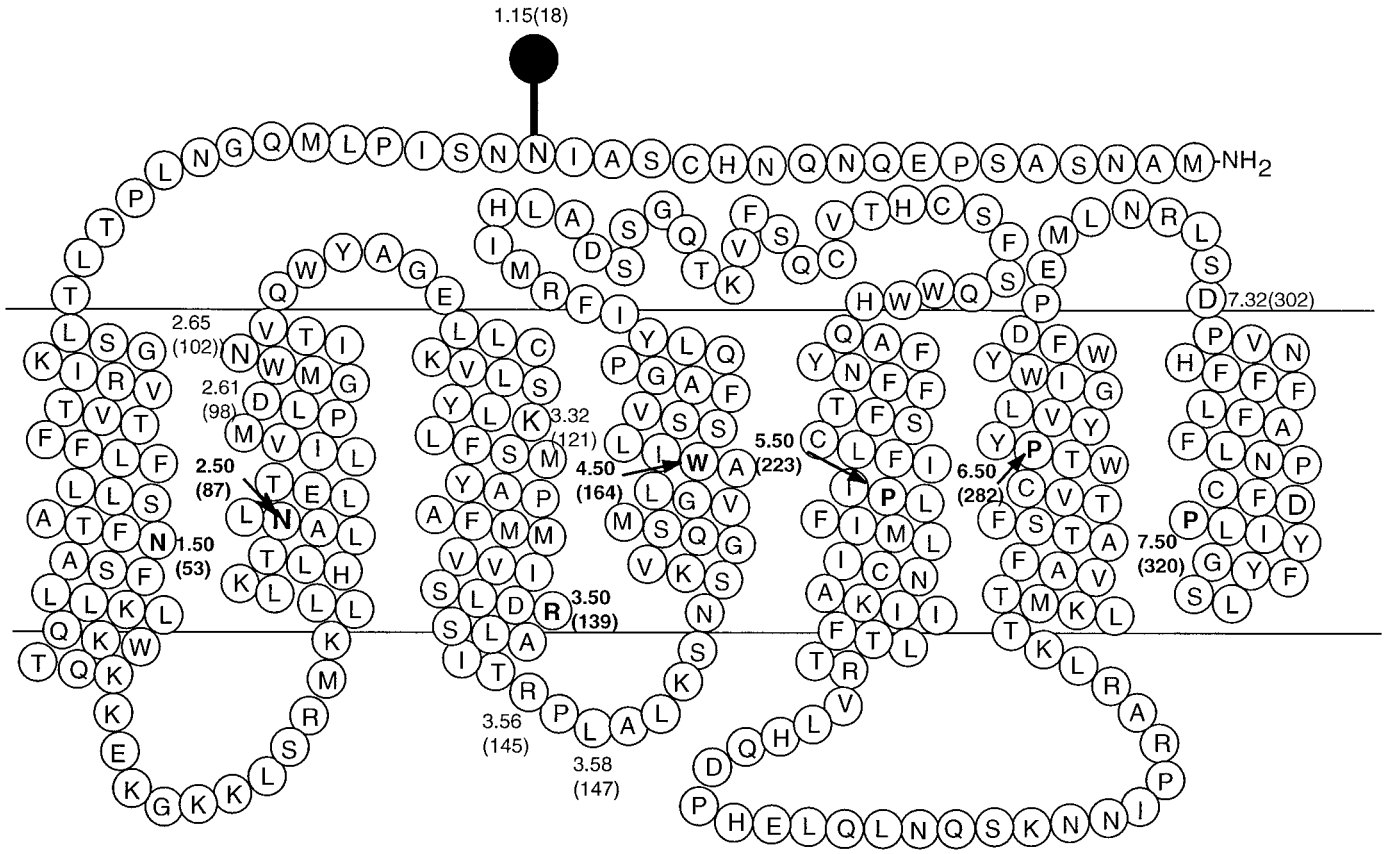


FIG. 3. Helical net representation of the human GnRH receptor. The known glycosylation site is marked, and certain key functional residues are numbered. To allow for the generalization of findings in the GnRH receptor to other GPCRs, we utilize a consensus numbering scheme that has been described elsewhere (39) and is used in the NIH GPCR mutation database available via World Wide Web at http://mgddk1.niddk.nih.gov:8000/MutationAnalysis.html (237). In this system, the most conserved residue in a particular TMD (*e.g.* helix 3) is designated by the index 50. Thus the conserved Arg at the cytoplasmic side of helix 3 is numbered 3.50. The other residues are numbered in relation to the conserved residue. For example, the Lys in TMD 3 is denoted as $Lys^{3.32(121)}$. The reference residue in each helix is indicated by *bold type*.

FIG. 4. Projection density map of bovine rhodopsin at 9 Å resolution. [Reprinted with permission from G. F. Schertler *et al.*: *Nature* 362: 770–772, 1993 (58). ©1993 Macmillan Magazines, Limited]

the amino terminus. The glycosylation at these sites of the mouse GnRH receptor was investigated by site-directed mutagenesis and photoaffinity labeling (66). Mutation of $Asn^{1.01(4)}$ or $Asn^{1.15(18)}$ in the N-terminal domain to Gln caused a lower apparent molecular weight in gel electrophoresis, whereas mutation of $Asn^{2.65(102)}$ in the putative first extracellular loop did not affect mobility. Whereas the ligand-binding affinities of the amino terminus domain mutants were unchanged, these receptors were expressed in transfected cells at a lower level than the wild type receptor. These results suggest that only the amino-terminal domain sites are glycosylated and that the glycosylation contributes to the level of receptor expression, consistent with earlier studies in which sialidase and tunicamycin were reported to decrease the level of receptor expression but not to alter affinity (65). The mutagenesis studies do not support an earlier suggestion, based on studying the effects of periodate on binding, that glycosylation contributes to high affinity binding (66). The receptor levels of glycosylation-deficient mutants show similar decreases in both membrane preparations and in whole cell assays (67). Thus, in contrast to the $\beta$-adrenergic receptor, for which glycosylation is required for proper transport of the receptor to the cell surface (62), in the case of the GnRH receptor the observed decrease in receptor number does not appear to represent altered receptor transport.

The possibility that differential glycosylation contributes to the differing level of expression observed with transfection of the mouse and human receptor has been examined by introducing a second glycosylation site into the human receptor sequence, thereby recreating the pattern of sites found in the mouse. The second site was found to be glycosylated, and its presence increased the level of receptor expression

(68). These studies indicate that the glycosylation of the GnRH receptor does not contribute to receptor affinity but does improve the level of receptor expression, possibly by decreasing the rate of receptor degradation.

*2. Phosphorylation sites.* Many intracellular serine and threonine residues are within phosphorylation consensus sequences, and phosphorylation could be involved in modulating receptor responsiveness or intracellular trafficking. While desensitization of the GnRH-induced responses in pituitary cells has been observed, it is not clear whether this occurs at the level of the receptor (for review see Refs. 13–15). Of note, however, a lack of rapid receptor-mediated desensitization of the GnRH receptor has been reported in αT3–1 cells and in transfected cells (69–71).

*3. Disulfide bridges.* Most GPCRs contain single conserved cysteines in the first and second extracellular loops that may form a disulfide bond to stabilize the structure of the functional protein. Mutation of these conserved cysteine residues disrupts the function of rhodopsin, muscarinic, β-adrenergic, and TRH receptors, suggesting that this disulfide bond is required for proper receptor folding (72–76). Experiments using site-directed mutagenesis and photoaffinity cross-linking support the presence of two extracellular disulfide bridges in the GnRH receptor. The presence of a conserved cystine bridge between $C^{3.25(114)}$-$C^{5.23(196)}$ was demonstrated in the mouse receptor, and evidence for a second disulfide bond between $C^{1.11(14)}$-$C^{5.27(200)}$ has been obtained for the human receptor (J. Davidson, personal communication).

### *D. Gene structure*

The chromosomal locations of the mouse, sheep, and human genes have been reported. The human gene was assigned to chromosome 4 by PCR analysis of somatic hybrid cell lines (77, 78) and to the 4q13.1–4q21.1 region using cell hybrid mapping panels (79). Using chromosomal *in situ* hybridization, three groups have reported the gene localization at band 4q13.2–13.3 (78, 80, 81) and one group at band 4q21.2 (82). Mapping the gene relative to 4q microsatellite markers in GnRH receptor YAC clones supports the 4q13.2–13.3 assignment (80). The mouse gene has also been mapped by linkage analysis to within 1.2 ± 1.2 centimorgans of the chromosome 5 marker Pmv-11 (79), and the sheep gene has been localized near the FecB locus of chromosome 6 (83).

The structures of the mouse and human GnRH receptor genes have been investigated. In contrast to the genes of many GPCRs, which are intronless and are believed to have arisen by retroposition (84), the GnRH receptor contains introns within the coding region. The mouse gene is composed of at least three exons spanning more than 22 kb (85). The open reading frame is distributed among the three exons, which encode amino acids 1–174, 175–247, and 248–327, respectively (see Fig. 1 for location of exon junctions). Variant transcripts of the mouse receptor that are generated by alternative splicing and do not encode functional receptors have been isolated from αT3–1 cells (85). The alternative transcripts found all include exon 1 but lack either exon 2 or 3. The alternative transcripts form a minority of the cDNAs isolated from an αT3–1 cell library, and the biological function of the proteins encoded by these cDNAs is not known. The human gene is also distributed over three exons that span 18.9 kb (77, 86). The amino acid locations of introns 1 and 2 are homologous to their positions in the mouse receptor gene. Intron I is located between amino acids 174–175 in the putative TMD 4 domain, and intron 2 is located between amino acids 248–249. Exon 2 is three nucleotides longer in the human gene in comparison with the mouse, reflecting the presence of an additional amino acid ($Lys^{4.77(191)}$) in the second extracellular loop of the human receptor (see Fig. 1). Southern blot analysis is consistent with the presence of a single gene in the mouse (85), rat (85), and human (77) genomes.

Fan *et al.* (86) have mapped the 5′- and 3′-flanking regions of the human receptor gene. Multiple initiation sites and multiple polyadenylation signals are present. Five consensus TATA sequences distributed over 669 bases are present in the 5′-flanking region. Primer extension analysis using human brain RNA indicates the utilization of several initiation sites. The longest extension was confirmed by PCR and represents a transcript with 1393 bp of 5′-untranslated sequence. A putative cAMP response element is found at −1490, a putative glucocorticoid/progesterone response element is located at −92, and consensus binding sites for several transcription factors are present. At the 3′-end of the gene, five polyadenylation signals are found, distributed over 800 bp. In the largest possible transcript, the 3′-untranslated sequence is 3.1 kb in length. Thus the exons of the human gene identified appear to account for the largest ~5-kb transcript identified on Northern blot analysis (31).

The 5′-flanking region of the mouse gene has been investigated by Albarracin and co-workers (87). In contrast to the human gene, the mouse gene appears to have a smaller 5′-untranslated segment. The major initiation site is found 62 bases upstream of the translation initiation site. Also in contrast to the human gene, the 5′-sequence of the mouse gene lacks TATA sequences. Preliminary studies on the regulation of a 1.2-kb fragment of the 5′-flanking region of the mouse gene have been reported (87).

## III. Structure-Activity Relations of GnRH Peptides

### *A. Overview*

A prerequisite for a meaningful investigation of ligand-receptor interactions is an understanding of the roles of ligand residues involved in receptor binding and in agonist activation of the receptor. This knowledge base for GnRH may be gleaned from the large number of structure-activity studies on synthetic GnRH analogs and on the 11 naturally occurring GnRH structural variants and their synthetic chimeras. The structural features of GnRH agonist and antagonist analogs were comprehensively reviewed a decade ago (21), and a number of more specialized reviews on antagonists and structural constraints have subsequently appeared (88–91). The structure-activity relations of naturally occurring vertebrate GnRHs have been extensively reviewed (23, 92–99). However, these reviews do not attempt to specifically and systematically analyze the roles of each of the individual

amino acids in GnRH. The comprehensive review of Karten and Rivier (21) addressed the substitutions that produced superactive agonists and antagonists, whereas the reviews on comparative activities of vertebrate GnRHs focused on the contributions of substitutions in positions 5, 7, and 8. In this section we shall attempt to synthesize these diverse sources of information into a more complete review of the roles of the constituent amino acids of GnRH.

Several thousand GnRH analogs have been synthesized to date, and information on their activities potentially provides a very large data base for the purpose of identifying functional residues in GnRH. However, interpretation is complicated by a number of factors:

1. Frequently, multiple substitutions have been incorporated simultaneously in single GnRH analogs, often without a systematic approach due to the large number of combinatorials involved.

2. Even the effects of single-amino acid substitutions may be difficult to interpret. A single substitution may alter affinity and agonist activity via modification of a side chain that interacts with the binding pocket and/or by altering the conformation of the peptide and thus affecting the presentation of other peptide moieties that interact with the receptor. Substitutions that have a conformational effect cannot be differentiated through structure-activity data alone from those that eliminate receptor contact sites. Any substitution may establish new contacts with the receptor and disturb the normal contacts by altering the families of conformations of the peptide. This difficulty in the interpretation of structure-activity data would be obviated by achieving a more complete understanding of the conformational effects of substitutions and by the analysis of ligand-receptor interactions in a structural context. Progress toward predicting the effects of amino acid substitutions on peptide conformation is described in the next section, whereas the development of three-dimensional models of receptor molecules is discussed in *Section V*.

3. While substitutions of residues that produce antagonists may remove a contact interaction, they most likely establish new compensatory contact sites, presumably with different sites in the receptor, to retain high-affinity binding.

4. For much of the available data, the activities of analogs cannot be rigorously compared because they have been tested in different assay systems (see Ref. 100 for review). The most commonly employed assays have been *in vivo* bioassays (*e.g*. inhibition of ovulation) in which activity is a composite of pharmacokinetics of absorption from the injection site, association with lipophilic compartments (*e.g*. fat and cell membranes), binding to plasma proteins, degradation, metabolic clearance (including renal clearance), receptor binding affinity, and efficacy. Ideally, comparative data on binding affinity and signal transduction (*e.g*. second messenger generation) are required. However, as these data are available for relatively few analogs, in adjudging the effects of single-amino acid substitutions we have relied extensively on *in vivo* data, particularly from early studies. It has been necessary, therefore, to consider possible pharmacodynamic contributions to the activity of the analogs when making inferences about receptor binding and receptor activation based on data obtained from *in vivo* bioassays (see below).

Even the direct measure of analog affinity in receptor-binding assays may yield misleading results. These assays are usually conducted on membrane preparations (which expose all receptors), as opposed to whole cells, and employ conditions (buffers, pH, temperature, etc.) optimized to give maximal binding. These nonphysiological conditions may affect the binding of a substituted analog or mutated receptor differently than physiological conditions. The radiolabeled GnRH analogs used in binding assays mostly rely on incorporating $^{125}I$ into $Tyr^5$ of GnRH analogs that have a D-amino acid in position 6. This incorporation of the large electron-withdrawing iodine atom considerably alters the properties of the ligand. Since a large, bulky side chain is allowable when a D-amino acid substitutes for $Gly^6$ in the superactive analogs (see below), we have attempted to overcome this problem by substituting the $Tyr^5$ with His (as in the active chicken GnRH II) and incorporating D-Tyr in position 6 (101). This analog has a higher affinity and increased total binding.

A feature of ligand-receptor complexing is that receptor interaction (affinity) and capacity of the bound ligand to activate the receptor (efficacy) are separable phenomena. Thus particular residues of GnRH are more critical for agonist activity (*e.g*. $His^2$, $Trp^3$), and others are critical for ligand binding (*e.g*. $Pro^9$). Various models have been proposed to explain the differing contributions of ligand substituents to affinity and efficacy (102). In the "conformational induction" model, agonists bind to an inactive receptor state and induce the receptor to assume an altered active state that leads to coupling with G proteins. In the "conformational selection" model, the receptor spontaneously fluctuates between inactive and active conformers, and agonists have a higher affinity for the active state whereas antagonists (or inverse agonists) have a higher affinity for the inactive state. Consequently, agonist binding causes the concentration of active receptor to increase by mass action, and inverse agonists have the opposite effect. The separate effects of ligand substitutions on affinity and efficacy can be interpreted within either model. In the case of the induction model, some receptor interaction sites are critical for ligand docking, whereas others are critical for inducing a change in the receptor. In the selection model, some receptor contact sites are accessible in both active and inactive states and thus contribute to affinity, whereas other contact sites are accessible or properly positioned for agonist complexing only when the receptor assumes an activated state. In the following sections, the term "activity" will be used to refer to data derived from functional assays, usually LH release. When radioligand binding data are available, the term "affinity" will be employed.

With the preceding caveats in mind, it is nevertheless possible and useful to review the extant data. When evaluated in concert with studies of the receptor-binding pocket and of analog conformation (reviewed in subsequent sections), the data on the structure-activity of analogs provide insight toward elucidating the interactions in the ligand-receptor complex. For this reason, we present here some indications of the roles of the individual constituent amino acids of GnRH in receptor binding and activation.

*B. Comparative structures and activities of vertebrate GnRHs*

Identification of the GnRHs present in more than 70 species (for review see Refs. 22, 23, 92–99, and 103–105) has demonstrated that two or more forms of GnRH are present in most vertebrate species and in a protochordate, the tunicate (22, 106–116) (Fig. 5). One form is represented by mammalian GnRH and its nonmammalian counterparts, which have a predominant function as hypophysiotropic peptides regulating the pituitary. The second form of GnRH, first identified in chicken brain ($His^5Trp^7Tyr^8$GnRH), is the most ubiquitous form in vertebrates, and most species have this form along with one or two other GnRHs. As $His^5Trp^7Tyr^8$GnRH is present in fish, amphibians, reptiles, birds, and mammals, referring to this peptide as "chicken GnRH II" is confusing. We propose that it be designated "GnRH II." The original mammalian GnRH is then "GnRH I." Specific chemical identification may be accomplished by designating the variable amino acids 5–8. Thus chicken GnRH I, salmon GnRH, and catfish GnRH would be YGLQ GnRH, YGWL GnRH, and HGLN GnRH, respectively.

The similarity in organization of genes encoding the different GnRHs indicates that the GnRHs arose from a common ancestral gene (see Ref. 23) for review). The distribution of GnRH II in the extrahypothalamic central nervous system and peripheral nervous system suggests a neuromodulatory role for the peptide. The most thoroughly documented effect of GnRH II is the inhibition of $K^+$ channels in the bullfrog sympathetic ganglion (117). The presence of GnRH II in bullfrog and *Xenopus* sympathetic ganglia was recently demonstrated (118). Using PCR, GnRH receptor sequences have been obtained from fish, amphibian, and reptile (B. Blackman, Y.-M. Sun, N. Illing, J. Hapgood, E. Rumbak and R. P. Millar, unpublished). Multiple GnRH receptors are present in these species, suggesting that the duplication of the GnRH gene was accompanied by a coordinated structural evolution of the cognate receptors.

All natural GnRH peptides isolated to date are highly conserved with respect to their length, to the $NH_2$-terminal domain (residues pGlu-His-Trp-Ser), and to the COOH-terminal domain (Pro-Gly.$NH_2$), suggesting that these domains are functionally essential. However, residue conservation does not invariably imply functional signficance. For example, $Ser^4$ is highly conserved and yet can be substituted with the retention of high activity at the mammalian receptor (see below). Among vertebrate GnRHs, position 8 is most variable, and positions 5 and 7 are highly variable (Fig. 5). Position 6 is invariably Gly in the higher vertebrates but varies considerably in the lamprey and tunicate GnRHs. No variation in GnRH sequence is found among mammalian GnRHs.

The comparative activities of the GnRH variants in vertebrates provide insight into structure-activity relations. In mammals, mammalian GnRH is highly active at low doses while the other vertebrate GnRHs, with the exception of GnRH II, have poor activity (binding affinity or $EC_{50}$ and/or maximal gonadotropin release) (107, 110, 119–121) (Fig. 6). Since the single residue that distinguishes mammalian GnRH from all of the other vertebrate GnRH structural variants is $Arg^8$ (Fig. 5), this residue was identified as being critical for high-affinity binding to the mammalian receptor. However, the substantial activity of GnRH II (20–30%) (120–123) suggests that the loss of activity when substituting a neutral amino acid for $Arg^8$ can be overcome by the simultaneous substitution of His in position 5 and Trp in position 7.

In contrast with the limited activity of most nonmammalian GnRHs at the mammalian receptor, all of the vertebrate GnRHs (with the exception of lamprey GnRHs, which have an acidic residue in position 6) have similar high activities in all of the nonmammalian vertebrates tested (107, 119–121, 124, 125). Thus, the nonmammalian vertebrate receptors are promiscuous in interacting well with most of the vertebrate GnRHs, whereas the mammalian pituitary GnRH receptor is selective for mammalian GnRH [(see reviews (23, 92–98)].

Differences in the pharmacology of the GnRH receptors in vertebrate species are illustrated in studies with certain mammalian GnRH antagonist analogs that are pure antag-

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MAMMAL | pGlu | His | Trp | Ser | Tyr | Gly | Leu | Arg | Pro | Gly | $NH_2$ |
| CHICKEN I | pGlu | His | Trp | Ser | Tyr | Gly | Leu | Gln | Pro | Gly | $NH_2$ |
| SEABREAM | pGlu | His | Trp | Ser | Tyr | Gly | Leu | Ser | Pro | Gly | $NH_2$ |
| CATFISH | pGlu | His | Trp | Ser | His | Gly | Leu | Asn | Pro | Gly | $NH_2$ |
| SALMON | pGlu | His | Trp | Ser | Tyr | Gly | Trp | Leu | Pro | Gly | $NH_2$ |
| DOGFISH | pGlu | His | Trp | Ser | His | Gly | Trp | Leu | Pro | Gly | $NH_2$ |
| CHICKEN II | pGlu | His | Trp | Ser | His | Gly | Trp | Tyr | Pro | Gly | $NH_2$ |
| LAMPREY III | pGlu | His | Trp | Ser | His | Asp | Trp | Lys | Pro | Gly | $NH_2$ |
| LAMPREY I | pGlu | His | Tyr | Ser | Leu | Glu | Trp | Lys | Pro | Gly | $NH_2$ |
| TUNICATE I | pGlu | His | Trp | Ser | Asp | Tyr | Phe | Lys | Pro | Gly | $NH_2$ |
| TUNICATE II | pGlu | His | Trp | Ser | Leu | Cys | His | Ala | Pro | Gly | $NH_2$ |

FIG. 5. Amino acid sequences of naturally occurring vertebrate and protochordate GnRHs. The conserved $NH_2$- and COOH-terminal domains are *boxed*. Only $Gly^6$ is conserved among the higher vertebrates in the central domain.

onists in mammals but exhibit agonist activity in chicken (126) and goldfish (127) gonadotropes. Intriguingly, some antagonists stimulate gonadotropin secretion while others release GH in the goldfish (127). It is apparent, therefore, that the structural requirements of GnRH receptors for activation by ligands are variable among vertebrates. Differences, albeit more subtle, between mammalian GnRH receptors in agonist and antagonist binding have also been noted (30, 31, 35, 51, 128).

The studies on GnRH chimeras have also revealed effects of amino acid substitutions on agonist efficacy at the mammalian GnRH receptor by comparing relative potencies of the chimeric analogs in stimulating LH release from sheep pituitary cells and in binding to sheep pituitary GnRH receptors (Fig. 7) (121). These data showed that $Arg^8$ substitution by neutral amino acids in mammalian GnRH resulted in a low binding potency but relatively higher LH-releasing potency (ratios of 10–150). In contrast, $Tyr^5$ substitution by His enhanced binding potency but reduced LH-releasing potency (ratios of 0.14–0.2). Thus, once bound, analogs with a neutral amino acid in position 8 are more efficient at activating the receptor. On the other hand, $His^5$ enhances binding, but reduces efficacy. The role of specific amino acids in affinity and activation of the mammalian receptor will be addressed in the subsequent section.

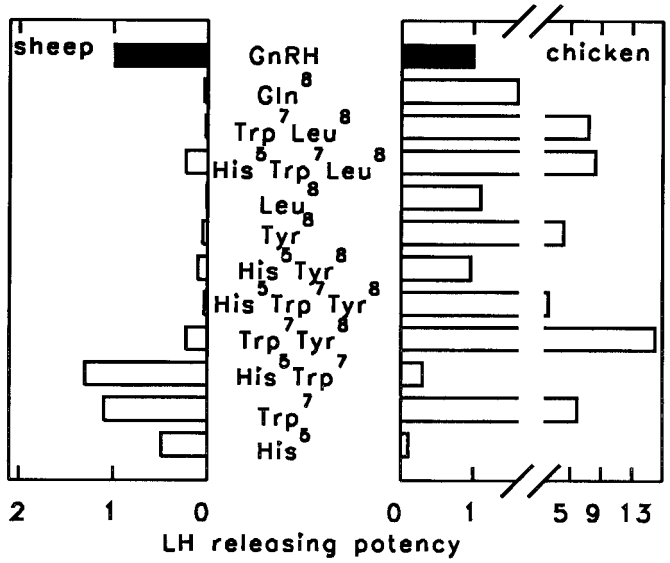


FIG. 6. Comparative LH releasing activities of chimeras of vertebrate GnRHs in sheep and chicken pituitary cells. [Derived from Ref. 121.]

### *C. Roles of individual amino acids in GnRH activity at the mammalian receptor*

The contributions of individual amino acids to GnRH receptor binding and activation may be explored by the systematic substitution of single amino acids. Although a considerable body of research was conducted along these lines in the early 1970s (*e.g.* see Ref. 129), this approach has not been revisited with the advent of specific binding assays and more recently with expressed cloned GnRH receptors.

The conservation of the $NH_2$- and COOH-terminal sequences of GnRH through vertebrate evolution and the conclusion that these domains are critically important for receptor binding and activation are substantiated by extensive structure-activity data. Indeed, cognizance of the evolutionary constraints on acceptable structures could have obviated much of the endeavor to produce agonist and antagonist analogs through empirical approaches. It is now clear that both the $NH_2$- and COOH-terminal domains are involved in receptor binding while the $NH_2$-terminal domain plays the major role in receptor activation (see reviews in Refs. 10 and 21). Although the lack of conservation of amino acids 5–8 suggests that these residues are not critical for ligand activity, this is not entirely so, as $Arg^8$ is important for high-affinity binding to the mammalian receptor (see below). This central domain is thus a determinant of receptor selectivity. The role of individual amino acids will be considered within these designated $NH_2$-terminal, central, and COOH-terminal domains.

#### *1. The $NH_2$-terminal domain (pGlu-His-Trp-Ser).*

*$pGlu^1$.* The essential requirement of pGlu was first noted with the loss of activity of native purified GnRH when treated with pyroglutamyl aminopeptidase and confirmed by a series of substitutions in this position. While $Leu^1$, $Gly^1$, $Pro^1$, $Gln^1$ and (O=)$Thr^1$-substituted analogs were essentially inactive in *in vivo* bioassays (129–132), several acylated

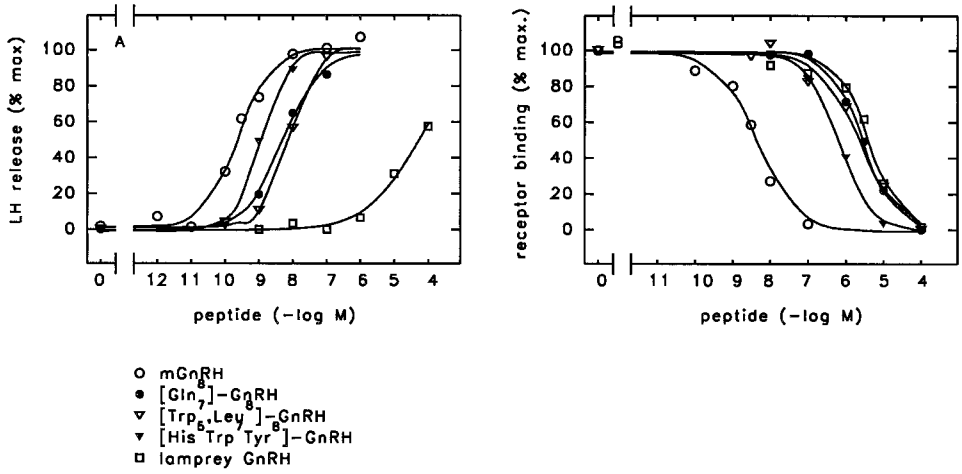


FIG. 7. LH stimulation and receptor binding of vertebrate GnRHs in sheep gonadotropes. [Derived from Ref. 121.]

$Gly^1$ analogs (formyl, acetyl, and propionyl) had low but significant activity (~1%), indicating that the -CO-NH-CHCO- group is the minimal structure required for activity (131). The cyclic (O=) $Ser^1$ structure (130), which resembles the pGlu structure, and D-pGlu (133) both had about 5% activity, suggesting that a change in conformation in the $NH_2$ terminus is not completely detrimental (134). Although a specific role for pGlu in binding and/or activation of the receptor has not been revealed by these early studies, the subsequent universal substitution of pGlu in GnRH antagonists (see reviews in Refs. 10, 21, and 88–91) identifies this residue as important in receptor activation.

*$His^2$*. $His^2$ of GnRH is a good candidate for interaction with the receptor (134). The imidazole ring of histidine has a number of features appropriate for participation in enzyme actions and also in hormone interactions with receptors. These include its aromatic character, hydrogen-bonding capacity, and acid-base properties. The very low activities of Ser-, Ile-, Leu-, Gln-, Gly-, Thr-, Ala-, Lys-, and Arg-substituted analogs (129, 130, 135) and substantial activity of $Phe^2$ GnRH (1–7%) (130, 135, 136), 3-Me $His^2$ (1%) (129), and $Trp^2$ (40%) (137) demonstrate the need for aromaticity and possibly the imidazole moiety. The lack of activity of $Gln^2$ GnRH indicates that the role of $His^2$ does not involve $\epsilon$-position hydrogen-bonding because, were that the case, Gln would be a suitable substitute. The high activity of $Trp^2$ GnRH has been confirmed by examining binding to the human GnRH receptor expressed in COS-1 cells (138). On the other hand, the acid-base and hydrogen-donor and hydrogen-acceptor capability of His can be modified with reasonable retention of activity, as demonstrated by I-$N^{im}$-$His^2$ (6%) (139) and $\beta$-pyrazolyl-3-alanine (19%) (140), although the latter is a much weaker base. A major breakthrough in understanding the function of $His^2$ evolved from the observations that the $Gly^2$ was a partial agonist and the des-$His^2$ analog was an antagonist (141). Bulky hydrophobics (*e.g.* D-4-Cl-Phe) have subsequently become the hallmark of substitutions of $His^2$ in GnRH antagonists (for review see Refs. 10, 21, and 88–91). It is therefore likely that $His^2$ plays a role in GnRH interaction with the receptor, which leads to signal propagation and G protein activation. The nature of this interaction appears to demand an element of aromaticity and may be enhanced by basicity/H-bonding capability. Mutagenesis studies identify $Lys^{3.32(121)}$ in the receptor as a possible site of interaction with $His^2$ (142) (see *Section V*).

*$Trp^3$*. $Trp^3$ is clearly a critical residue in GnRH. $Trp^2$ $His^3$ GnRH and Des-$Trp^3$ GnRH were inactive (143). Substitution with nonaromatic amino acids (*e.g.* Gly, Leu, and Ala) gives rise to very low activity (129, 143–145), whereas some activity is present with Tyr (0.1%), as well as Phe (0.5%) substitution, and this is increased substantially in pentamethyl-Phe GnRH (30–70%) (129, 143). Notably, the latter residue resembles Trp in its ability to form $\pi$-$\pi$ complexes with aromatic molecules (134). The introduction of an electron-withdrawing fluorine atom of similar atomic radius in position 5 of $Trp^3$ leads to a marked reduction in activity (6%), presumably due to reorienting the dipole and forming hydrogen bonds itself so that an aromatic interaction does not occur (145). The role of an aromatic side chain in this position is further emphasized by the natural substitution of Tyr for $Trp^3$ in lamprey GnRH I (Fig. 5). Even 2-napthyl-Ala substitution results in 50% activity (129). Since D-$Trp^3$ GnRH has poor gonadotropin-releasing activity (133) but has been commonly incorporated in antagonists, it is possible that $Trp^3$ plays a role in receptor activation. The altered stereochemistry evidently has a critical effect on agonistic activity. The role of $Trp^3$ in receptor activation is further suggested by an early study demonstrating antagonist activity of $Leu^3$ GnRH (145) and recent work showing that incorporation of NMe in the amino acid in position 3 converts the peptide to an antagonist (146).

*$Ser^4$*. The last of the conserved residues in the $NH_2$-terminal domain, $Ser^4$, can be substituted with a number of amino acids (Ala, Thr, Gln, NMeSer) with reasonable retention of activity (10–20%) (129, 130, 132, 143), yet this is the most conserved residue in the empirically generated analogs. Because substitution with larger amino acids such as Ser (But) and Leu is very detrimental (129), it appears that spatial constraints are paramount. Recent work has shown that constraint of the peptide bond with NMe does not decrease activity (146, 147) unlike most other positions in GnRH. The conclusion from early studies that large side chains are not tolerated is supported by the recent observation that biotinylated $Ser^4$ GnRH is inactive (148).

*2. The COOH-terminal domain (Pro-Gly·$NH_2$).*

*$Pro^9$*. The conservation of $Pro^9$ in the natural GnRHs and the expected conformational limitations imposed by Pro on the peptide chain suggest that substitution would not be readily tolerated. Sarcosine$^9$ GnRH and $Ala^9$ GnRH had low activity (<1%) while N-Me-$Gly^9$ had 10% activity (129, 132). The exchange of amino acids 8 and 9 ($Pro^8$ $Arg^9$ GnRH) also results in very low activity (129). The discovery that $Pro^9$ may be hydroxylated in fetal brain and decreases activity to 10% (149) underlines the importance of this conserved residue and suggests that this may be a regulatory mechanism.

*Gly-amide$^{10}$.* Removal of the amide to yield the free acid of GnRH results in very low activity (150). This has recently been confirmed for the human GnRH receptor expressed in COS-1 cells (67). Replacement of the Gly-$NH_2$ moiety with Ala resulted in a mild reduction in activity (10%) (130), and a similar reduction was observed with Gly-$NMe_2$ (14%) (132). On the other hand, substitution of Gly-$NH_2$ with alkylamides maintained (methylamide and ethanolamide) or increased activity up to 600% (propylamide and ethylamide) (150, 151) whereas substitution with larger amides (pyrolidineamide and morpholineamide) (150) or D-amino acids (129) decreased activity. The incorporation of electron-withdrawing fluorine atoms into the ethylamide (2,2,2-trifluoroethylamide) further enhanced activity to about 900% (151). These findings suggest that the terminal Gly-$NH_2$ is not essential for activity and that small, uncharged moieties are acceptable at the COOH terminus. Larger groups are inhibitory, possibly by sterically hindering ligand access to the binding site. The findings also suggested that the total chain length might have an important role in the binding of GnRH to its receptor (150, 151). Recent mutagenesis of a receptor site ($N^{2.65(102)}\rightarrow A$) has demonstrated a much greater decrease in binding affinity of Gly-$NH_2$ ligands than *N*-ethylamide ligands (152) (see *Section V*).

*3. The central nonconserved domain (Tyr-Gly-Leu-Arg).*

*Try*$^5$. In accordance with the lack of conservation of Tyr$^5$ in vertebrate GnRHs, substitution in this position is well tolerated. The 44–64% activity of Phe$^5$ (143, 153) demonstrated that the hydroxyl group is not required. Interestingly this substitution has yet to be found in naturally occurring GnRHs, although it would require only one base change. Substitution of the hydroxyl group of tyrosine resulted in activities of 37% (amino), 24% (methoxy), and 5% (nitro) (154). NMe Tyr substitution, which has been proposed to constrain the peptide backbone and to eliminate one of two postulated H bonds with Arg$^8$ in a $\beta$-II turn conformation, led to a reduction in binding affinity to 10–20% (155) Interestingly, mono-iodo-Tyr-GnRH (129) and mono-chloro-Tyr-GnRH (130) had activities of 30–80% and 10%, respectively, while di-iodo-Tyr$^5$ GnRH and di-chloro-Tyr GnRH were devoid of LH-releasing activity (129, 130). His$^5$ GnRH has very high binding affinity for mammalian GnRH receptors (121). These findings demonstrate that the hydroxyl group of Tyr$^5$ is not required, and that simply an aromatic side chain (Trp, Phe, or His) is adequate for high LH-releasing activity. The findings suggest that Tyr$^5$ contributes only to receptor binding and does not play a role in the process of receptor activation. However, substitution of Tyr$^5$ with His, as in GnRH II, results in an analog with high receptor potency (aromaticity maintained) but reduced LH release (partial agonism; (121). Partial agonism is also observed with His$^5$ D-Trp$^6$ GnRH and in His$^5$ D-Tyr$^6$ GnRH and Arg$^5$ D-Tyr$^6$ GnRH (R. P. Millar, unpublished). However, when Arg$^8$ is substituted by Tyr in analogs with His or Arg in position 5, efficacy is restored. Thus the motif His$^5$/Arg$^5$-Xxx-Xxx-Arg$^8$ produces compounds with high binding but diminished receptor activation, indicating that the Tyr$^5$ does play a role, albeit possibly indirect, in receptor activation in the mammalian ligand-receptor complex.

*Gly*$^6$. Gly$^6$ is conserved in all vertebrates except the ancient jawless lamprey and is also absent in the tunicate GnRHs (Fig. 5). The presence of this small residue in this position allows for flexibility and the assumption of the postulated $\beta$-II-type bend and the preferred conformation for receptor binding (see below and *Section IV*). This bend would be energetically unfavorable in analogs with larger L-amino acid substitutions for Gly$^6$, and Ile, Val, and Ala analogs were found to have low activity (132, 137). However, the folded conformation is favored by the stereochemistry of D-amino acid substitutions (10, 21, 88–91, 137). The proposal of a $\beta$-II-type bend for the active conformation of GnRH was first proposed by Monahan *et al.* (137) after demonstrating that D-Ala$^6$ substitution increased activity to about 400%. This seminal work led to the exploration of numerous substitutions with D-amino acids in this position (see reviews in Refs. 10, 21, and 88–91). In general, substitution with D-amino acids having bulky hydrophobic side chains, particularly aromatics, was most effective (10, 21), and this has been confirmed in numerous binding studies using pituitary membranes and, more recently, with receptors expressed in COS-1 cells. A correlation between hydrophobicity (HPLC retention time) of the D-amino acid and potency has been noted (100). It appears that there is a large "allowable space" facing away from the $NH_2$- and COOH-domains which interacts with the receptor, and this will accommodate the D-amino acids with large side groups (147). In addition to further favoring the $\beta$-II-type conformation, the large side chains of the D-amino acids may interact with nearby residues in the receptor, thereby enhancing the binding affinity. These potential alternative interactions probably account for species differences and are likely to be prevalent in GnRH antagonists with numerous unusual side chains, often aromatic. These features must be taken into account when analyzing the effects of mutagenesis of receptor residues on the binding of these analogs.

*Leu*$^7$. The comparative studies of activities of vertebrate GnRHs indicate that substitutions of Leu$^7$ with uncharged L-amino acids with varying size side chains are generally well tolerated. This supposition is confirmed by the demonstration that Val, Ile, Nle, Ser, ethoxycarbonyl-Lys, butoxycarbonal-Lys, and Boc-Lys all had high activities (16–45%) (129, 130) while Ala and Gly had lower (3–6%) activities (129, 130). Potential disruption of conformation by D-amino acid D-Leu (133) or Pro (129) substitution resulted in very low activities as did substitution with basic residues (Lys, Arg) (see Ref. 129). Tolerance of the large, bulky substitution of Trp for Leu$^7$ was recently demonstrated by the high LH-releasing activity (110% in sheep pituitary cells) and receptor binding (37% for sheep and 230% for rat) for this analog (121, 122). The original proposal of a type-II $\beta$ turn conformation of GnRH also envisaged a hydrogen bond between the C=0 of Ser and NH of Leu. However, substitution of Leu$^7$ with N-Me-Leu, which would eliminate this H bond, did not reduce activity (155a).

*Arg*$^8$. Comparative activities of vertebrate GnRHs had indicated that Arg$^8$ is required for high-affinity binding to mammalian receptors (91, 105, 119–121). A number of early studies had shown that D-Arg, Gln, Leu, Orn, His, diaminobutyryl, and Cit substitution for Arg$^8$ results in a substantial decline in activity (1–6%) while homoArg, Narg, and Lys retained good activity (10–20%) (92, 119, 129, 130, 133, 156–161). A systematic study on the LH-releasing activities from sheep pituitary cells of Gln-, Ser-, Tyr-, Phe-, Glu-, His-, Leu-, Lys-, Ile-, and Trp-substituted analogs confirms the requirement of a basic amino acid in position 8 for high activity (92, 93). Since receptor-binding is correlated with LH-releasing activity in all position 8-substituted analogs studied, it appears that the role of Arg$^8$ may be in receptor binding. However, as noted above, analogs with neutral amino acid substitutions display improved efficacy. Two hypotheses may be invoked to explain the basis of the higher affinities of Arg$^8$-containing GnRHs. An ionic interaction of Arg$^8$ with one or more negatively charged residues, either an amino acid side-chain (162) or a polysaccharide sialic acid residue (163) in the receptor, were proposed. An alternative or additional possibility was that the side chain of Arg$^8$ affects the structure of the ligand, stabilizing the active conformation of GnRH by hydrogen bonding with the side chains of His$^2$ and Tyr$^5$ (159, 160). Low pK values were measured for His$^2$ and Tyr$^5$ in GnRH, and it was suggested that the more acidic nature of these amino acid side chains was due to their proximity to the cationic side chain of Arg$^8$ (160). GnRH analogs with neutral substitutions, Gln and $\omega$-nitro-Arg (159), in position 8, exhibited normal pK values for His$^2$ and Tyr$^5$ and extended titration ranges. These results were in-

terpreted as indicating a decreased interaction of the $His^2$ and $Tyr^5$ side chains with the neutral substituents in position 8 (161). The decreased side-chain interaction was proposed to decrease stabilization of the bioactive conformation and thus cause the lower bioactivity in the neutral GnRH analogs. Based on these findings, a folded conformation of GnRH was proposed (159, 160) similar to models of GnRH that were based on energy minimization and database sequence comparison (137, 164–167).

The role of $Arg^8$ in determining the preferred conformation of GnRH and in receptor interaction is explored in detail in the following sections. In recent mutagenesis studies, an acidic amino acid residue in extracellular loop III was shown to convey specificity for $Arg^8$ such that mutation to an isosteric amide resulted in the loss of the preferential binding of $Arg^8$ GnRH compared with GnRH with a neutral amino acid in this position (see *Section V*). The requirement for Arg in position 8 and the acidic residue in the receptor is obviated if the GnRH structure is constrained by incorporation of a D-amino acid in position 6 (see *Section V*). A recent reexploration of the role of $Arg^8$ in antagonists concluded that this residue may be significant for receptor binding (168) while the demonstration in another study that (Orn(2,4-NAPS0)$^8$ GnRH cross-linked with the receptor (169) was interpreted to support the proposal that $Arg^8$ interacts with receptor moieties.

### *D. Conclusions from peptide structure-activity data*

We have attempted to identify individual residues in GnRH that are involved in receptor binding and receptor activation, as this information is critical in undertaking receptor mutagenesis studies directed at defining ligand contact sites. Although thousands of GnRH analogs have been synthesized and biologically characterized, the complexity of most analogs and the predominance of *in vivo* testing have complicated the task of clearly identifying the roles of individual amino acids in ligand conformation and in receptor binding and activation. Although a more controlled and systematic examination of the functions of GnRH residues using expressed cloned receptors has begun (see *Section V*), relatively few analogs have been studied to date. Nevertheless it is possible to generate working hypotheses about ligand requirements of the mammalian pituitary GnRH receptor for binding and activation (Fig. 8). The following generalizations may be proffered:

1. It is evident that no single residue is crucial for GnRH activity.
2. The $NH_2$- and COOH-terminal domains are the most important in receptor binding and activation.
3. Although both domains are involved in receptor binding, residues in the $NH_2$-terminal domain are predominantly responsible for receptor activation.
4. The only residues for which good evidence exists for a role in receptor activation are $His^2$ and $Trp^3$, but $pGlu^1$ may also be involved.
5. Substitution of residues outside of the $NH_2$-terminal domain can affect receptor activation, possibly through effects on the conformation that change the presentation of activating residues, or through restrictions in dynamic ligand conformation changes that occur on binding to the receptor.
6. Nonconserved residues of the central domain are less critical, but $Arg^8$ is required for high-affinity binding to the mammalian receptor. However, the requirement for $Arg^8$ may be obviated in conformationally constrained analogs with D-amino acids in position 6, and also when $His^5$ is present as in chicken GnRH II.
7. The achiral Gly or D-amino acids are required in position 6, presumably to allow assumption of the folded active conformation.
8. Nonmammalian GnRH receptors have different require-

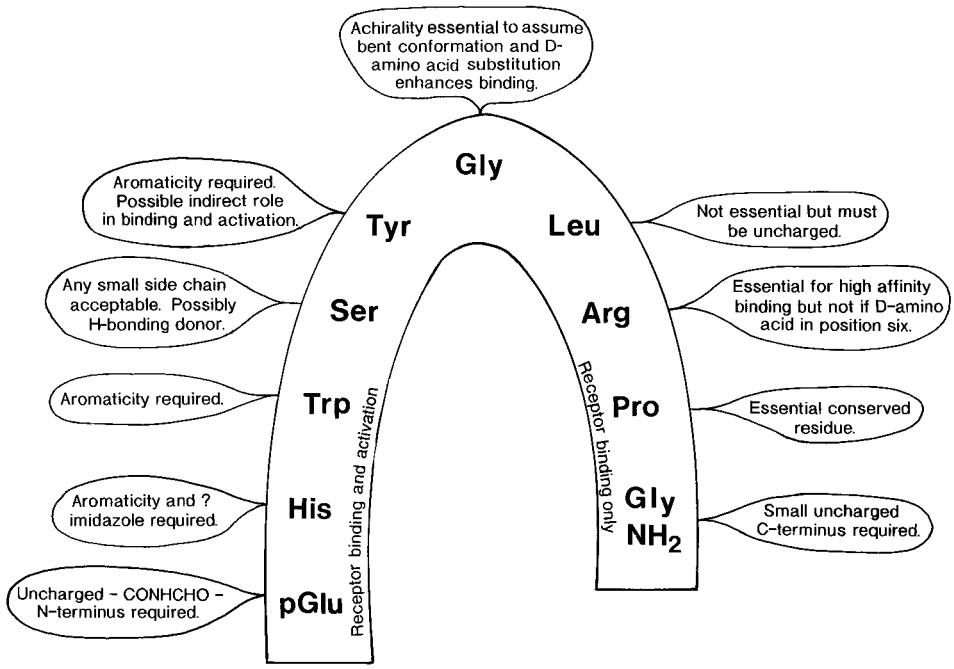


FIG. 8. Schematic of GnRH summarizing functional properties of individual amino acid residues.

ments for the nonconserved residues in the central domain. Examples include the lack of requirement for a basic residue in position 8 and the nonacceptance of $His^5$ substitution when $Arg^8$ is present. Nonmammalian GnRH receptors also tend to be less dependent on conformational constraint, and D-amino acid substitution may not enhance activity to the same degree as in mammalian receptors.

## IV. Structure and Conformation of GnRH and Its Analogs

### A. Early studies of GnRH conformation

The structure-activity data reviewed in the previous section provide important hypotheses about the role of specific groups in GnRH binding and receptor activation. However, it is becoming increasingly likely that the elucidation of structure-activity relations for GnRH, and of the molecular basis for agonist and antagonist properties at the GnRH receptor, will not be possible at the level of the ligands alone (170). To understand the action of GnRH and its congeners from a structural perspective, it may become necessary to gain an understanding of the structural and dynamic properties of the ligands, as well as of the ligand/receptor complexes. Such insights are sought from experimental explorations of structure-function relations, as well as with novel computational methods that focus on the structural properties of both the receptor and the ligands.

The inherent flexibility of the hormone decapeptide makes it likely that interactions with various sites in the receptor-binding pocket will affect the conformation of any GnRH analog and reduce the ability to define a single "biologically relevant" conformation for the isolated peptide. Moreover, the pharmacophoric patterns of different peptides depend on the specific residues available for interaction with the various receptor sites, even if their conformations are the same. Consequently, the structural determinants for action on the GnRH receptor will have to be sought from a comprehensive characterization of all the conformations accessible to the peptides under given conditions of temperature and environment, *i.e.* the conformational space of these peptides, as well as of the three-dimensional pattern of the pharmacophoric elements that their amino acids present to the receptor. If the ability to adopt certain conformations determines the receptor activity of peptides with similar pharmacophoric elements, then differences in their conformational spaces can reveal the conformations required for receptor interactions. The conformational properties of the most active peptides can thus serve to define the spatial and dynamic requirements for optimal interaction with the receptor. A useful ranking of structure-activity characteristics can be constructed on this basis, provided that peptides with pharmacologically distinct activities such as agonism and antagonism are differentiated. For these reasons, it is not realistic to expect a full understanding to emerge entirely from structure-activity data obtained from probing the activities of various synthetic analogs without specific analysis of their conformational properties. Rather, the mechanistic insights are more likely to emerge when the powerful approaches offered by current experimental and computational methods for conformational analysis (for reviews see Refs. 171 and 172; also Refs. 166, 173, and 174) are applied to the exploration of peptide structure and design.

Pioneering efforts were undertaken to achieve such a comprehensive exploration of the conformation of GnRH and its active analogs by computational methods (164, 165, 175). The impetus for such studies continues to be the assumption that if the peptide conformation recognized by the receptor (*i.e.* the bioactive conformation) corresponds to the most abundant form of the peptide in solution, then the peptide will have high affinity for the receptor. Because the most abundant conformers in solution are those corresponding to the lowest free energy, the computational approaches concentrated on the calculation of the conformational energies of the peptides. The early studies (164, 165, 175) identified low-energy conformations of GnRH that were considered to occur also in solution, although it was not possible at the time to account for the effects of aqueous solvation. In spite of the significant limitations of the methods for energy-based evaluation of peptide conformations (for a review of methods see Ref. 176) that were available at the time for studies of peptide molecules of the size of GnRH, the early studies identified the central characteristic of the bioactive conformation of GnRH, the $\beta$-bend involving the Tyr-Gly-Leu-Arg in positions 5–8 (see Fig. 9).

### B. Integrated computational and experimental studies

Subsequently, the most incisive studies of structure-activity relationships used in the design of GnRH analogs combined the computational conformational studies with a variety of experimental (mostly spectroscopic) approaches to characterize the structural features of the peptides (for illustrations and reviews see Refs. 177–179). Such studies often took advantage of cyclization as a means of restricting the conformational freedom of the peptide molecules to reduce the complexity of the problem. The initial inferences from conformational analysis of GnRH that residues $Tyr^5$-to-$Arg^8$ may form a $\beta$-type turn supported the efforts to produce cyclic analogs in the search of high-affinity ligands for the GnRH receptor. Much attention was devoted to the conformational properties of these cyclic analogs in computational studies. It was quickly recognized that, even in these cyclic

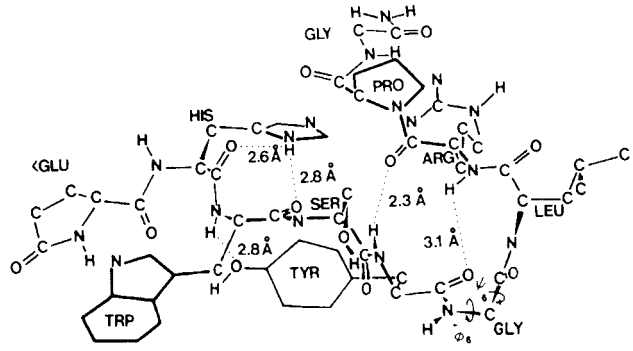


FIG. 9. View of hinged conformer of GnRH obtained using empirical energy calculations. [Reprinted with permission of the publisher from F. A. Momany: *J Am Chem Soc* 98:2990–2996 (165). ©1976 American Chemical Society.]

analogs, the intramolecular motions were complex, and conformational variability persisted. The overall conformational properties of the cyclic analogs were found to be generally in good agreement with the results from energy-based calculations and with the information on the structure and dynamics of the analogs obtained from nuclear magnetic resonance (NMR) (173, 179). The points of agreement include the all-*trans* nature of the peptide bonds in the major conformational families of the analogs and the $\beta$-type turn structure between residues 4–8 (177) (Fig. 10). However, the computational simulations of molecular conformation and dynamics indicated the large variability in the structural options of the uncyclized 1–3 fragment, as well as the fact that several different conformations with equivalent energies and geometrical features compatible with the NMR data, can be adopted even by the constrained dicyclic compound (177).

The insights from computational studies were confirmed by results from NMR spectroscopy (*e.g.* Refs. 177–179 and references therein), but uncertainty persisted with respect to the exact location of the $\beta$-type turn and to the spatial orientation of the side chains assumed to be involved in affinity-determining interactions with receptor sites (180). Thus, *cyclo*(1–10) analogs were compared with 4–9 and 4–10 bridged analogs (170) and more recently with the dicyclic analogs (4–10; 5–8) designed to incorporate the conformational constraints of both classes of cyclic analogs (177). It is quite clear from these studies that the multiple conformational forms exist even for the most constrained analogs (170, 178) and that these analogs must be studied with detailed conformational approaches. To be most useful, such conformational studies must not only identify the lowest energy conformations, but must be able to provide reliable information on the probability distribution of the various conformational families, *i.e.* their *relative abundance* in solution (174, 181).

Early attempts to decompose the structural determinants for activity of cyclized GnRH analogs into contributions from the length of the bridge and the orientation of certain functional groups (*e.g.* amide bonds considered to be involved in direct interactions with receptor sites) were not successful (170). A main reason is the residual flexibility of the cyclized GnRH analogs, which lose only a portion of their conformational freedom. This insight (*e.g.* see Refs. 170 and 178) led to the suggestion that a complete structural characterization, rather than the mere identification of minimal energy conformations, will be necessary for both linear and cyclic analogs (170).

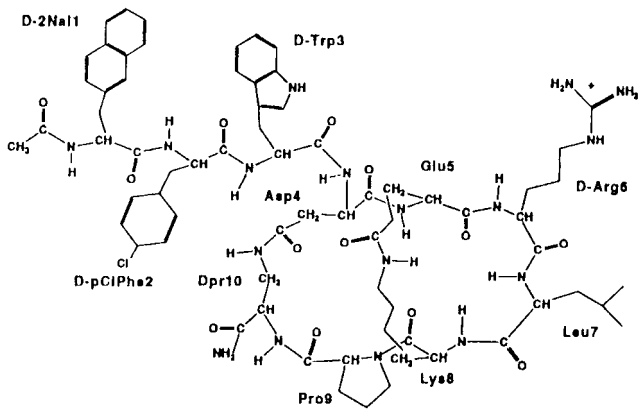


FIG. 10. Schematic representation of dicyclic GnRH analog studied using two-dimensional nuclear magnetic resonance and molecular dynamics simulation. [Reprinted with permission of the publisher from R. J. Bienstock *et al.*: *J Med Chem* 36:3265–3273, 1993 (177).]

### *C. Exploration of the entire conformation space of GnRH analogs*

The results from experimental and computational studies of the structure and conformation of GnRH analogs emphasize the importance of a complete exploration of the conformational space of the peptides (176). Whether the analogs are conformationally constrained or linear, the complete structural characterization is necessary before a reliable consensus on the structural features most important for receptor interaction can be reached (174). Current methods (174, 181) make possible such extensive explorations of both the conformational and dynamic properties of the peptides, offering the ability to explore the entire conformational space of decapeptides such as GnRH and its analogs (174). Specifically, the application of a recently developed technique of Conformational Memories (182) to the study of GnRH conformational properties illustrates the first complete exploration of the entire conformational space of the peptide using a method of simulation that includes a satisfactory model for the aqueous solvent.

The novel method overcomes some of the shortcomings of modern molecular dynamics approaches to the study of the peptide hormone: although the molecular dynamics techniques are useful for their ability to describe the molecular motions of the peptide in short time scales, they are unable to explore all the conformational states of the peptide in solution, and hence are not able to characterize their relative abundance. In contrast, the Conformational Memories method utilizes a two-stage process of computation to map, and then characterize, the conformational space of a flexible molecule (174). In the first, exploratory stage, repeated runs of the Monte Carlo method (183) combined with simulated annealing (184) are carried out to map the entire conformational space of a flexible molecule by heating it to very high temperatures and cooling it slowly to body temperature. Once the Conformational Memories are established, the method proceeds to a new Monte Carlo search of the conformations of the peptide, performed at 310K and sampling only from the populated regions. Because only about 50% of the torsional space of the 35 bonds of GnRH is populated at 310K, the two-tiered approach reduces by many orders of magnitude the conformational space that must be explored in this second phase (174). The configurations sampled from the Conformational Memory can be any part of the *populated* space of dihedral angles defining the conformations of the peptide. Consequently, the notion of a barrier restricting access to any part of the conformational space is eliminated in this procedure without approximations.

*1. Conformational families of GnRH.* In the application of the conformational memories approach to GnRH, the second step of the procedure involved 500,000 steps (174). Structures of the peptide obtained from the run were clustered in con-

formational families, resulting in the five basic structures depicted in Fig. 11. Notably, families of conformations having a β-turn between residues 5–8 occur in GnRH with a frequency of approximately 70%. A distribution showing a superimposition of 70 of these structures is illustrated in Fig. 12; the β-type turn common to all the structures in this family is clearly evident. In contrast, families that have an extended backbone occur with a frequency of about 5%. The distribution of side-chain orientations of $Arg^8$ in all conformational families was found to be wider than that of any other residues in GnRH.

*2. Conformational families of $Lys^8$-GnRH.* The conformational properties of the $Lys^8$ analog of GnRH that served to explore the role of $Arg^8$ in the receptor interactions of GnRH (21, 121) were compared with the conformational profile of GnRH. In contrast to the wild type hormone, the major conformational family of the $Lys^8$-GnRH congener was found to have an extended backbone, while the β-turn conformation exists as a very minor family (174). A backbone trace of a representative of each family is shown in Fig. 11. The family of conformations with an extended backbone has an abundance of more than 70%, while the β-type turn conformation of $Lys^8$-GnRH, which is virtually identical to the major conformational family of the GnRH, has a probability of only about 3%. A distribution of the members of the predominant $Lys^8$-GnRH family superimposed upon each other is shown in Fig. 13, with the entire molecule shown in *red*, except for $Lys^8$, which is colored *green*. Because $Lys^8$-GnRH is a low affinity agonist for the GnRH receptor, adoption of a large population of β-type turn conformation appears to be a key requirement for hormone-receptor recognition. This inference agrees with earlier proposals in the literature (*e.g.* see Ref. 173) and is supported by results from additional Conformational Memories simulations on the structural characterization of eight other GnRH analogs that exhibit different distributions between the β-turn like structures and the fully extended conformations of the backbone (F. Guarnieri, S. C. Sealfon, and H. Weinstein, unpublished).

To test the key inference regarding a direct correlation between the abundance in solution of the β-type turn structure and the GnRH receptor affinity, the most populated conformational family of GnRH obtained from Conformational Memories was compared with a structurally constrained cyclic decapeptide GnRH analog (185) in which residues 6 and 7 were shown from NMR data to form a type II′ β-turn and residues 1 and 2 formed a type II β-turn, a weak hydrogen bond was identified between the $Arg^8$-NH and the $Tyr^5$-CO, and a stronger hydrogen bond was observed between the D-$Trp^3$-NH and the β-$Ala^{10}$-CO. All computationally derived structures were found to superimpose on the cyclic analog with a very small root mean square deviation (0.6–0.8 Å) in the region of residues 5–10 (174). However, the structures were found to diverge between the N terminus and residue 4, indicating the flexibility of that region.

Thus, the results of the first conformational study capable of overcoming energy barriers efficiently and achieving a complete sampling of the conformational space of GnRH support a relation between the β-turn structure identified as the major conformational family of GnRH in solution and high affinity for the GnRH receptor. These inferences support the results from the earlier investigations of conformationally restricted GnRH analogs (164, 173, 178) and provide unbiased support for this mechanistic hypothesis based on a complete exploration of the conformational space of the peptide hormone itself and its unconstrained congeners. Because the method seems to have produced the lowest energy con-

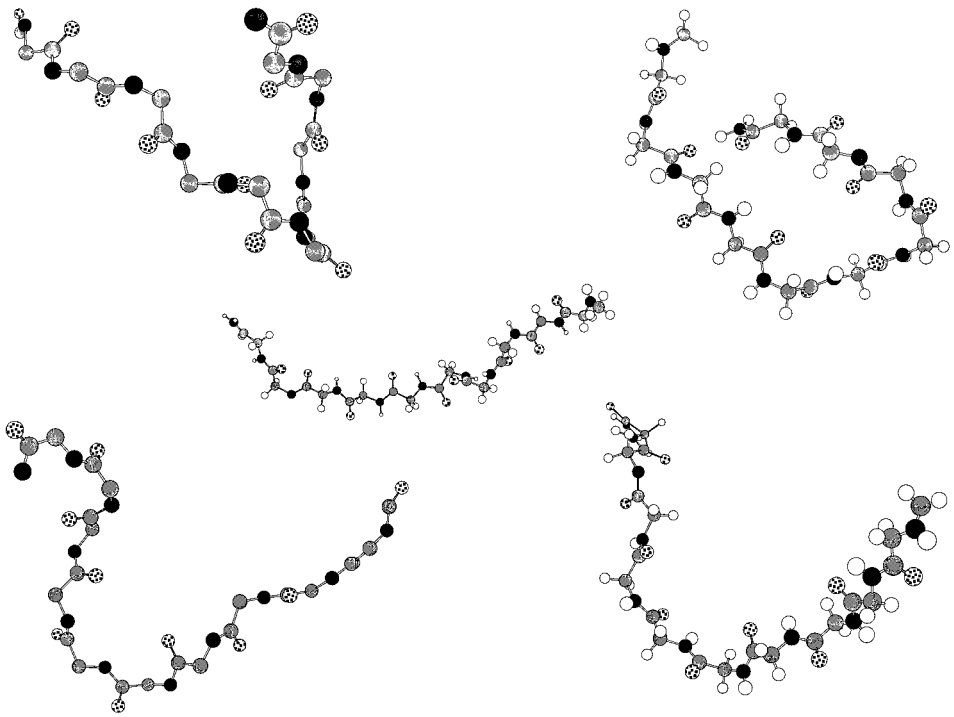

FIG. 11. Backbone trace of representative members of the conformational families of GnRH obtained from Conformational Memories.

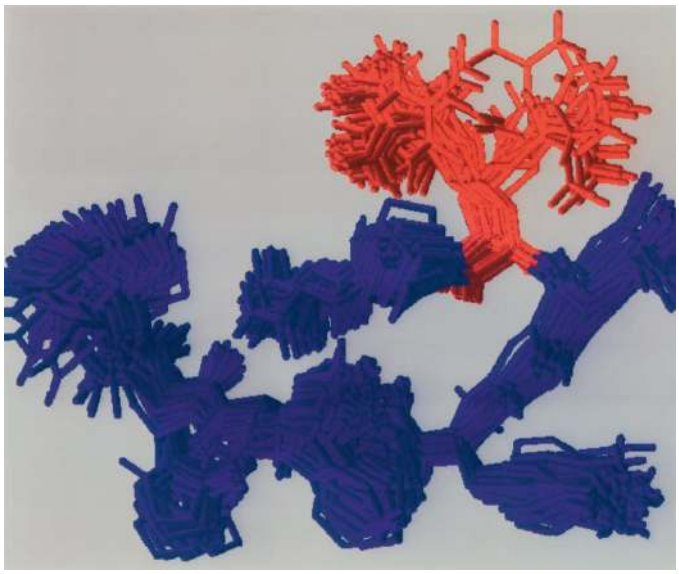

FIG. 12. Superimposition of structures that make up the major conformational family of GnRH obtained from Conformational Memories. GnRH is colored in *purple*, with $Arg^8$ colored in *red*. The $\beta$-turn common to all structures is evident.

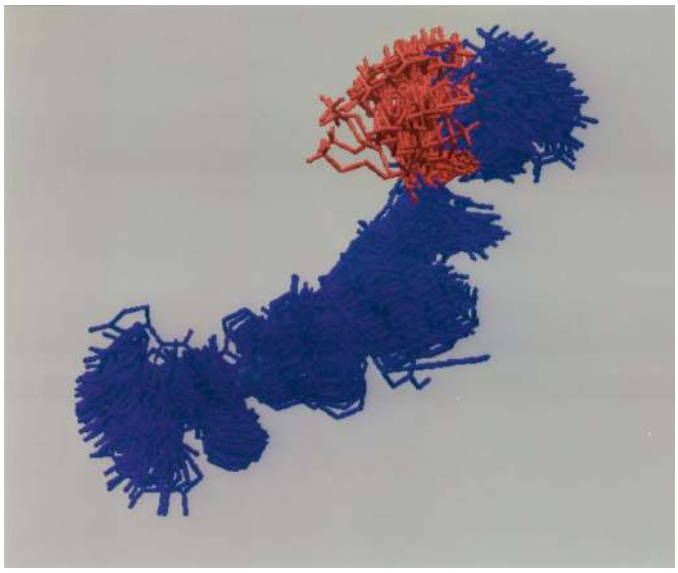

FIG. 13. A distribution of the members of the predominant Lys8-GnRH family superimposed upon each other is shown. Note the straightened backbone structure in comparison with the bent GnRH structure shown in Fig. 12.

formers reported for GnRH (174) from a full exploration that is economical and practical, its continued application to the study of structure-function relations of GnRH analogs should produce important mechanistic insights and powerful guides for ligand design.

## V. Functional Structure of the Receptor and Ligand-Receptor Complex

### *A. Extracellular domains*

A driving force of investigations into the structure of the GnRH receptor and its interaction with GnRH is the anticipation that this information will provide the basis for the rational design of novel analogs. To understand the structure-activity properties and conformational family perturbations of the various GnRH analogs, insight into the "binding pocket" for GnRH must be obtained and the precise sites of interaction of receptor and ligand determined. As the direct structural data available on GPCRs are limited to low resolution cryoelectron microscopy of rhodopsin (58) (Fig. 4), studies of the binding pocket have relied on indirect approaches, particularly the study of the functional effects of site-directed mutagenesis and the construction of computational three-dimensional molecular models of the ligand-receptor complexes. Through these approaches, a view of the receptor and its mode of ligand binding is emerging. Side chains required for high-affinity binding of peptide ligands have been identified in both the extracellular and transmembrane domains, and specific helix-helix proximities within the receptor have been determined.

As described above, the basic $Arg^8$ of GnRH is critical for high-affinity agonist activity at the mammalian receptor. Substitutions at $Arg^8$ [as in Chicken I GnRH ($Gln^8$GnRH)] cause a marked reduction in the affinity of binding to the mammalian receptor (119, 121). Based on cation competition experiments, Hazum (162) originally suggested that the $Arg^8$ of GnRH may interact with carboxylic groups on the receptor (162). The possibility that $Arg^8$ of GnRH forms an ionic interaction with an acidic residue of the GnRH receptor was investigated by site-directed mutagenesis (186). The chicken GnRH receptor shows little discrimination between $Arg^8$-GnRH (mammalian GnRH) and $Gln^8$-GnRH (chicken I GnRH). All acidic residues on the receptor were mutated to their isosteric amine, and a mutant that failed to discriminate between $Arg^8$-GnRH and $Gln^8$-GnRH was sought. One mutant receptor was identified, $Glu^{7.32(301)}$-Gln, which had decreased affinity for mammalian GnRH in comparison with the wild type receptor. The affinity of this mutant for $Gln^8$-GnRH and for other GnRH analogs with an uncharged residue in the eight position, however, was relatively unchanged or improved (see Fig. 14). Most significant is the marked increase in activity of $Glu^8$-GnRH in the mutant. These results support a role for the $Glu^{7.32(301)}$ residue, located in the third extracellular domain, in conferring the preference of the mammalian receptor for $Arg^8$ GnRH. A GnRH analog that is conformationally restricted by having a D-amino acid in position 6 (D-$Trp^6$,$Pro^9$-NHEt)GnRH and its $Gln^8$ congener (D-$Trp^6$,$Gln^8$,$Pro^9$-NHEt)GnRH have more similar affinities for both the wild type and $Gln^{7.32(301)}$ mutant receptors. Thus conformationally restricted analogs do not seem to require Arg in position 8, suggesting that the role of $Glu^{7.32(301)}$ in the receptor is to help induce or select the optimum conformation of the ligand for high-affinity interaction.

A critical role of $Asn^{2.65(102)}$, located at the extracellular surface of TMD 2, for high-affinity interactions with some GnRH analogs has been identified (152). Mutation of this site to Ala caused a 2- to 3-order of magnitude loss of potency for GnRH and analogs with the naturally occuring glycinamide (NH-CH2-CO-$NH_2$) C terminus in stimulating hydrolysis of phosphoinositides. However, this mutation had much less effect on the potency of ethylamide (NH-$CH_2$-$CH_3$)-modified

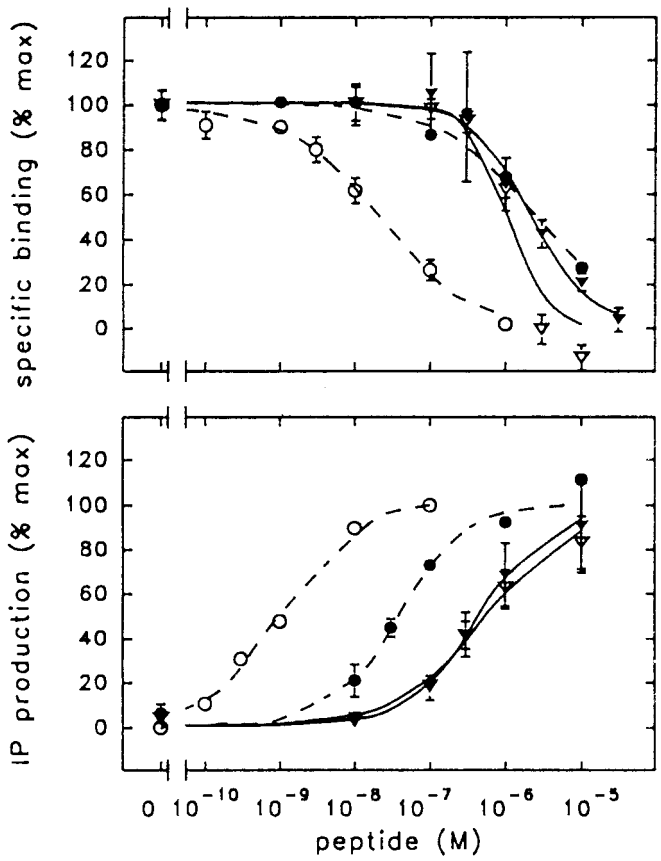


FIG. 14. GnRH and Gln$^8$-GnRH ligand binding and phosphoinositol hydrolysis in COS-1 cells transfected with wild-type GnRH receptor and Gln$^{7.32(301)}$ GnRH receptor. Binding of $^{125}$I-[D-Ala$^6$,N-Me-Leu$^7$,Pro$^9$NEt]GnRH in the presence of various concentrations of GnRH (○, ●) and [Gln$^8$]-GnRH (●,▼) to membranes from wild-type (*broken line*) or mutant (*unbroken line*) receptors. [Reprinted with permission of the publisher from C. A. Flanagan *et al.*: *J Biol Chem* 269:22636–22641, 1994 (186).]

C-terminal agonists. These results are consistent with the hypothesis that the carbonyl of glycinamide analogs forms a hydrogen bond with this locus.

### *B. Helix domains*

The receptors for the biogenic amine neurotransmitters all contain an Asp at a homologous location in TMD 3. Mutagenesis studies on several of these receptors suggest that the Asp anionic side chain serves as a counterion required for high-affinity interaction with the cationic head group of the ligand (187–193). All cloned GnRH receptors have a lysine at the corresponding 3.32 position (Fig. 1). The role of Lys$^{3.32(121)}$ in ligand binding and activation of the human receptor was studied by introducing a series of mutations at this position (142). Substitution of Arg at this position preserved high affinity agonist binding, whereas Gln at this position reduced agonist binding to below the limit of detection. Leu and Asp at this locus abolished both binding and detectable signal transduction. The $EC_{50}$ of concentration-response curves for coupling to phosphatidyl inositol hydrolysis obtained with the Gln$^{3.32(121)}$ receptor was more than 3 orders of magnitude higher than that obtained for the wild-type receptor (see Fig. 15). Receptor inactivation studies confirmed that this increase in $EC_{50}$ represented a large decrease in agonist affinity. In contrast, an antagonist had comparable high affinities for the wild type, Arg$^{121}$, and Gln$^{121}$ mutants. This study indicated that a charge-strengthened hydrogen-bond donor is required at this locus for high-affinity agonist binding, but not for high-affinity antagonist binding. Based on the available structure-activity data of GnRH analogs, His$^2$ in the ligand is a potential candidate for interaction with this locus (see *Section III*). Although the Lys$^{3.32(121)}$ in the GnRH receptor appears to serve a function analogous to Asp$^{3.32}$ in the neurotransmitter receptors, this correspondence does not extend to all peptide GPCRs. The corresponding position of the mouse TRH receptor, Gln$^{3.32(105)}$, appears to make only a modest contribution to ligand affinity (194). In contrast, two other TMD 3 side chains in that receptor, Tyr$^{3.33(106)}$ and Asn$^{3.37(110)}$, have been identified as major determinants of agonist affinity (194). Thus, while the side chain at the 3.32 locus is not a critical determinant of affinity in all receptors, the local properties of this region of TMD 3 seem generally important for ligand-receptor interaction (195).

Mutagenesis studies have also indicated the importance of Asp$^{2.61(98)}$ in high-affinity interactions of certain agonists. Mutation of this locus to Glu caused a large increase in the $EC_{50}$ for GnRH but had little affect on the $EC_{50}$ for [Trp$^2$]-GnRH (195a). This dependence of the effect of mutation at the 2.61(98) locus on the identity of the residue in the second position of GnRH is consistent with an interaction between His$^2$ of GnRH and the side chain at the 2.61(98) locus of the receptor.

As noted above (see *Section II.B*), an unusual feature of the GnRH receptor, observed in all mammalian species, is the presence of Asn$^{2.50(87)}$ in the second putative transmembrane helix at the location of a highly conserved Asp in the GPCR family, and of Asp$^{7.49(318)}$ in the putative seventh transmembrane helix where nearly all other GPCRs have Asn (see Fig. 2). This apparent interchange of conserved residues in the native receptor raised the possibility that these residues in-

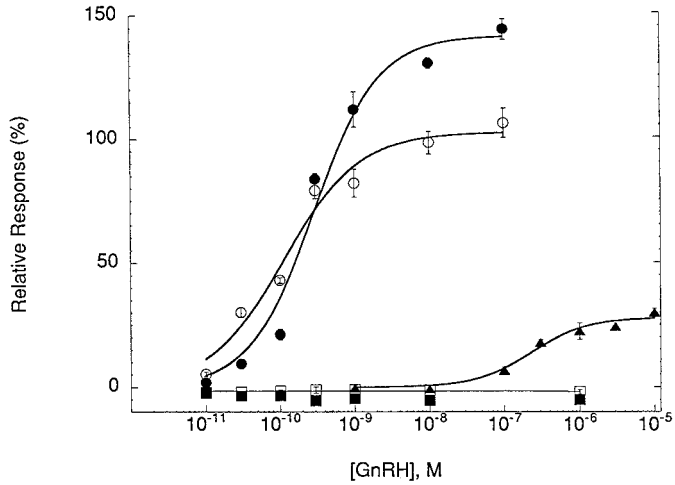


FIG. 15. GnRH-stimulated phosphoinositol hydrolysis in COS-1 cells expression Lys$^{3.32(121)}$ mutant receptors. Vector ■; Leu$^{3.32(121)}$-receptor □; Gln$^{3.32(121)}$-receptor ▲; Arg$^{3.32(121)}$-receptor ●; wild-type receptor ○. [Reprinted with permission of the publisher from W. Zhou *et al.*: *J Biol Chem* 270:18853–18857, 1995 (142).]

teract. This possibility was investigated by expressing mutant receptors in which each residue was replaced by the other or both residues were exchanged (51). The results showed that the $Asp^{2.50(87)}$ mutant had no detectable binding. The double mutant $Asp^{2.50(87)}$ $Asn^{7.49(318)}$, which recreates the arrangement found in other GPCRs, regained high-affinity agonist and antagonist binding (see Fig. 16). The restoration of binding by a second reciprocal mutation indicates that these two specific residues in TMD 2 and TMD 7 are adjacent in space (Fig. 17) and provides an empirical basis for refining the structural parameters in the model of the receptor's transmembrane helix bundle (see below). Cook and co-workers (196) reported that the interchange mutation in the rat GnRH receptor did not show ligand binding. However, these investigators have subsequently confirmed that this construct is functional (K. Eidne, personal communication), and two other groups have confirmed the results with the mouse receptor (K. Catt, personal communication and Ref. 197).

The relationship of the same two postions in TMD 2 and TMD 7 has also been tested in the serotonin 5-$HT_{2A}$ receptor, which has the more typical arrangement of amino acids at the two loci (198). A related role of the two positions was also confirmed In the 5-$HT_{2A}$ receptor. However, the pattern of residues that are functionally tolerated at the two positions differed in the GnRH and serotonin receptors. Whereas in the GnRH receptor the TMD 2 Asp/TMD 7 Asp receptor was not functional, in the 5-HT receptor this construct was well coupled. The (TMD 2/TMD 7) Asn/Asn GnRH receptor was functional, whereas the Asn/Asn 5-HT receptor had no detectable coupling. Thus, in both receptors a functional and spatial relationship of these two side chains is supported by the demonstration of a function-restoring interchange mutation. However, the differences in the residues that are functionally tolerated at each position most likely reflect differences in the microenvironment of the two receptors caused by differences in receptor loci other than at these two positions. One group has reported that the $CCK_B$ receptor has wild-type function with an Asp at both positions and has reduced coupling with an Asn at each position (199). However, their conclusion that the two positions do not interact is uncertain, especially as the effects of an interchange mutation were not reported.

### *C. Intracellular loop domains*

Mutagenesis, chimera, and deletion studies indicate that the intracellular domains of GPCRs are involved in mediating G protein coupling. Particularly important domains are the membrane-proximal segments of the third intracellular loop, the second intracellular loop, and the membrane-proximal segment of the carboxy-terminal domain (for review see Refs. 49, 50, and 200). The second intracellular loop domains of the mouse and human receptors have been investigated by site-directed mutagenesis (60, 201). The GnRH receptor has a Ser at position $3.51^{(140)}$, a locus where most receptors have the Tyr of the Asp-Arg-Tyr ("DRY") motif. Mutation of this locus to the consensus Tyr by Arora and co-workers (60) caused an increase in agonist affinity and in rates of internalization in comparison to the wild-type receptor. Mutation of the second intracellular loop $Leu^{3.58(147)}$ to Ala or Asp was found to impair receptor coupling and internalization.

A specific conformational structure of this loop domain appears to be required for efficient G protein coupling. Mutation of $Arg^{3.56(145)}$ to Pro has been found to disrupt significantly the efficiency of coupling to signal transduction (201). This mutation introduces a Pro-Pro motif that disrupts most

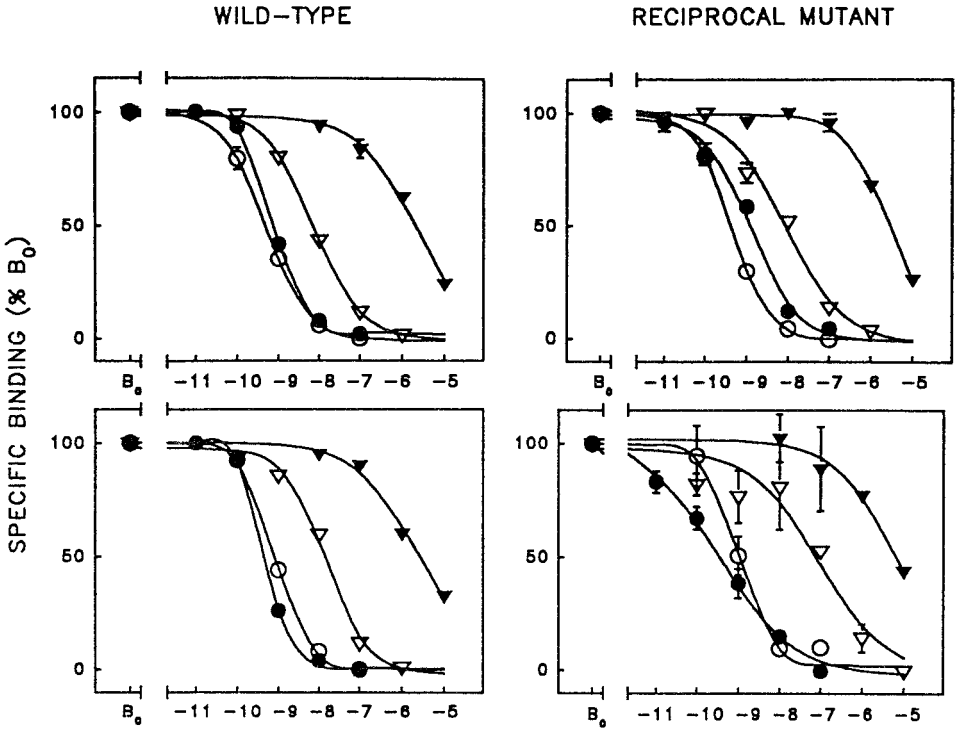


FIG. 16. Receptor binding in COS-1 cells transfected with wild-type (*left*) and $Asp^{2.50(87)}Asn^{7.49(318)}$ reciprocal-mutant (*right*) receptor constructs. *Top panel* shows competition binding of GnRH peptides with $^{125}$I-labeled GnRH agonist ($^{125}$I-[D-Ala$^6$,N-Me-Leu$^7$,Pro$^9$NEt]GnRH; GnRH-A). *Bottom panel* shows competition binding of GnRH peptides with $^{125}$I-labeled GnRH antagonist ([Ac-D4-Cl-Phe$^{1,2}$-D-Trp$^3$-D-Lys$^6$-D-Ala$^{10}$-$NH_2$]GnRH; Antagonist 26). GnRH-A, ○; antagonist 26, ●; Gln$^8$-GnRH, ▼; GnRH, △. [Reprinted with permission of the publisher from W. Zhou *et al.*: *Mol Pharmacol* 45:165–170, 1994 (51).]

known secondary structure. We have performed computational simulations and protein database searches with the the wild-type and Pro-Pro mutant receptor loop segment sequences. By comparing the structures accessible to the loop segments in the wild-type and mutant receptors, the conformations likely to be preferred for G protein coupling have been identified. We find by incorporating these results into the computational model of the receptor that an association of the second intracellular loop with other loop domains may be required for efficient receptor coupling (F. Guarnieri, L. Chi, V. Rodic, L. Ballesteros, H. Weinstein, and S. C. Sealfon, unpublished data).

One feature of the mammalian GnRH receptor, unique among the several hundred GPCRs cloned to date, is the complete absence of an intracellular C-terminal domain. In most rhodopsin-family GPCRs, this domain contains a cysteine that has been shown to be palmitoylated in several receptors (202–205). This domain also contains sites involved in phosphorylation-mediated regulation and desensitization of several GPCRs (206, 207). In the TRH receptor, for example, agonist-induced receptor internalization requires specific domains in the C terminus (208). Truncation or mutation of specific C-terminal domains has been reported to diminish agonist-mediated internalization of the gastrin-releasing peptide receptor (209) and the angiotensin receptor (210, 211), and to diminish desensitization of the LH receptor (212) (see, however, Ref. 213), the substance P receptor (214), the neurokinin receptor (215), and the $\alpha$-1B-adrenergic receptor (216). Nonetheless, it is likely that the functional role of the carboxyl terminus domain is not identical in different GPCRs. For example, truncation of the $\beta$-adrenergic receptor was reported not to affect sequestration (217), and truncation of the FSH receptor was found not to alter desensitization (218). While the ultimate response elicited after GnRH receptor stimulation (*e.g*. LH release) undergoes desensitization (reviewed in Refs. 13–15), it has not been demonstrated that this desensization occurs at the level of the receptor. In view of the role of the missing carboxyl-terminal domain in desensitization of a number of receptors, it is interesting to note that minimal rapid desensitization of the phosphoinositol response mediated by the endogenous $\alpha$T3–1 GnRH receptor or the cloned GnRH receptor expressed in several cell lines has been observed (69–71).

### *D. Computational modeling of three-dimensional receptor structure*

Inferences from the probing of the GnRH receptor with biochemical approaches, mutagenesis, and biophysical considerations, as described above, have validated its initial structural classification as a member of the family of rhodopsin-like GPCRs. Although no direct structural information at atomic resolution is available for any GPCR, strong inferences about structural characteristics of the transmembrane portion of the GnRH receptor rest on the projection map of the electron density of bovine rhodopsin (Fig. 4) and the results of extensive probing of the other members of the rhodopsin-like GPCR family. The extension of these inferences to the GnRH receptor is based on the extensive and pervasive sequence homologies and identities of specific motifs observed among the various rhodopsin-like receptors that include the GnRH receptors. Such sequence comparisons are used to identify the likely determinants for the structural commonalty expressed in the template of protein families, such as the seven loop-connected transmembrane helix bundles of the GPCRs (39, 219).

It has been shown that such sequence comparisons are useful in the characterization of structural properties and can also serve to identify the basis for the different functional properties of receptor proteins, *e.g*. those that determine ligand binding as well as the response of the GPCR to the actions of a large variety of ligands (for a review see Ref. 39). Not surprisingly, sequence alignments of the rhodopsin-family GPCRs are often the first steps in the modeling process of probing structure-function relations of these proteins, and in the construction of three-dimensional molecular models of specific receptors (39). The basic assumptions underlying the extraction of structural information about GPCRs from a set of aligned sequences are that they all share a structural framework, and that highly conserved residues can be considered essential for the structural and/or functional integrity of the receptor. Sequence sites observed to have a lower degree of conservation are considered to play a lesser role in determining the structure and/or function of the GPCR. The criteria guiding the construction of a sequence alignment of GPCRs have been reviewed, together with the conceptual and practical limitations of the sole use of sequence alignments for the prediction and construction of three-dimensional molecular models of GPCRs (39).

Using a complex array of interrelated criteria after a set of well defined methods for the construction and computational probing of such models (39), a three-dimensional model of the transmembrane helix bundle of the GnRH receptor has been developed (51) (Fig. 18). Specific criteria in the construction of this model included the structural inferences derived from the analysis of sequence conservation patterns (220, 221), the physico-chemical properties of conserved and partially conserved residues (219, 222), and specific protein motifs such as Pro-kinks (223–225), the projection map of rhodopsin (58, 219), and experimental results. The predicted helix boundaries take into account the role of Arg and Lys residues at the membrane-cytoplasm interface, where these residues belong, as described (39), to the transmembrane helix acting as an anchor to the membrane through ionic pairs with phospholipid head-groups (224). The model is consistent with the overall template of rhodopsin-family GPCRs (39), including the proposed interactions between helix 2 and 7 (51, 198), the mutual orientation of TMDs 1 and 7 (226), the counter-clockwise connectivity of the TMD domains when viewed from the extracellular side (227, 228), as well as the detailed deployment of sites in the interior of the TMD bundle and in the extracellular loops that have been suggested by experiments to contribute to the ligand-binding pocket. As illustrated by model-based investigations of structure-function relations of other GPCRs, both in the family of neurotransmitter receptors (195, 229) and in peptide receptors (194, 230–232), the discrete molecular models of the GnRH receptor should provide key insight into mechanisms of receptor specificity and activation (*e.g*. see Refs. 51, 142, 195, and 198).

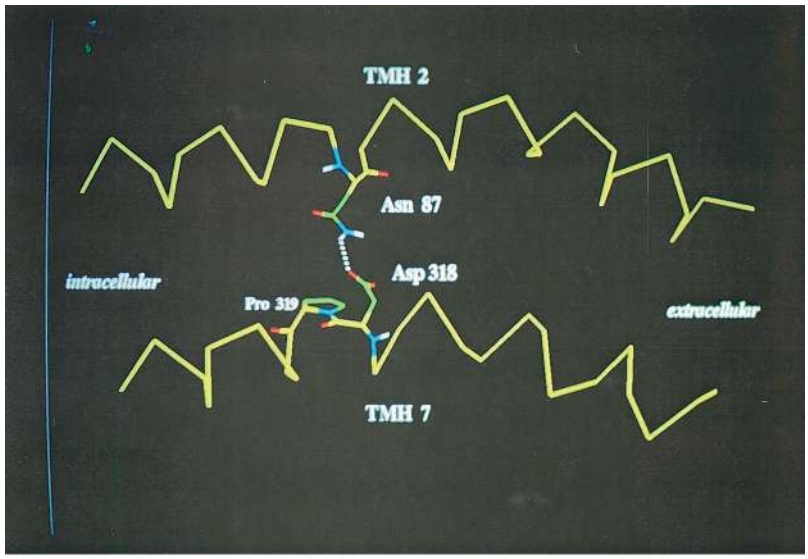


FIG. 17. Molecular modeling of the GnRH receptor. View parallel to the membrane of a partial GnRH receptor model showing spatial proximity of $Asn^{2.50(87)}$ and $Asp^{7.49(318)}$ [Reprinted with permission of the publisher from W. Zhou *et al.*: *Mol Pharmacol* 45:165–170, 1994 (51).]

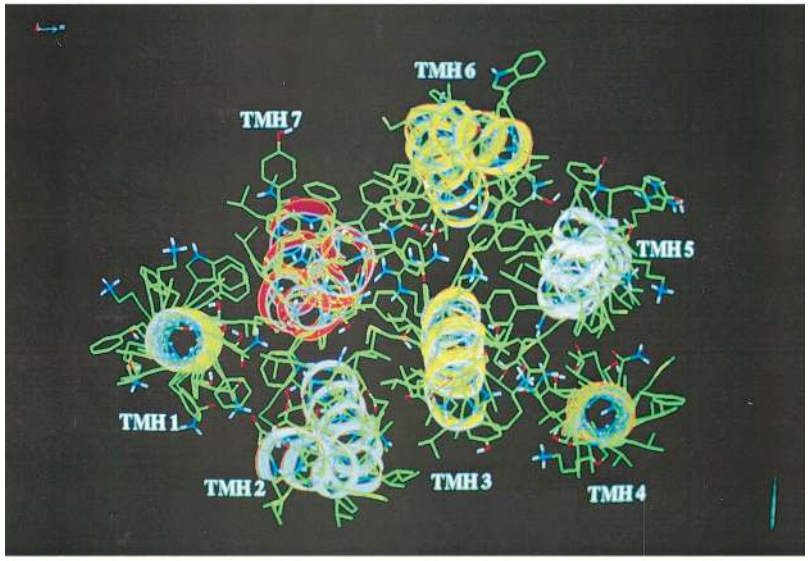


FIG. 18. Three-dimensional model of the transmembrane portion of the GnRH receptor, viewed from the extracellular side. [Reprinted with permission of the publisher from W. Zhou *et al.*: *Mol Pharmacol* 45:165–170, 1994 (5).]

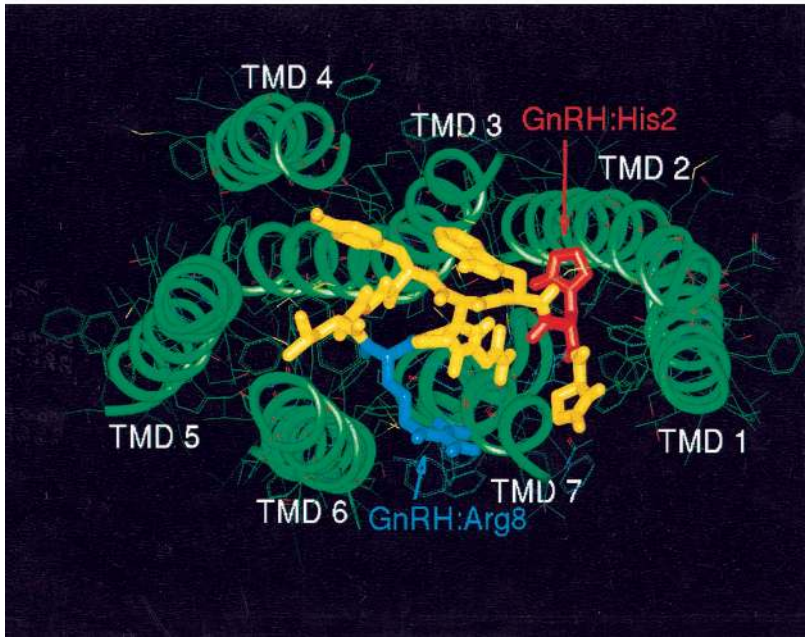


FIG. 19. Three-dimensional computational model of the GnRH receptor transmembrane helix bundle and GnRH. GnRH is shown in *yellow* except for $His^2$ (*red*) and $Arg^8$ (*blue*). The transmembrane domains of the receptor are highlighted with a *green* ribbon.

Using this model (51) as a starting point and incorporating the available experimental work on peptide conformation and ligand interaction sites, as reviewed in the preceding sections, a schematic that accomodates the computational and experimental data can be generated (Fig. 19). The computational model of GnRH is based on the predominant conformer observed in conformational family simultations of the peptide (174), and the ligand positioning reflects the following assumptions derived from experimentation: 1) $Arg^{8}$ of GnRH is in proximity to $Asp^{7.32(302)}$ (186); 2) the C terminus glycinamide of GnRH may interact with $Asn^{2.65(102)}$ (152); 3) $His^{2}$ of GnRH may interact with $Lys^{3.31(121)}$ (142) and/or $Asp^{2.61(980}$ (195a) of the receptor.

As illustrated in this review, the present model is undergoing continual evolution to reflect new experimental and computational data. Significant issues that are under study include the need to define the lipid-aqueous interface computationally, to incorporate models of the loop domains, and to predict receptor rearrangements that accompany ligand-complexing. The present iteration of the model GnRH-GnRH receptor complex serves to represent current experimental and computational insight into the molecular details of the ligand-receptor complex and to guide the design of ongoing experimentation.

## VI. Conclusions

The cloning of the mammalian GnRH receptors prompts an explanation of the abundant structure/activity data on GnRH analogs in terms of the specific interactions occurring in the ligand-receptor complex. Achieving this understanding requires a definition of the molecular organization of the receptor in terms of helix-helix proximities and the nature of the side chains that contribute to ligand selectivity and receptor conformational change. Through experiments based on evolutionary and computational considerations, much information about the properties of the receptor and its interaction with GnRH congeners is emerging and is being incorporated into computational molecular models of the ligands and receptor. Testing hypotheses that emerge from such studies and computational constructs by cross-validation of data obtained through molecular biological, pharmacological, and computational simulation approaches is leading to an understanding of receptor function by a characterization of the molecular events underlying the interaction with GnRH and its congeneric ligands.

## Acknowledgment

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