Exploring deltorphinII binding to the third extracellular loop of the delta opioid receptor*

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The abbreviations used are: hDOR, human delta opioid receptor; ECLIII, third extracellular loop; TFE, trifluoroethanol; HFIP, hexafluoroisopropanol; SDS, sodium dodecyl sulfate; NMR,
nuclear magnetic resonance; CD, circular dichroism; HPLC, high performance liquid chromatography; TOCSY, total correlated spectroscopy; NOE, nuclear Overhauser enhancement
SUMMARY

The third extracellular loop of the human δ-opioid receptor (hDOR) is known to play an important role in the binding of δ↑ selective ligands. In particular mutation of three amino acids including Trp-284, Val-296 and Val-297 to alanine significantly diminished δ-opioid receptor affinity for δ-selective ligands. In order to assess the changes in conformation accompanying binding of the endogenous opioid peptide deltorphinII to the δ-opioid receptor at both the receptor and ligand level, as well determine points of contact between the two, an in depth spectroscopic study was initiated which addressed these points. Fragments of the δ-opioid receptor of variable length and containing residues in the third extracellular loop were synthesized and studied by NMR and CD spectroscopy in a membrane mimetic milieu. The receptor peptides examined included hDOR(279-299), hDOR(283-299), hDOR(281-297) and hDOR (283-297). A helical conformation was observed for the longest receptor fragment between Val-283 and Arg-291 whereas a nascent helix occurred in a similar region for hDOR(281-297). Further removal of N-terminal residues Val-281 and Ile-282 abolished helical conformation completely. Binding of the δ-selective ligand deltorphinII to hDOR(279-299) destabilized the helix at the receptor peptide N-terminus. Dramatic changes in the alpha proton chemical shifts of hDOR(279-299) residues Trp-284 and Leu-286 also accompanied this loss of helical conformation. Large up-field displacement of alpha proton chemical shifts were observed for residues Leu-295, Val-296 and Val-297 within hDOR(279-299) following its interaction with deltorphinII thus identifying a gain in β-conformation at the receptor peptide
C-terminus. Similar changes did not occur for the shorter peptides hDOR(283-299) and hDOR(283-297). A hypothesis describing the conformational events accompanying selective deltorphinII binding to the δ-opioid receptor is presented.
INTRODUCTION

Opioid receptors are widely recognized for their role in mediating pain. Recent cloning of the cDNAs encoding the 3 opioid receptor subtypes (κ, μ, δ) defines them as belonging to the superfamily of G protein coupled receptors. When comparing the different opioid receptor subtypes, considerable amino acid identity is found in the trans membrane and intracellular regions with a 60% receptor homology over all (1-4). Greater sequence diversity occurs in the extracellular loops and towards the amino- and carboxy- termini.

The δ-opioid receptor (DOR) represents a particularly attractive target for the development of pain therapeutics due to its known ability to mediate analgesia without inducing opiate physical dependence. Furthermore, δ-opioid receptor-selective drugs may possess potential clinical benefits over those currently targeted towards the μ-opioid receptor (5-8). These advantages include greater relief of neuropathic pain, reduced respiratory depression, and constipation, as well as a minimal potential for the development of physical dependence (9).

Deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂) originally isolated from frog skin represents the most δ-opioid receptor-selective ligand amongst the endogenous opioid peptides discovered so far (10). Structure activity relationship studies centered on the delorphins have revealed a number of key elements within the peptide sequence that are important for affinity and or selectivity (9). A negative charge on the Glu-4 side chain is critical for δ-receptor selectivity but not for receptor affinity (11,12) while the presence of hydrophic
residues at position Val-6 and Val-7 is required for both high affinity binding and δ-receptor selectivity (13,14,15). Removal of the Tyr-1 hydroxyl group results in a loss of δ-receptor affinity (16). High δ-receptor selectivity may also be attributed to a compact peptide conformation which is postulated as preferred by the δ-opioid receptor (17).

Evidence gathered from both site-directed mutagenesis and chimeric receptor studies, as well as subsequent molecular modeling studies have pointed to a number of residues located in the transmembrane helices and extracellular loops of hDOR that are potentially involved in the binding of both δ-agonists and antagonists (18-21). In particular, simultaneous mutation of three residues, Trp-284, Val-296 and Val-297 to alanine, in the third extracellular loop of hDOR significantly altered the normal binding of δ-selective ligands SNC-80, DPDPE, deltorphin II and naltrindole (19). Others studies have suggested that Leu-300 and Arg-291 as well as the hydrophobic nature of hDOR(295-300) are important for δ-selective binding. (21).

In this study, we have undertaken the structural characterization of the third extracellular loop of the human δ-opioid receptor (hDOR ECLIII) both alone in solution and in a complex with deltorphin II. The goal was to investigate changes occurring at both the receptor peptide and ligand level following formation of the complex that may explain events following deltorphin II binding to hDOR. Four peptides of different length, which contain the third extracellular loop and some of the transmembrane amino acids of hDOR, were synthesized. The conformations of these peptides in 88% HFIP/12% H2O were assessed using CD and NMR
spectroscopy. The results suggest that the interaction of deltorphin-II with hDOR ECLIII generally destabilizes a helix at the N-terminus of the extracellular loop and promotes a β-structure at its C-terminus.

EXPERIMENTAL PROCEDURES

The chemical structures of opioid receptor ligands used in this study are shown in Fig. 1. DeltorphinII and dynorphin A(1-13) were obtained from BACHEM (CA, USA). High purity endomorphin 1 and 2 were purchased from BACHEM Bioscience Inc (PA, USA). U50,488 and NaPi are from Sigma (MO, USA). TFE (trifluoroethanol) and HFIP (hexafluoropropanol) were obtained from Aldrich chemical Inc (ON, CN). Sodium dodecyl sulfate (SDS) was purchased from BioRad.

Peptide synthesis: Linear hDOR ECLIII peptides: hDOR(283-297), hDOR(283-299), hDOR(281-297) and hDOR(279-299) (Figure 2) were synthesized on solid support using a Symphony multiple peptide synthesizer and standard Fmoc chemistry procedures with HATU as coupling reagent. The polymeric starting material was CLEAR amide resin and TentaGel R RAM (Peptides International) with 0.4 mMol/g and 0.22 mMol/g of resin loading capacity, respectively. The crude peptides were cleaved from the resin by a mixture of TFA/triisopropylsilane/thioanisole/H2O (94:2:2:2) then isolated by ether precipitation. Peptides
were subsequently purified to homogeneity by preparative chromatography on a Hitachi HPLC instrument equipped with a Jupiter RP-4 column (250x22mm). The synthesized products were obtained in high yield with purity $\geq 96\%$ as determined by analytical HPLC on a Jupiter RP-18 and a Vydac 218TP54 column and by LC-MS. Electrospray mass spectrometry using a LCT mass spectrometer (micromass) provided the correct molecular weight for each peptide. All peptides were amidated at the C-terminus.

**Sample preparation:** Samples for CD spectroscopic studies were prepared by dissolving sufficient amount of peptide in 500 µl of one of the following solvent systems: TFE/H$_2$O (440 µl/60 µl); HFIP/H$_2$O (440 µl/60 µl); SDS in aqueous buffer (50 mM NaPi/SDS 200 mM; pH 6.0) to provide a peptide final concentration of 2 mM, unless otherwise stated. NMR samples contained 2 mM peptide in HFIP/H$_2$O (440 µl/60 µl). All non-aqueous reagents used for NMR experiments were fully deuterated. The solvents systems were chosen in order to provide a membrane mimetic environment and for maximizing peptide solubility.

For studies aimed at examining the interaction between opioid ligands and various hDOR ECLIII peptides a 2 mM sample of the chosen receptor peptide was first prepared in HFIP/H$_2$O as described above. The ligand was then added to a peptide concentration of 2 mM.

**Circular Dichroism Spectroscopy:** CD spectra were recorded on a Jasco J710 spectropolarimeter.
at room temperature. Ten scans were collected for each sample over a wavelength range of 180-260 nm, using a 0.2 nm resolution, a 1.0 nm bandwidth, a 100 nm/min. scan speed, and a 0.25 s response time. The CD cell path length employed for all experiments was 0.01 cm. The collected spectra were improved through background subtraction and smoothing and then converted to units of molar ellipticity per residue (deg cm² dmol⁻¹). Estimates of the helical fraction of the peptide were calculated by the method of Greenfield and Fasman (22) using the value of [θ] at 222 nm.

**NMR Spectroscopy:** All NMR spectra were acquired on a Bruker Avance-600 spectrometer at 300K. 3,3,3-trimethylsilylpropionate (TSP) was used as an internal reference (0.0ppm). ¹H signals for each peptide were assigned from analysis of the cross-peaks in TOCSY spectra acquired with a 50 ms mix times and from the sequential connectivities determined from NOESY spectra obtained with a mixing time of 200 ms. Two-dimensional TOCSY and NOESY experiments were carried out in the phase sensitive mode using the States-TPPI method. In cases where TFE/H₂O and HFIP/H₂O solvent mixtures were employed the solvent signals appeared as two broad peaks, one originating from the water protons and the other from the residual alcohol OH. Chemical exchange between the 2 labile proton sites resulted in similar solvent peak intensities. The best overall solvent suppression was thus achieved by presaturating one of the solvent peaks for a period of 1s prior to the first pulse of each fid acquisition.
The amide proton exchange rates in deltorphin II were measured by first incubating a 2mM solution of peptide in HFIP/D$_2$O (440µl/60µl) for 24 hours then lyophilizing the sample. The lyophilized sample was then resuspended in a mixture of HFIP/H$_2$O (440µl/60µl) and 2D TOCSY spectra acquired at 15 minute intervals immediately following dissolution. After 3 hours of incubation hDOR (279-299) was added to the HFIP/H$_2$O sample at 2mM concentration and the spectral monitoring continued.

A second amide exchange rate experiments involved first incubating the receptor peptide hDOR(281-297) at 2 mM concentration in HFIP/H$_2$O, then lyophilizing the sample. Samples were then dissolved in HFIP/D$_2$O (440µl/60µl). Disappearance of the amide proton signals was monitored through acquisition of 2D TOCSY spectra every 15 minutes following sample preparation. The experiment was repeated with deltorphinII also contained in the sample at 2 mM concentration.
RESULTS

CD Spectroscopy:

i) optimization of organic solvent conditions

A general study was performed in which the percentage of organic solvent (TFE or HFIP) in the alcohol/water solvent systems was varied and the solubility of the hDOR ECLIII peptides monitored. Fluorinated alcohol/water solvents systems have been widely employed for examining peptide conformations in a membrane like environment. As well, TFE and HFIP are structure promoting solvents that are particularly effective in stabilizing helical conformations without inducing intermolecular aggregation (23-25).

Optimal solubility conditions were noted when the fraction of organic solvent reached 88%. Sample stability over time was marginally better when HFIP was employed as the organic solvent. To ensure that the conformation of the hDOR ECLIII peptides were not affected by a change in peptide concentration CD spectra were obtained for the longest peptide hDOR(279-299) in HFIP/H2O (440 µl/60 µl) using four different peptide concentrations ranging from 2 mM to 0.1 mM. The CD spectra did not change over the entire concentration range studied. Therefore, the peptide was not aggregating at 2 mM. These controls were repeated for the remaining peptides leading to the same conclusions.

ii) Conformational analyses
To assess the overall conformational preference of hDOR ECLIII peptides CD spectra were first acquired for the longest peptide hDOR(279-299) in a number of solvent systems. As seen in Figure 3, the CD spectrum of this peptide in HFIP/H$_2$O contains a well-resolved shoulder near 222 nm, a negative peak at 208 nm, and a positive peak at 192 nm. These spectral properties are consistent with a partial helical peptide. Almost an identical spectrum was observed when the peptide was dissolved in TFE/H$_2$O (Figure 3). However, the percentage helicity marked by the absorbance at 222 nm is more pronounced in this case. In SDS, the shape of the CD curve was typical of a peptide with $\beta$-sheet structure and contained a broad band centered at 216 nm (Figure 3). The $\beta$ conformation likely reflects the presence of an aggregated peptide system in the detergent environment. Indeed support for this statement stems from the observation of a white precipitate in the micellar sample.

When CD spectra were acquired for the remaining hDOR peptides in HFIP/H$_2$O and compared to that obtained for hDOR(279-299) a number of similarities and differences were noted (Fig. 4). The Spectrum for the medium length peptide hDOR(281-297) displayed a partial $\alpha$-helical conformation as judged by the observation of two negative peaks at 222 nm and 202 nm and a positive peak at 190 nm (Fig. 4). The left shift of the negative band at 202 nm indicated a destabilization of the helix compared to that associated with hDOR(279-299). Deletion of the N-terminal Ile-279, Phe-280 motif and C-terminal Ala-298, Ala-299 motif within hDOR(279-299) thus had the effect of moderately destabilizing the peptide’s secondary structure.
spectra acquired for the remaining medium sized peptide hDOR(283-299) and smallest analog hDOR(283-297) suggested less helix stability was associated with these two molecules. This result implies that further removal of N-terminal residues Val-281 and Ile-282 abolishes a helical conformation. Perhaps there is no longer a critical number of amino acids available for establishment of 2 helical turns along the peptide backbone. However, some conformational preference for the pair was evident from the shoulder at 222 nm and a positive peak at 190 nm in their associated CD spectra (Fig.4).

iii) DOR peptides:ligand peptide complexes

Changes in the CD spectrum of hDOR(279-299) in HFIP/H2O produced by adding opioid receptor ligands (figure 1) were investigated. The CD spectra acquired for hDOR (279-299) in the presence of deltorphin II exhibited a reduced negative ellipticity at 222 nm and a shift in the absorption maximum from 208 to 205 nm compared to that obtained for the receptor peptide alone (Fig.5a). Interestingly adding an endogenous ligand of the µ-opioid receptor, endomorphin-(1 and 2) to a sample of hDOR(279-299) also resulted in a reduced negative ellipticity at 222 nm (Fig.5 b and c respectively). The addition of a selective κ-receptor peptide agonist, dynorphin A(1-13) , to a hDOR(279-299) actually increased very marginally the negative absorbance at 222 nm suggesting a helix stabilization occurred (Fig. 5 d). No change was observed in the hDOR(279-299) CD spectrum when a small
molecule kappa agonist U50,488, was added.

Addition of deltorphin II to samples of the other three hDOR ECLIII peptides in general did not change their CD spectra (data not shown). Only a small decrease was observed in the negative ellipticity of hDOR(281-279) at 202 and 222 nm when deltorphin II was added again reflecting a destabilization of helical conformation.

**NMR Spectroscopy:** More detailed conformational properties of the hDOR ECLIII peptides were examined by NMR spectroscopy. Initially a search for secondary structure was performed using the chemical shift analysis method developed by Wishart et al. (26). As stipulated by this method, observed pronounced upfield \( \alpha H \) chemical shift deviations (> 0.1 ppm) from random coil values within a stretch of four or more residues signals the presence of a helical structure. Conversely a stretch of three or more pronounced \( \alpha H \) downfield resonance shifts defines a region of \( \beta \)-structure. Analysis of the \( \alpha H \) chemical shifts for the longest loop peptide hDOR(279-299), indicated there is a helix in the region Val-283-Arg-291 (Fig. 6 a). No such feature was observed in any of the other 3 peptides according to the chemical shift data alone (Figs 6 b-d). However a short density of up field shifted alpha proton frequencies (> 0.1 ppm) was noted in the region Asp-289-Arg-291 in the case of hDOR(281-297) (Fig. 6 b). It is likely that a nascent helix populates this region of the peptide since the CD spectrum acquired for the same sample supports the presence of a weak helical structure. The main difference between hDOR(279-299) and hDOR(281-297) at the N-
terminal end is the absence of two trans membrane domain amino acids (Ile-279 and Phe-280). Therefore, a Ile-279-Phe-280 residue sequence may play an important role in stabilizing a N-terminal helix in the hDOR third extracellular loop.

A second noteworthy observation was the β conformation formed at the C-terminus of hDOR peptides hDOR(281-297) and hDOR(283-297) (Fig. 6 b and d). Supporting evidence for this assessment was obtained from the NOE results. Two i,i+2 NOEs including Asp-293 αH to Leu-295 NH and Pro-294 αH to Val-296 NH were observed only in the case of hDOR(281-297) and hDOR(283-297). These data suggest the presence of a C-terminal β-turn structure. Since the remaining 2 peptides hDOR(279-299) and hDOR (283-299) end with an Ala-298, Ala-299 motif it may be hypothesized that the presence of these 2 amino acids destabilizes the β-structure.

When the αH chemical shift analysis was repeated for the longest peptide hDOR(279-299) in the presence of deltorphinII considerable changes were noted. According to the αH chemical shift data a β-structure formed at the C-terminus, between Asp-293 and Val-297. Moreover, the binding of deltorphin-II destabilized the helix originally present in hDOR (279-299) (Fig. 7 a). Dramatic changes in the hDOR(279-299) αH chemical shifts occurred for five receptor amino acids, including Trp-284, Leu-286, Leu-295, Val-296 and Val-297.

Addition of deltorphin-II to a sample of hDOR(281-297) also resulted in an overall destabilization of helical conformation between residues Asp-288 and Arg-291. This is indicated by the shift in αH chemical shift indices to more positive values and above the 0.1
ppm helix cutoff for amino acids in this region (Fig. 7b). When deltorphinII was added to samples of hDOR(283-299) and hDOR(283-297) no changes in their respective αH chemical shifts occurred and hence peptide secondary structure was not affected in either case (data not shown).

When the focus of NMR analysis was shifted from the receptor peptides to the ligand deltorphinII, some interesting observations were made. A sample of deltorphinII was first incubated in HFIP/D$_2$O to allow a NH to ND exchange amongst the amide protons. The sample was then freeze-dried and resuspended in HFIP/H$_2$O. The degree to which each amide proton was shielded from the aqueous solvent was determined by examining the rate of reappearance of the NH signals in TOCSY spectra over time. Within a short period of time (15 mins.) after dissolving the peptide all the NH signals reappeared with reasonable intensity except for the amide proton signal associated with Glu-4. Absence of a Glu-4 NH signal was still apparent three hours following dissolution of the peptide in the HFIP/H$_2$O milieu (Fig. 8a). This result suggests that the amide proton of Glu-4 is engaged in a hydrogen bond or is otherwise inaccessible to the surrounding water molecules. When the receptor peptide hDOR(279-299) was added to the same sample at the three hour time point the Glu-4 NH signal intensified to the same level as those of the other amide protons. Disruption of the shielding process around this amide proton therefore occurred as a result of a deltoprhinII-receptor peptide interaction.

The complex formed between deltorphinII and the hDOR ECLIII surrogate was
examined in more detail. hDOR(281-297) was chosen over hDOR(279-299) for these experiments since it was easier to work with in terms of sample stability. Furthermore, hDOR(281-297) exhibited similar changes in its CD spectrum following an encounter with deltorphinII as the longer hDOR ECLIII peptide. A sample of hDOR(281-297) was first lyophilized from HFIP/H₂O (440µl/60µl) then resuspended in HFIP/D₂O at the same solvent ratio. After 1.5 hours all of the amide proton signals had disappeared except for those belonging to Val-287 and Val-296 (Fig. 9). When the experiment was repeated this time with both hDOR(281-297) and deltorphinII contained in the sample a number of receptor peptide amide protons exhibited slow NH to ND exchange. In particular the amide proton signals associated with Thr-285, Leu-286, Val-287, Leu-295, Val-296 and Val-297 were still visible 1.5 hours following sample preparation. In the case of deltorphinII only the Gly-7 amide proton exhibited slow exchange (Fig. 9).

**DISCUSSION**

In the present study, the conformations of four synthetic peptides representing the third extracellular loop of the human δ-opioid receptor were examined in solution both alone and in the presence of opioid receptor selective ligands. Each receptor mimic peptide involved in the study contained the entire extracellular portion of hDOR ECLIII and some of the hydrophobic trans-membrane amino acid. The purpose of this study was first to determine the propensity of
hDOR ECLIII to form secondary structures and second to gain insight into the mechanism of complex formation between hDOR and deltorphinII. The study focused on three critical hDOR ECLIII amino acids Trp-284, Val-296 and Val-297 as previously determined through molecular biology experiments (19).

The results presented here suggest that the longest hDOR ECLIII peptide (hDOR(279-299)) adopts a well-defined helical conformation within the N-terminal portion of its amino acid sequence. Elimination of the Ile-279-Phe-280 motif from the N-terminus of hDOR(279-299) destabilized the helical conformation. It is thus conceivable that an aromatic or hydrophobic interaction between the side chains of Phe-280 and Trp-284 is required for stabilizing the helix whose start point is Val-283 according to alpha proton chemical shift data (Fig. 6). Residues Phe-280 and Trp-284 are separated by three amino acids and hence their respective side chains are appropriately positioned for interaction assuming a helical conformation exists. A side-chain hydrophobic interaction is more likely the case however, since mutating Phe-280 to an alanine in hDOR did not appreciably affect the binding of δ-opioid receptor ligands (19). Interestingly a complete abolishment of helical conformation was evident following further removal of four additional N-terminal amino acids within hDOR(279-299). This result suggests that the shortened peptide hDOR(283-299) no longer has the critical number of amino acids required for promoting an N-terminal helical fold.

The interaction of deltorphin II with the longest hDOR peptide hDOR(279-299) resulted in a destabilization of the helix (Fig 6,7a). Dramatic displacement of the αH chemical shift for
residues Trp-284 and Leu-286 accompanied this change indicating an interaction between the receptor loop peptide and deltorphinII in the vicinity of Trp-284 (Fig. 6,7 a). It has been demonstrated through mutagenesis experiments that Trp-284 plays a major role in the hDOR selective binding of opioid ligands (19). Previous structure activity relationship investigations have also provided evidence indicating that the size and hydrophobic nature of Phe-3 in addition to the negative charge associated with Glu-4 of deltorphinII are critical determinants for hDOR binding. In particular the acidic function associated with Glu-4 is considered a discriminating factor for δ-opioid receptor selectivity (11,12, 27-31). In comparing the amino acid sequences in the vicinity of ECLIII for both the δ↑ and µ↑opioid receptors one notices two positively charged residues in the hDOR sequence (Arg-291 and Arg-292) whereas the µ opioid receptor is void of any acidic residues in this region. An intensive site directed mutagenises study of hDOR also identified Arg-291 as being an important residue for the binding of deltorphinII suggesting that the Glu-4 side-chain of deltorphin II may interact with Arg-291 of the receptor (19). The closest distance of approach between the side-chains of Trp-284 and Arg-291 measured from a crude helical model of hDOR(279-291) (data not shown) is 4.7 Å. It can be hypothesized then that deltorphinII residues Phe-3 and Glu-4 interact respectively with hDOR Trp-284 and Arg-291. The loss of a helical conformation may arise from the disruption of helix stabilizing forces including possibly interruption of a Phe-280-Trp284 interaction, resulting from the ligand receptor interaction. Further support for this hypothesis stems from the results of amide proton exchange experiments. When a ligand receptor interaction was allowed to occur,
the amide protons of Thr-285, Leu-286 and Val-287 exhibited a slow exchange with its surrounding aqueous milieu. By contrast these same protons exchanged rather quickly with deuterium when deltorphinII was absent. This data suggest the amide protons close to Trp-284 become more shielded from the solvent in the presence of deltorphinII presumably due to an interaction between deltorphinII and hDOR(281-297) around Trp-284 of the receptor peptide. It should be pointed out that the amide protons of the receptor peptide alone should also be well-shielded from the solvent and exhibit a slow NH to ND exchange if a very stable helix is present. The fact that fast exchange was observed for hDOR(281-297) amide protons in this case reflects the nascent helix determined for this particular receptor peptide.

From the ligand point of view, some changes were noted around Glu-4 following a ligand receptor-peptide interaction. A weak Glu-4 amide proton signal intensity was consistently found in TOCSY spectra acquired over time for deltorphin II in solution (Fig. 8a). This data reflected a slow ND to NH exchange of the Glu-4 amide proton. The same amide proton signal was greatly intensified when hDOR(281-297) was added to the sample (Fig. 8b). This result demonstrates a deshielding of the deltorphinII Glu-4 amide proton when encountering the hDOR peptide. A likely explanation for this event is the breaking of an internal hydrogen bond in deltorphinII involving the Glu-4 NH. Indeed, some groups have reported deltorphinII adopting a U conformation that results in the N and C-termini of the peptide approaching one another (32,33). In particular a reverse turn at Val-5 has been proposed through NMR and modeling experiments (17). A hydrogen bond involving the Glu-4 NH would
stabilize this type of turn (34). Binding to hDOR could thus open up the deltorphinII U shaped structure and lead to a breaking of the hydrogen bond.

Shifting focus to the C-terminal end of hDOR ECLIII one notices a number experimental pieces of evidence, which explain events occurring in this region of the receptor following ligand binding. First of all, a β-structure at the C-terminus of hDOR ECLIII peptides was observed when the peptide sequence ends in Val-297 but was absent when Ala-298, Ala-299 were included. The NOEs observed between Asp-293 and Leu-295 and between Pro-294 and Val-296 for peptides hDOR(281-297) and hDOR(283-297) suggest formation of the β-structure is the result of a reverse turn centered on Pro-294 (35). A Chou-Fasman protein conformational profile plot was made for different variants of the C-terminal sequence after and including Arg-292 using the program Peptide Companion (CoshiSoft/PeptiSearch V. 1.25). A good propensity to adopt either a helical or β conformation was noted post Pro-294 when Ala-298 and Ala-299 were present whereas only a β-structure was calculated for this domain when the 2 alanines were removed (data not shown). Lengthening the C-terminus with two additional alanines thus potentially allows for competing C-terminal secondary conformations that prevent a stable turn at Pro-294. This would explain the results observed for the receptor peptides presented here.

Regarding the intact receptor it has been demonstrated that a hydrophobic segment from Leu295-Leu-300 is an important element for δ-selective ligand binding (21). Much supporting information for this was obtained from the results presented here. According to alpha proton chemical shift measurements hDOR(279-299) gained a C-terminal β-conformation (Fig. 7a)
upon complexation with deltorphinII. In particular the alpha proton chemical shifts of the
important residues Val-296 and Val-297 moved significantly up field as a result of
deltorphionII binding (Fig. 6,7 a). A slow amide proton exchange with the surrounding water
was also observed for hDOR(281-297) residues Leu-295, Val-296 and Val-297 when
deltorphinII was present whereas only the NH of Val-296 appeared shielded from the aqueous
environment in the absence of deltorphinII (Fig. 9). Likewise the amide proton of Gly-7 in
deltophinII was protected from the solvent in the presence of hDOR(281-297) (Fig. 9). One can
conclude from these data that there is some hydrophobic interaction between Leu-295, Val-296
and Val-297 of hDOR(281-297) and the C-terminal address domain of deltorphinII. Indeed,
many groups have reported that the hydrophobicity associated with residues Val-5 and Val-6
is critical for δ-opioid receptor binding and selectivity (13-15). Therefore it is possible that
Val-5 and Val-6 of deltophinII interact with the Leu-295-Val-297 segment of hDOR. Support
for this hypothesis stems from the observed dramatic displacement of alpha proton chemical
shifts for Val-296 and Val-297 when deltorphinII was added to a sample of hDOR(279-299)
(Fig. 6,7 a).

When a Chou-Fasman calculation was repeated for the hDOR segment Arg-292 to Ala-
299 with alanine residues replacing the two valines a helix appeared as the only possible
conformation available to the Leu-295-Ala-296-Ala-297-Ala-298-Ala-299 sequence (data
not shown). Mutating Val-296 or Val-297 to alanine experimentally also abolished deltorphinII
binding thus suggesting that a helical conformation in the important hDOR hydrophobic domain
(Leu-295 to Leu-300) is unfavorable for deltorphinII binding. All of the evidence taken together would indicate that a turn centered on Pro-294 may form following deltorphinII binding to hDOR Leu-295-Leu-300 in an extended conformation.

Loss of helical conformation unexpectedly also occurred for hDOR(279-299) following the addition of μ-selective peptides endomorphin-1,2. The residue sequences for these 2 peptides contain a Tyr-Xaa-Phe (or Tyr-Xaa-Trp in the case of endomorphin-1) motif commonly associated with the message component of opioid ligands (28). It is therefore possible that the endomorphins bind weakly to the δ-opioid receptor at levels undetectable in conventional binding assays in the vicinity of Trp-284. Yet, these peptides possibly lack the additional molecular determinants that form the address domain of δ-selective ligands. When the selective kappa peptide agonist dynorphin A(1-3) was allowed to interact with the hDOR ECLIII peptide a very small stabilization in helix was noted. Dynorphin A(1-13) does not contain a N-terminal Tyr- Xaa-Phe motif as the other peptides studied but rather a Tyr-Gly-Gly-Phe sequence. Based on the CD data it may be suggested that dynorphin A(1-13) interacts very weakly with the third extracellular loop of hDOR but in a manner distinct from that of deltorphin II due to its additional Gly spacer in the message domain.

The small molecule κ agonist U50,488 did not elicit a conformational change in the hDOR peptide. This is expected considering the small number of functional groups in the molecule and its established lack of binding to hDOR.

In conclusion one may hypothesize conformational adjustments, which accompany
delta-endorphin binding to the third extracellular loop of the human \( \delta \)-opioid receptor. The spectroscopic evidence presented here suggests that Phe-3 and Glu-4 of delta-endorphin interact with Trp-284 and Arg-291 respectively of the \( \delta \)-opioid receptor leading to a destabilization of helical structure at the N-terminal end of the receptor third extracellular loop. Coincidently, important residues Val-5 and Val-6 in the address domain of delta-endorphin interact with Val-296 and Val-297 at the C-terminal end of the hDOR third extracellular loop in an extended conformation and possibly induce a turn centered on Pro-294.
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FIGURE LEGENDS

FIG. 1. Chemical structures of opioid receptor ligands used in this study. DeltorphinII is a selective delta opioid receptor agonist while the andomorphins are selective for the mu opioid receptor. Dynorphin A(1-13) and U50,488 are kappa opioid receptor selective agonists.

FIG. 2. Linear hDOR ECLIII peptides examined in the present study. All peptides were synthesized on solid support using standard Fmoc chemistry procedures with HATU as coupling reagent. Peptides were subsequently purified to homogeneity by preparative chromatography. Final purities of all peptides were >95%.

FIG. 3. Circular Dichroism spectra acquired for hDOR(279-299) in three different solvent systems including HFIP/H₂O (440 μl/60 μl), TFE/H₂O (440μl,60μl) and 200 mM SDS in aqueous buffer (50 mM NaPi pH 6.0). Spectra were acquired with a cell path length of 0.01 cm and a wavelength range of 180-260 nm. Ten scans were collected for each sample.

FIG. 4. Circular dichroism spectra acquired for hDOR ECLIII peptides hDOR(283-297), hDOR(283-299), hDOR(281-297) and hDOR(279-299) in HFIP/H₂O (440 μl/60 μl) at 2 mM concentration. Ten scans were collected for each sample over a wavelength range of 180-260 nm. The employed cell had a 0.01 cm path length.
FIG. 5. Circular dichroism spectra of hDOR(279-299) in presence of a) delta selective peptide deltorphin II b) mu selective peptides endomorphin-1, and c) endomorphin-2 and d) kappa opioid receptor agonist dynorphin A(1-13). The solid line in each figure corresponds to hDOR(279-299) alone in solution. Ten scans were collected for each sample over a wavelength range of 180-260 nm.

FIG. 6. Alpha proton chemical shift indices determined for hDOR ECLIII peptides in HFIP:H₂O (440 µl/60 µl) according to the method of D.S. Wishart et al Biochemistry 1992: a) hDOR (279-299) b) hDOR(281-297) c) hDOR(283-299) and d) hDOR (283-297). A stretch of residues where index values are more negative than 0.1 ppm for at least 4 residues and containing no points with values > 0.1 ppm, defines a helix region whereas a similar series of index points > 0.1 ppm uninterrupted by a -0.1 ppm point indicates a beta conformation. Residue numbers and chemical shift indices (δ ppm) are plotted on the x and y axes respectively.

FIG. 7. Alpha proton chemical shift indices for hDOR ECLIII peptide in HFIP/H₂O following addition of deltorphin II: a) hDOR(279-299) b) hDOR(281-297). All peptides are at 2 mM concentration. Residue numbers and chemical shift indices (δ ppm) are plotted on the x and y axes respectively.
FIG 8. TOCSY spectra acquired for deltorphinII in HFIP:H₂O (440 µl/60 µl) at 2 mM concentration a) 3 hours after sample preparation alone in solution b) immediately following addition of 2 mM hDOR(279-299) at the 3 hour deltorphinII incubation time point. Intensity of the backbone amide proton cross-peaks were monitored.

Fig 9: Portion of the TOCSY spectrum acquired for a 2 mM hDOR(281-297) sample containing deltorphinII in HFIP/H₂O (440 µl/60 µl). Residues with visible amide protons signals in the TOCSY spectrum 1.5 hours after sample preparation are indicated at the right hand side of the figure in bold face type.
Fig. 1

Tyr-Pro-Trp-Phe-NH₂
endomorphin-1

Tyr-Pro-Phe-Phe-NH₂
endomorphin-2

U50,488

H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂
deltorphin II

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH
dynorphin A (1-13)
Fig. 2

hDOR(283-297) V-W-T-LV-D-I-D-R-R-D-P-L-V-V-NH₂
hDOR(281-297) V-I-V-W-T-L-V-D-I-D-R-R-D-P-L-V-V-NH₂
Fig. 4

[Diagram showing experimental data for hDOR(279-299), hDOR(281-297), hDOR(283-299), and hDOR(283-297).]

Wavelength [nm]

[θ] (Deg cm² dmol⁻¹)

20000

-20000

180

260