Role of Aspartate7.32(302) of the Human Gonadotropin-Releasing Hormone Receptor in Stabilizing a High-Affinity Ligand Conformation

BERNARD J. FROMME, ARIEH A. KATZ, ROGER W. ROESKE, ROBERT P. MILLAR, and COLLEEN A. FLANAGAN

Division of Medical Biochemistry (B.J.F., A.A.K., R.P.M., C.A.F.) and Department of Medicine (C.A.F.), University of Cape Town Faculty of Health Sciences, Observatory, South Africa; Indiana University School of Medicine, Indianapolis, Indiana (R.W.R.); and Medical Research Council Human Reproductive Sciences Unit, Edinburgh, Scotland, UK (R.P.M.)

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ABSTRACT

Mammalian gonadotropin-releasing hormone (GnRH) receptors preferentially bind mammalian GnRH, which has Arg in position eight. The Glu7.32(301) residue, which determines selectivity of the mouse GnRH receptor for Arg-containing GnRH, is Asp7.32(302) in the human GnRH receptor. We have confirmed that Asp7.32(302) confers selectivity of the human GnRH receptor for Arg of GnRH and investigated the mechanism of this specificity using site-directed mutagenesis and ligand modification. We find that although Arg and Asp7.32(302) are required for high-affinity binding of GnRH, conformationally constrained peptides, with D-amino acid substitutions in position six or with a 6,7 γ-lactam, bind the human GnRH receptor with high affinity, which is independent of the presence of Asp7.32(302) in the receptor or Arg in the ligand. The ability of the ligand constraints to compensate for the absence of both Arg and Asp7.32(302) indicates that these residues both have roles in stabilizing a high affinity ligand conformation and that their roles are complementary. This suggests that the Arg and Asp7.32(302) side chains interact to induce a high affinity conformation of native GnRH. Thus, Asp7.32(302) of the human GnRH receptor determines selectivity for mammalian GnRH by its ability to induce a high affinity conformation of its native ligand. However, this initial interaction seems not to contribute to the final ligand-receptor complex. We propose that Arg interacts transiently with Asp7.32(302) to induce a high-affinity ligand conformation of GnRH, which then interacts with a binding pocket that is common for both constrained and unconstrained analogs of GnRH.

Gonadotropin-releasing hormone [GnRH, also called luteinizing hormone releasing hormone or luteinizing hormone releasing hormone] is a decapeptide that is synthesized in the hypothalamus and interacts with GnRH receptors on gonadotrope cells in the anterior pituitary. GnRH stimulates the biosynthesis and release of luteinizing hormone and follicle-stimulating hormone, which in turn are required for steroidogenesis and gametogenesis, respectively. Because of this central role in reproduction, GnRH analogs have been used in a variety of therapeutic applications (Millar et al., 1987).

Although an X-ray diffraction analysis of rhodopsin has recently been published (Palczewski et al., 2000), most other G protein-coupled receptors (GPCRs) are more difficult to purify. Consequently, understanding of the structure of these GPCRs is likely to depend on indirect methods, such as computational modeling and mutagenesis, for some time to come. Considerable advances have been made in understanding how GnRH interacts with its receptor. In the human GnRH receptor, residues Asp2.61(98), Trp2.64(101), Lys3.32(121), and Asp5.61(212) (residue numbering is described under Materials and Methods) have been shown to have roles in ligand binding (Zhou et al., 1995; Davidson et al., 1996; Flanagan et al., 2000; Hoffmann et al., 2000). Some of these receptor residues have been proposed to form part of the ligand binding pocket, interacting with the amino and carbonyl termini of GnRH in a computational model of the receptor-ligand complex (Sealfon et al., 1997). Asp7.32(302) is proposed to interact with His5 of GnRH, whereas Asn2.65(102) interacts with Gly10-NH2. In the mouse GnRH receptor, Glu7.32(301) was shown to have a role in recognizing the Arg residue of GnRH (Flanagan et al., 1994). However, Glu7.32(301) is not completely conserved in mammalian GnRH receptors. In the human and other nonrodent GnRH recep-

ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; HPLC, high-performance liquid chromatography; PEI, polyethyleneimine; IP, inositol phosphate; antagonist 26, [Ac-α-4-Cl-Pheβ-6-γ-Trpβ-6-Lysβ-6-Alaγ-H9280 glycine]-GnRH; antagonist 26, [Ac-α-3-(2-naphthyl) alanineβ-4-Cl-Pheβ-6-γ-Trpβ-6-(3-pyridyl)alanineβ-6,7 γ-lactam, lpyr-Lysγ-6-Alaγ-H9254 glycine]-GnRH; Emax, maximal agonist-stimulated inositol phosphate production; lpyr-Lysγ, N-isopropyllysine; GnRH II, [Hisβ, Trpβ, Tyrβ]-GnRH.
tors the equivalent residue is Asp\textsuperscript{7,32/302} (Kakar et al., 1992; Chi et al., 1993; Illing et al., 1993; Sealfon et al., 1997; Cui et al., 2000). Although this is a conservative substitution, it is surprising that such a functionally important residue is not absolutely conserved. In the monoamine receptors, the Asp\textsuperscript{3,32} residue, which is important for ligand binding, is conserved as Asp not only in different species but also in different receptor subtypes that recognize the same ligand and in different receptors that recognize distinct ligands ranging through acetylcholine, adrenaline, serotonin, and histamine (Probst et al., 1992).

In GnRH, Arg\textsuperscript{8} is required for high-affinity binding to mammalian GnRH receptors. Substitution of this residue decreases GnRH potency and affinity for the receptor (Millar et al., 1989). Mutation of the Glu\textsuperscript{7,32/301} residue of the mouse GnRH receptor to Gln decreased the receptor affinity for GnRH, but not for analogs with substitutions for Arg\textsuperscript{8} (Flanagan et al., 1994). Subsequent models of GnRH receptor-ligand complexes have incorporated an interaction of the acidic residue of the receptor with Arg\textsuperscript{8} of the ligand (Chauvin et al., 2000; Flanagan et al., 2000; Hoffmann et al., 2000). However, a GnRH analog with d-Trp substituted in position six showed only a small decrease in affinity for the Glu\textsuperscript{7,32/301} Gln mouse receptor. This suggested that although Glu\textsuperscript{7,32/301} determines selectivity for native GnRH, the mechanism by which it does so may be more complex than a simple electrostatic interaction of Glu\textsuperscript{301} with Arg\textsuperscript{8}. It also indicates a need for caution in extrapolating experimental results to molecular models of GPCRs.

The lack of conservation indicates a need to determine whether Asp\textsuperscript{7,32/302} has the same function in the human GnRH receptor as Glu\textsuperscript{7,32/301} has in the mouse receptor. Furthermore, the incorporation of a direct interaction of Glu\textsuperscript{7,32/301}/Asp\textsuperscript{7,32/302} with Arg\textsuperscript{8} in models of receptor-ligand complexes, despite evidence that a direct interaction may not always occur, shows that better definition of the mechanism by which the Asp\textsuperscript{7,32/302} determines binding specificity for GnRH is needed. We now show that mutating Asp\textsuperscript{7,32/302} in the human GnRH receptor decreases affinity for GnRH, but not for analogs with substitutions for Arg\textsuperscript{8}. In contrast, a series of peptides with different structural constraints that stabilize a high-affinity conformation of GnRH retain high affinity for the mutant receptor. This indicates that an electrostatic interaction is not involved in the binding of these constrained analogs. We also show that Arg\textsuperscript{8} is not required for high-affinity binding of constrained analogs. We interpret these results in terms of a sequential binding mechanism in which the Arg\textsuperscript{8} side chain of native GnRH interacts transiently with Asp\textsuperscript{7,32/302}, before interacting with a final ligand binding pocket that also binds constrained analogs.

**Materials and Methods**

**Consensus Residue Numbering Scheme.** A consensus numbering scheme is used to facilitate the comparison of equivalent amino acid residues in the different rhodopsin-like GPCRs (Ballesteros and Weinstein, 1995). Amino acids were numbered relative to the most conserved residue in each transmembrane domain, which is assigned the number 50 (Fig. 1). Individual amino acid residues are identified by a generic identifier consisting of the transmembrane helix number, followed by the number representing its position relative to the most conserved residue in the helix. This is followed by its sequential number in the particular GPCR. For example, the most conserved residue in helix seven of the GnRH receptor is Pro, which is designated Pro\textsuperscript{7,50}. In the GnRH receptor, Pro\textsuperscript{7,50} is residue number 320 and is designated Pro\textsuperscript{7,50/320}. Asp\textsuperscript{302} is 18 amino acids closer to the amino-terminal than Pro\textsuperscript{7,50} and is therefore designated Asp\textsuperscript{7,32/302}.

**Site Directed Mutagenesis.** A polymerase chain reaction-based mutagenesis method was used to replace Asp\textsuperscript{7,32/302} with Asn in the human GnRH receptor. Primers contained the desired mutation and a silent restriction endonuclease site flanked by 12 bases of the wild-type receptor sequence on either side. Polymerase chain reaction products were digested with appropriate restriction enzymes, ligated using T4 DNA Ligase (Amersham Pharmacia Biotech, Piscataway, NJ), subcloned into the EcoRI and XhoI sites of the mammalian expression vector pcDNAI/AMP (Invitrogen, Carlsbad, CA), and transformed into competent XL-1 blue Escherichia coli. Plasmid DNA was extracted (Nucleobond Kit; Macherey-Nagel, Duesseldorf, Germany) from ampicillin-resistant clones and the mutation was confirmed by DNA sequencing (Epigencentre Technologies, Madison, WI).

**Transfection and Cell Culture.** COS-1 cells were transiently transfected using the DEAE-Dextran method (Koem et al., 1990), as described previously (Millar et al., 1995). After transfection, COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (Delta Bioproducts, Kempton Park, South Africa) and antibiotics (2 mg/ml streptomycin sulfate, 4000 U/ml sodium benzylpenicillin) in a 10% CO\textsubscript{2} incubator at 37°C.

**GnRH Analogs.** GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH\textsubscript{2}), [His\textsuperscript{8},d-Trp\textsuperscript{3},d-Thr\textsuperscript{2},d-Me-Leu\textsuperscript{5},Pro\textsuperscript{3},NHEt\textsuperscript{6}]-GnRH, [d-Trp\textsuperscript{3},Pro\textsuperscript{3},NHEt\textsuperscript{6}]-GnRH, [d-Trp\textsuperscript{3},Pro\textsuperscript{6},NHEt\textsuperscript{6}]-GnRH, [d-Trp\textsuperscript{3},Pro\textsuperscript{6},Gln\textsuperscript{6},Pro\textsuperscript{3},NHEt\textsuperscript{6}]-GnRH, [Gln\textsuperscript{6}]-GnRH, GnRH II ([His\textsuperscript{8},d-Trp\textsuperscript{3},d-Thr\textsuperscript{2}]-GnRH), and antagonist 27 ([Ac-d-3-(2-naphthyl)alanine\textsubscript{1},d-Me-4-Cl-Phe\textsubscript{2},d-Trp\textsubscript{3},Ipr-Lys\textsubscript{5},d-Thr\textsuperscript{2},d-Ala\textsuperscript{10},NH\textsubscript{3}]-GnRH) were prepared by conventional solid phase methodology and purified by preparative C-18 reverse phase high-performance liquid chromatography in our Cape Town laboratory. Antagonist 26 ([Ac-d-3-(2-naphthyl)alanine\textsubscript{1},d-Me-4-Cl-Phe\textsubscript{2},d-Trp\textsubscript{3},3-(3-pyridyl)alanine\textsubscript{5}\textsuperscript{6,7}-y-lactam,Ipr-Lys\textsubscript{5},d-Ala\textsuperscript{10},NH\textsubscript{3}]-GnRH) and [Glu\textsuperscript{8}]-GnRH were prepared by solid phase synthesis on a 4-methylenebzydridylamine HCl resin using Boc/Benzyl chemistry. The Boc-y-lactam (Freidinger et al., 1980) was added as one amino acid unit. After removal from the resin by hydrogen fluoride, the peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography on a C-18 preparative column. Antagonist 26 ([Ac-d-4-Cl-Phe\textsubscript{1},d-Trp\textsubscript{3},d-Lys\textsubscript{6},d-Ala\textsuperscript{10},NH\textsubscript{3}]-GnRH) was a gift from David Coy (Tulane University School of Medicine, New Orleans, LA). [6,7 y-lactam]-GnRH was a gift from Roger Freidinger (Merck Co., West Point, PA).

**Phosphatidyl Inositol Hydrolysis.** Transfected COS-1 cells (2 x 10\textsuperscript{6} cells/well) in 12-well plates were incubated overnight with

![Fig. 1. Schematic diagram of the GnRH receptor. Circles indicate residues that are important for ligand binding. Squares indicate the most conserved residues in each transmembrane domain, which are reference residues in the consensus numbering scheme.](image-url)
myo-[2-3H]inositol (1 μCi/well; Amersham) in 0.5 ml medium 199 (Invitrogen) with antibiotics. Labeled cells were incubated with various concentrations of ligand for 1 h at 37°C in the presence of LiCl as described previously (Millar et al., 1995). Aspirating the medium and addition of 10 mM formic acid (1 ml/well) terminated the incubation. Inositol phosphates were extracted from the formic acid extract on DOWEX-1 ion exchange columns and eluted into scintillation liquid (QuickSafe; Zinsser Analytical, Frankfurt, Germany) and the radioactivity was counted.

Radioiodination of GnRH analogs was performed as described previously (Flanagan et al., 1998). Specific activity ranged between 900 and 1800 μCi/μg and 69% of the radioactivity could be bound by GnRH receptors. Using this high-affinity label allowed accurate determination of IC50 values for the mutant receptor, which had low total binding (Flanagan et al., 1998). Transfected COS-1 cells were homogenized in binding buffer (1 mM EDTA, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin) and centrifuged at 15,000 g for 20 min. In the assay (Millar et al., 1995). The agonist peptide, [His5,D-Tyr6]-GnRH, was radioiodinated by the Chloramine-T method as described previously (Rodbard, 1983; Klotz, 1982; Motulsky, 1999). The high nonspecific binding of the radioligand (IC50) was used to determine the low IC50 value for that homologous competition binding assay (Munson and Rodbard, 1980) results from the slightly decreased affinity of the mutant receptor reported in initial experiments (Flanagan et al., 1998) results from the slightly decreased affinity for 125I-[His5,p-Tyr6]-GnRH.

In competitive ligand binding assays, the affinities of the wild-type human GnRH receptor for uncharged, [Gln8]-GnRH (Kd = 923 ± 222 nM) and negatively charged, [Glu8]-GnRH (Kd = 10,000 nM) were lower than that for Arg8-containing GnRH (Kd = 6.79 ± 1.08 nM) (Table 1, Fig. 2). This shows that the human GnRH receptor preferentially binds Arg8-containing GnRH.

**Results**

**Mutation of Asp7.32(302) Decreases Affinity for GnRH.**

The wild-type GnRH receptor bound [His5,p-Tyr6]-GnRH, which was used as a radiolabeled ligand, with high affinity (Kd = 0.35 ± 0.06 nM). The Asp7.32(302)Asn mutant receptor showed 2.8-fold lower affinity for [His5,p-Tyr6]-GnRH (Kd = 0.99 nM ± 0.01 nM). Receptor number was unaffected by the Asp7.32(302) mutation (wild-type, 1.3 ± 0.23 × 105 sites/cell; Asp7.32(302)Asn mutant, 1.31 ± 0.06 × 105 sites/cell). The similarity expression suggests that the lower total binding of the mutant receptor reported in initial experiments (Flanagan et al., 1998) results from the slightly decreased affinity for 125I-[His5,p-Tyr6]-GnRH.

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**GnRH Analog Substitutions in Position Six of GnRH Enhance Affinity and Overcome**

**Table 1**

<table>
<thead>
<tr>
<th>GnRH analog</th>
<th>Wild-Type</th>
<th>Mutant</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Gln8]-GnRH</td>
<td>6.79 ± 1.08</td>
<td>212 ± 40.6**</td>
<td>31.2</td>
</tr>
<tr>
<td>[Glu8]-GnRH</td>
<td>923 ± 222</td>
<td>1240 ± 227</td>
<td>1.35</td>
</tr>
<tr>
<td>[His5,p-Tyr6]-GnRH</td>
<td>&gt;10 000</td>
<td>&gt;10 000</td>
<td></td>
</tr>
<tr>
<td>[His5,N-Me-Leu7,Pro9-NHEt]-GnRH</td>
<td>0.48 ± 0.12b</td>
<td>1.36 ± 0.20b</td>
<td>2.83</td>
</tr>
<tr>
<td>[His5,p-Tyr6]-[Thr21,Glu22]-GnRH</td>
<td>0.65 ± 0.35</td>
<td>1.55 ± 0.09</td>
<td>1.60</td>
</tr>
<tr>
<td>[His5,γ-lactam]-GnRH</td>
<td>0.35 ± 0.04</td>
<td>0.27 ± 0.06</td>
<td>1.80</td>
</tr>
<tr>
<td>6.7 γ-lactam]-GnRH</td>
<td>1.86 ± 0.19</td>
<td>1.67 ± 0.43</td>
<td>0.82</td>
</tr>
<tr>
<td>GnRH II ([His5,Trp7,Tyr8]-GnRH)</td>
<td>1.37 ± 0.07</td>
<td>4.86 ± 0.36**</td>
<td>3.40</td>
</tr>
<tr>
<td>Antagonist 1229</td>
<td>193.7 ± 28.5</td>
<td>213.5 ± 72.4</td>
<td>1.10</td>
</tr>
<tr>
<td>Antagonist 26</td>
<td>0.86 ± 0.21</td>
<td>1.52 ± 0.28</td>
<td>1.76</td>
</tr>
</tbody>
</table>

**Notes:**

* Significantly different from wild type, p < 0.05.

** Significantly different from wild type, p < 0.001.


Kd values are reported for the homologous ligand, [His5,p-Tyr6]-GnRH.
the Absence of Arg⁸ and/or Asp⁷.32(302). We found previously that binding of the conformationally constrained analog, [d-Trp⁶,Pro⁹-NHEt]-GnRH, was not affected by the Glu⁷.32(301) Gln mutation in the mouse GnRH receptor (Flanagan et al., 1994). To test whether this is a general phenomenon in mammalian GnRH receptors, binding affinities of a series of GnRH analogs with different d-amino acid substitutions in position six were characterized in the wild-type human receptor and in the Asp⁷.32(302)Asn mutant (Fig. 3). As expected for the wild-type receptor (Karten and Rivier, 1986; Sealfon et al., 1997), three GnRH analogs, [His⁶,d-Tyr⁶]-GnRH, [d-Ala³,N-Me-Leu⁷,Pro⁹-NHEt]-GnRH, and [d-Trp⁶,Pro⁹-NHEt]-GnRH, showed higher affinity (7.0- to 45-fold) than native GnRH (Tables 1 and 2, Fig. 3). The affinity of the wild-type receptor for the uncharged but conformationally constrained analog [d-Trp⁶,Gln⁸,Pro⁹-NHEt]-GnRH (Kᵢ = 1.9 ± 0.19 nM) was 486-fold higher than the affinity for [Gln⁸]-GnRH (Kᵢ = 923 ± 222 nM) (Table 1). This shows that, in the wild-type receptor, incorporation of a d-amino acid in position six enhances the affinity of [Gln⁶]-GnRH more than the affinity of Arg⁸-containing GnRH and thus compensates for the absence of Arg⁸ (Table 2). However, the affinity of [d-Trp⁶,Gln⁸,Pro⁹-NHEt]-GnRH (Kᵢ = 1.9 ± 0.19 nM) remained 13-fold lower than the affinity of [d-Trp⁶,Pro⁹-NHEt]-GnRH (Kᵢ = 0.15 ± 0.04 nM).

All four GnRH analogs with d-amino acid substitutions in position six had similar high affinity for the Asp⁷.32(302)Asn mutant receptor compared with the wild-type receptor (Table 1). In the mutant receptor, the affinities of the three analogs with d-amino acids in position six and Arg in position eight were 137- to 784-fold higher than the affinity for (native) GnRH (Table 2). The affinity of the uncharged [d-Trp⁶,Gln⁸,Pro⁹-NHEt]-GnRH was 744-fold higher than for [Gln⁸]-GnRH (Fig. 3, Table 2).

Consistent with the previous report (Freidinger et al., 1980), [6,7 γ-lactam]-GnRH exhibited higher affinity than GnRH for the wild-type GnRH receptor (5.0-fold; Table 2). This result is similar to the increase found with d-amino acid substitutions in position six of GnRH (Table 2). [6,7 γ-Lactam]-GnRH also had high affinity for the Asp⁷.32(302)Asn mutant GnRH receptor (Kᵢ = 4.66 ± 0.36 nM; Table 1), which was 45.5-fold higher than the affinity for native GnRH (Table 2). This shows that the γ-lactam constraint enhances the affinity of GnRH for both the wild-type and mutant receptors (Fig. 4, Table 1). [6,7 γ-Lactam]-GnRH had similar affinity for both the wild-type receptor (Kᵢ = 1.37 ± 0.07 nM) and the mutant receptor (Kᵢ = 4.66 ± 0.36 nM, Table 1). Similar to the d-amino acid substitution in position six, incorporation of 6,7 γ-lactam enhanced binding affinity more in the mutant receptor than in the wild-type receptor. These results show that when the conformation of GnRH is constrained (6,7 γ-lactam or d-amino acid in position six), Asp⁷.32(302) of the

Incorporation of a 6,7 γ-Lactam Enhances Binding to the Receptor. Because d-amino acid substitutions in position six are thought to stabilize a high-affinity conformation of GnRH (Monahan et al., 1973), the high affinity of the mutant receptor for peptides with d-amino acids in position six suggests that conformationally constrained peptides may be less sensitive to the Asp⁷.32(302) mutation. However, part of the enhanced affinity may be contributed by an interaction of the amino acid side chain (e.g., d-Trp) with a receptor residue. To test whether the high affinity is caused predominantly by the conformational constraint of the d-amino acid, peptides with a conformational constraint, in which there is no side chain, were examined. Introduction of a γ-lactam moiety in place of residues six and seven is reported to impose a peptide conformation, which is similar to that stabilized by d-amino acid modifications (Freidinger et al., 1980).

![Fig. 2. Competition binding of GnRH and GnRH analogs in human wild-type and Asp⁷.32(302)Asn mutant GnRH receptors. COS-1 cell membranes expressing the human wild-type (top) and Asp⁷.32(302)Asn mutant (bottom) GnRH receptors were incubated with [¹²⁵I]His⁶,d-Tyr⁶]-GnRH in the presence of various concentrations of GnRH (○), [Gln⁸]-GnRH (□), and [Glu⁷]-GnRH (▼). Data are presented as mean ± S.E. of representative experiments performed in triplicate and expressed as percentage of specific binding in the absence of unlabeled ligand (T).](image1)

![Fig. 3. Competition binding of GnRH analogs with d-amino acid substitutions in position six. COS-1 cell membranes expressing the human wild-type (top) and Asp⁷.32(302)Asn mutant (bottom) GnRH receptors were incubated with [¹²⁵I]His⁶,d-Tyr⁶]-GnRH in the presence of various concentrations of GnRH (○), [d-Trp⁶,Pro⁹-NHEt]-GnRH (■), and [d-Trp⁶,Gln⁸,Pro⁹-NHEt]-GnRH (▲). Data are presented as mean ± S.E. of representative experiments performed in triplicate and expressed as percentage of specific binding in the absence of unlabeled ligand (T).](image2)
receptor is not required for high-affinity binding. Furthermore, the high affinity of [d-Trp⁶,Gln⁸,Pro⁹-NH²Et]-GnRH, which has a constraint but no Arg⁸, shows that Arg⁸ also is not required for high-affinity binding of conformationally constrained agonists.

Another class of conformationally constrained GnRH analogs includes the GnRH antagonists. The novel antagonist, antagonist 129–62, which has a 6,7 γ-lactam, and antagonist 26, which has d-Lys⁶, had similar high affinities for the wild-type and mutant receptors (Table 1, Fig. 5).

GnRH II, which occurs naturally in the human brain (White et al., 1998) does not contain Arg⁸, but binds the GnRH receptor with higher affinity ($K_i = 193.7$ ± 28.5 nM) than [Gln⁸]-GnRH ($K_i = 923$ ± 222 nM; Table 1, Fig. 4) or [Tyr⁷]-GnRH (Millar et al., 1989). This suggests that the combination of substitutions in positions five, seven, and eight allows the peptide to bind with relatively high affinity, independently of interactions that involve Arg⁸, possibly by stabilizing a high affinity conformation (de L Milton et al., 1983). GnRH II had similar affinity for the wild-type ($K_i = 193.7$ ± 28.5 nM) and mutant receptors ($K_i = 213.3$ ± 72.4 nM; Table 1). Consequently, the Asp⁷.32(302)Asn mutant receptor retained higher affinity for GnRH II ($K_i = 213.3$ ± 72.4 nM) than for [Gln⁸]-GnRH ($K_i = 1240$ ± 227 nM).

In summary, these results show that mutating Asp⁷.32(302) of the human GnRH receptor to Asn decreases the affinity for GnRH in a manner that is specific to an interaction with Arg⁸, and that a specific conformation of GnRH is important for high-affinity binding of GnRH to its receptor.

**Decreased IP Production in the Asp⁷.32(302)Asn Mutant GnRH receptor.** The Asp⁷.32(302)Asn mutant GnRH receptor coupled to the IP signaling pathway (Fig. 6). GnRH displayed a 44-fold decrease in potency at the mutant receptor ($EC_{50} = 12.6$ ± 1.78 nM) relative to the wild-type receptor ($EC_{50} = 0.29$ ± 0.07 nM; Fig. 6). This decrease in potency is consistent with the decreased affinity of the mutant receptor for native GnRH. However, the mutant receptor also exhibited a decreased $E_{\text{max}}$ value for GnRH (Fig. 6, Table 3), suggesting that the mutation may induce partial uncoupling of the receptor from intracellular signaling. Surprisingly, ligands that showed no decrease in affinity for the mutant receptor also exhibited decreased IP production in the mutant receptor (Table 3, Fig. 6). This shows that the effect of the mutation on cytosolic signaling is distinct from its effect on binding affinity for GnRH.

**Discussion**

The basic Arg residue in position eight of GnRH is required for high-affinity binding to mammalian GnRH receptors (Millar et al., 1989). The proposal that Arg⁸ may be involved in an electrostatic interaction with an acidic residue in the GnRH receptor (Hazum, 1987) was examined in the mouse GnRH receptor, where it was found that the Glu⁷.32(301) residue confers specificity for GnRH with Arg in position eight (Flanagan et al., 1994). Despite the demonstrated functional importance of Glu⁷.32(301), this residue is not conserved in the human GnRH receptor, which has Asp⁷.32(302) instead (Chi et al., 1993). The carboxyl side chain of both residues suggests that Asp⁷.32(302) can potentially perform the same functions in the mouse receptor and computational models of both rodent and human receptors have

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**TABLE 2**

Enhancement of GnRH affinity by conformational constraints

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Wild-Type $K_i$ (nM)</th>
<th>Fold enhancement</th>
<th>Mutant $K_i$ (nM)</th>
<th>Fold enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>GnRH</td>
<td>6.8*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[d-Ala⁶,N-Me-Leu⁷, Pro⁹-NH²Et]-GnRH</td>
<td>0.97*</td>
<td>7.0</td>
<td>1.55*</td>
<td>137</td>
</tr>
<tr>
<td>[His⁵,d-Tyr⁶]-GnRH</td>
<td>0.48*</td>
<td>14.2</td>
<td>1.36*</td>
<td>156</td>
</tr>
<tr>
<td>[d-Trp⁶,Pro⁹-NH²Et]-GnRH</td>
<td>0.15*</td>
<td>45.3</td>
<td>0.27*</td>
<td>784</td>
</tr>
<tr>
<td>[6,7 γ-lactam]-GnRH</td>
<td>1.37*</td>
<td>5.0</td>
<td>4.66*</td>
<td>45.5</td>
</tr>
<tr>
<td>[Gln⁸]-GnRH</td>
<td>923</td>
<td></td>
<td>1240</td>
<td></td>
</tr>
<tr>
<td>[d-Trp⁶,Gln⁸,Pro⁹-NH²Et]-GnRH</td>
<td>1.86**</td>
<td>485.5</td>
<td>1.67**</td>
<td>744</td>
</tr>
</tbody>
</table>

* $K_i$ values are the same as presented in Table 1.
* * Significantly different from GnRH, $p < 0.01$.
** ** Significantly different from [Gln⁸]-GnRH, $p < 0.001$.

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**TABLE 3**

Summary of GnRH receptor agonist-stimulated IP accumulation

<table>
<thead>
<tr>
<th>GnRH analog</th>
<th>$EC_{50}$ (nM)</th>
<th>Fold change</th>
<th>$E_{\text{max}}$</th>
<th>% wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>GnRH</td>
<td>0.29 ± 0.07</td>
<td></td>
<td>43.5</td>
<td>68.7 ± 8.32*</td>
</tr>
<tr>
<td>[Gln⁸]-GnRH</td>
<td>5.19 ± 1.13</td>
<td></td>
<td>3.3</td>
<td>69.3 ± 4.20*</td>
</tr>
<tr>
<td>GnRH II</td>
<td>1.75 ± 0.55</td>
<td></td>
<td>3.8</td>
<td>71.3 ± 7.70*</td>
</tr>
<tr>
<td>[d-Ala⁶,N-Me-Leu⁷, Pro⁹-NH²Et]-GnRH</td>
<td>0.06 ± 0.002</td>
<td></td>
<td>5.2</td>
<td>76.0 ± 2.89</td>
</tr>
</tbody>
</table>

* * Significantly different from wild-type receptor, $p < 0.05$.
** ** Significantly different from wild-type receptor, $p < 0.001$. 

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incorporated interactions of Glu or Asp with Arg. However, the study in the mouse receptor suggested that the mechanism of receptor selectivity for Arg-containing GnRH may be more complex than a simple electrostatic interaction in the receptor ligand complex. This demonstrates a need for detailed investigation of the mechanism by which Asp determines specificity for Arg. We show that an electrostatic interaction is not required for high-affinity binding of certain GnRH analogs and we propose that Asp may stabilize a high-affinity conformation of GnRH.

Asp of the Human GnRH Receptor Determines Selectivity for GnRH. The decreased affinity of the Asp Asn mutant for native GnRH, but not for [Gln]-GnRH shows a loss of specificity for Arg of GnRH. This shows that, like the Glu residue of the mouse GnRH receptor, the Asp side chain determines receptor preference for the Arg side chain of GnRH. However, the 31-fold decrease in the affinity of the Asp Asn mutant for GnRH is smaller than would be expected for a loss of an electrostatic interaction (Wells et al., 1987). The low affinity of the negatively charged ligand, [Glu]-GnRH could potentially result from a repulsive interaction with the negatively charged Asp side chain of native GnRH. Consequently, high-affinity binding of native GnRH may not arise from an electrostatic interaction of the Arg and Asp side chains.

Conformational Constraints Enhance GnRH Binding to the Wild-Type GnRH Receptor. Arg is proposed to stabilize an active conformation of GnRH (Shinitzky et al., 1976), which consists of a β-II-bend involving the Tyr-Gly-Leu residues (Monahan et al., 1973). Incorporation of a D-amino acid in position six (Momany, 1976) or a γ-lactam in positions six and seven (Freidinger et al., 1980) is proposed to stabilize this conformation and results in an increased GnRH potency (Monahan et al., 1973; Freidinger et al., 1980). The current study has shown that GnRH analogs with D-amino acid substitutions in position six, or with a γ-lactam, have higher affinities than native GnRH for the wild-type human GnRH receptor. This shows that the human GnRH receptor has enhanced affinity for the conformational state of GnRH that is stabilized by incorporating either constraint.

To test whether preferential binding of Arg-containing GnRH is retained in the presence of a conformational constraint in the wild-type receptor, affinities of conformationally constrained peptides with Arg or Glu (β-Trp, Pro, NHEt-GnRH and β-Trp, Glu, Pro, NHEt-GnRH) were compared. The modification enhanced the affinity of [Gln]-

Fig. 4. Competition binding of GnRH analogs with and without 6,7 γ-lactam conformational constraint. COS-1 cell membranes expressing the human wild-type (top) and Asp Asn mutant (bottom) GnRH receptors were incubated with [H]-[35S]-[D-Tyr]-GnRH in the presence of various concentrations of GnRH ( ), [6,7 γ-lactam]-GnRH ( ), and GnRH II ( ). Data are presented as mean ± S.E. of representative experiments performed in triplicate and expressed as percentage specific binding in the absence of unlabeled ligand (T).

Fig. 5. Competition binding of GnRH antagonists. COS-1 cell membranes expressing the human wild-type (top) and Asp Asn mutant (bottom) GnRH receptors were incubated with [H]-[35S]-[D-Tyr]-GnRH in the presence of various concentrations of GnRH ( ), antagonist 26 ( ), and antagonist 129–62 ( ). Data are presented as mean ± S.E. of representative experiments performed in triplicate and expressed as percentage specific binding in the absence of unlabeled ligand (T).

Fig. 6. GnRH-stimulated IP production. COS-1 cells transiently transfected with the wild-type human GnRH receptor (filled symbols) and with the Asp Asn mutant (open symbols) were incubated with various concentrations of GnRH ( , ) and GnRH II ( , ). Data are presented as mean ± S.E. of a representative experiment performed in duplicate and expressed as percentage of E stimulated by each peptide in the wild-type receptor. EC values in this experiment were 0.38 nM for GnRH in the wild-type, 15.1 nM for GnRH in the mutant, 1.36 nM for GnRH II in the wild-type, and 6.7 nM for GnRH II in the mutant.
GnRH more than that of GnRH (Table 2). Thus, a conformational constraint not only enhances the affinity of [Gln⁸]-GnRH but also compensates for the absence of Arg⁸. This shows that Arg⁸ is not required for high-affinity binding of conformationally constrained analogs to the wild-type human GnRH receptor.

Constrained Ligands Retain High Affinity for the Asp⁷.₃₂(₃₀₂)Asn Mutant. In contrast to native GnRH, GnRH analogs with conformational constraints retained high affinity for the Asp⁷.₃₂(₃₀₂)Asn mutant receptor. Because of the lower affinity of the mutant receptor for GnRH, the enhancement of the affinity of the conformationally constrained analogs, compared with GnRH, was greater in the mutant receptor than in the wild-type receptor. The preservation of high affinity for constrained peptides in the mutant receptor shows that the Asp⁷.₃₂(₃₀₂) side chain is not required for high-affinity binding of conformationally constrained GnRH analogs.

Ligand Constraint Compensates for the Absence of Both Arg⁸ and Asp⁷.₃₂(₃₀₂). The high-affinity binding of the Gln⁸-containing analog, [b-Trp⁶,Gln⁸,Pro⁹,NH₂]-GnRH, to the mutant receptor shows that the conformational constraint can compensate for the simultaneous absence of both Arg⁸ in the ligand and Asp⁷.₃₂(₃₀₂) in the receptor. This result suggests that native GnRH interacts with the receptor differently than conformationally constrained GnRH analogs. Thus, native GnRH and conformationally constrained GnRH analogs may occupy different (although overlapping) binding pockets on the receptor. Two other GnRH receptor residues, Asp².₆₁(₁₉₈) and Asp².₆₅(₁₀₂), determine recognition of His² and Gly-NH₂ of GnRH, respectively (Davidson et al., 1996a; Flanagan et al., 2000). Comprehensive analysis shows that the interaction of these receptor residues with native GnRH is similar to their interaction with constrained analogs. Mutation of these residues decreases receptor recognition of native and constrained analogs of GnRH to the same extent, suggesting that both native GnRH and constrained analogs interact with these residues (Davidson et al., 1996; Flanagan et al., 2000). Thus, the ability of the ligand conformational constraint to overcome a receptor mutation is specific for the Asp⁷.₃₂(₃₀₂)Asn mutant.

Asp⁷.₃₂(₃₀₂) and Arg⁸ Induce a High-Affinity Conformation of GnRH. We have shown that substituting Arg⁸ of native GnRH or Asp⁷.₃₂(₃₀₂) of the receptor decreases binding affinity. The lack of an additive effect when both substitutions are combined suggests that these side chains interact with each other. However, conformational constraint of the ligand reverses the loss of affinity due to substitution of Arg⁸ and/or Asp⁷.₃₂(₃₀₂). This suggests that both residues have roles in stabilizing a high-affinity conformation of unconstrained GnRH. Arg⁸ has been proposed to have two distinct roles in high-affinity binding: an intramolecular interaction that stabilizes a high-affinity peptide conformation (Shinitzky et al., 1976) and an intermolecular electrostatic interaction with an acidic group in the receptor (Hazum, 1987; Flanagan et al., 1994). The current results suggest that Arg⁸ both stabilizes peptide conformation and interacts with Asp⁷.₃₂(₃₀₂), and that Asp⁷.₃₂(₃₀₂) also affects peptide conformation. This, in turn, suggests that an interaction of Arg⁸ with Asp⁷.₃₂(₃₀₂) affects peptide conformation. The similar affinities of constrained peptides for the wild-type and mutant receptors (with and without Asp⁷.₃₂(₃₀₂)) suggest that once the ligand is in a high-affinity conformation, the putative Arg⁸-Asp⁷.₃₂(₃₀₂) interaction does not contribute to the binding energy of the final ligand-receptor complex. Although our results suggest that an interaction between Arg⁸ and Asp⁷.₃₂(₃₀₂) may be required to induce a high-affinity conformation in unconstrained, native GnRH, the absence of this interaction with constrained analogs suggests that the interaction that induces the high-affinity conformation is transient. It has been suggested that residues on the extracellular surface of the TRH receptor form a final ligand recognition site (Perlman et al., 1997) and that TRH binds sequentially with the surface binding site, and then with the transmembrane binding pocket (Colson et al., 1998). GnRH may interact initially with Asp⁷.₃₂(₃₀₂) and then move to a final binding pocket, which involves Asn².₆₅(₁₀₂) and Asp².₆₁(₁₉₈) (Davidson et al., 1996; Flanagan et al., 2000), after assuming a high affinity conformation. Thus, contrary to the initial hypothesis of an electrostatic interaction in the ligand-receptor complex, we show that the basis of receptor selectivity for mammalian GnRH seems to be the ability of Asp⁷.₃₂(₃₀₂) to induce a high-affinity conformation in native GnRH.

Cytosolic signaling assays showed that GnRH had decreased potency at the Asp⁷.₃₂(₃₀₂)Asn mutant receptor, consistent with its decreased affinity. However, the $E_{\text{max}}$ value for GnRH was lower in the mutant receptor, suggesting that the mutation destabilizes the activated receptor conformation (Samama et al., 1993). Several agonists, which showed no decrease in affinity for the mutant receptor, nevertheless showed decreased stimulation of IP production. This suggests that the Asp⁷.₃₂(₃₀₂) side chain has a role stabilizing the activated receptor conformation. The apparent decreased efficacy of ligands that had unchanged affinity suggests that this function is distinct from its role in ligand selectivity. A previous study reported that mutagenesis of extracellular loop three of the β₂-adrenergic receptor also affected receptor activation (Zhao et al., 1998).

In conclusion, the wild-type human GnRH receptor recognizes and binds ligands in a specific conformation that can be stabilized by ligand modifications. We show that the Asp⁷.₃₂(₃₀₂) side chain determines selectivity for Arg⁸-containing GnRH. However, certain ligand conformational constraints overcome the decrease in affinity that results from substitution of Arg⁸ and Asp⁷.₃₂(₃₀₂). This suggests that Asp⁷.₃₂(₃₀₂) determines selectivity for Arg⁸-containing GnRH by its ability to induce a high-affinity conformation in the ligand. We propose that unconstrained, Arg⁸-containing, native GnRH interacts transiently with Asp⁷.₃₂(₃₀₂), which induces a high affinity conformation in the ligand, before it interacts with a final ligand binding pocket, which excludes Asp⁷.₃₂(₃₀₂). This further definition of the mechanism of ligand recognition improves our conceptual models of GnRH receptor-ligand interaction.

Acknowledgments

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References


Chen Y and Probst WC (1997) Relationship between the bound agonist constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22(16):3099–3108.


Address correspondence to: Dr. Colleen A. Flanagan, Division of Medical Biochemistry, University of Cape Town Faculty of Health Sciences, Anzio Road, Observatory, 7925, South Africa. E-mail: flanagan@curie.uct.ac.za