Exploring Deltorphin II Binding to the Third Extracellular Loop of the δ-Opioid Receptor*

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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 (Trp284, Val296, and Val297) to alanine significantly diminished δ-opioid receptor affinity for δ-selective ligands. To assess the changes in conformation accompanying binding of the endogenous opioid peptide deltorphin II to the δ-opioid receptor at both the receptor and ligand levels as well as to determine points of contact between the two, an in-depth spectroscopic study that addressed these points was initiated. 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 Val283 and Arg291, whereas a nascent helix occurred in a similar region for hDOR-(281–297). Further removal of N-terminal residues Val281 and Ile282 abolished helical conformation completely. Binding of the δ-selective ligand deltorphin II to hDOR-(279–299) destabilized the helix at the receptor peptide N terminus. Dramatic changes in the α-proton chemical shifts for Trp284 and Leu296 in hDOR-(279–299) also accompanied this loss of helical conformation. Large upfield displacement of α-proton chemical shifts was observed for Leu286, Val286, and Val297 in hDOR-(279–299) following its interaction with deltorphin II, thus identifying a gain in β-conformation at the receptor peptide C terminus. Similar changes did not occur for the shorter peptide hDOR(281–297). A hypothesis describing the conformational events accompanying selective deltorphin II binding to the δ-opioid receptor is presented.

Opioid receptors are widely recognized for their role in mediating pain. Recent cloning of the cDNAs encoding the three opioid receptor subtypes (κ, μ, and δ) defined them as belonging to the superfamily of G protein-coupled receptors. Upon comparison of the different opioid receptor subtypes, considerable amino acid identity has been found in the transmembrane and intracellular regions, with a 60% receptor homology overall (1–4). Greater sequence diversity occurs in the extracellular loops and toward the N and C termini. The δ-opioid receptor represents a particularly attractive target for the development of pain therapeutics because of its known ability to mediate analgesia without inducing opiate physical dependence. Furthermore, δ-selective drugs may possess potential clinical benefits over those currently targeted toward the μ-opioid receptor (5–8). These advantages include greater relief of neuropathic pain and reduced respiratory depression and constipation as well as a minimal potential for the development of physical dependence (9).

Deltorphin II (Tyr-δ-Ala-Phe-Glu-Val-Gly-NH₂), originally isolated from frog skin, represents the most δ-selective ligand among the endogenous opioid peptides discovered so far (10). Structure-activity relationship studies centered on the deltorphins have revealed a number of key elements within the peptide sequence that are important for affinity and selectivity (9). A negative charge on the Glu1 side chain is critical for δ-receptor selectivity, but not for receptor affinity (11, 12), whereas the presence of hydrophobic residues at Val6 and Val7 is required for both high affinity binding and δ-receptor selectivity (13–15). Removal of the Tyr4 hydroxyl group results in 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 from subsequent molecular modeling studies have pointed to a number of residues located in the transmembrane helices and extracellular loops of human δ-opioid receptor (hDOR) that are potentially involved in the binding of both δ-selective agonists and antagonists (18–21). In particular, simultaneous mutation of three residues (Trp284, Val296, and Val297) to alanine in the third extracellular loop of hDOR significantly alters the normal binding of the δ-selective ligands SNC-80, Tyr-δ-Pen-Gly-Phe-n-δ-Pen-OH (DPDPE), deltorphin II, and naltrindole (19). Others studies have suggested that Leu286 and Arg291 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 (ECLIII) of hDOR 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 levels following formation of the complex that may explain events following deltorphin II binding to hDOR. Four peptides of different length containing the ECLIII and some of the transmembrane amino acids of hDOR were syn-

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The abbreviations used are: hDOR, human δ-opioid receptor; ECLIII, third extracellular loop; HFIP, hexafluoroisopropl alcohol; TFE, trifluoroethanol; Fmoc, N-(9-fluorenyl)methoxycarbonyl; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; HATU, N-(hydroxyaminomethyl)hexafluorophosphate N-oxide.
were cleaved from the resin by trifluoroacetic acid/triisopropylsilane/0.22 mmol/g resin loading capacity, respectively. The crude peptides resin and TentaGel R RAM (Peptides International Inc.) with 0.4 and synthesizer and standard Fmoc chemistry procedures with HATU as were synthesized on solid support using a Symphony multiple peptide

and 60 NMR samples contained 2 mM peptide in 440 l of HFIP/H2O. All non-aqueous reagents used for NMR experiments were fully tropolarized a helix at the N terminus of the extracellular loop and promotes a β-structure at its C terminus.

EXPERIMENTAL PROCEDURES

Materials—The chemical structures of the opioid receptor ligands used in this study are shown in Fig. 1. Deltorphin II and dynorphin A(1–13) were obtained from Bachem California. High purity endomorphin-1 and -2 were purchased from Bachem Bioscience Inc. U-50488 was from Sigma. Trifluoroethanol (TFE) and HFIP were obtained from Aldrich. SDS was purchased from Bio-Rad. Materials—The linear hDOR ECLIII peptides hDOR-(283–297), hDOR-(283–299), hDOR-(291–297), and hDOR-(279–299) (Fig. 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 Inc.) with 0.4 and 0.22 mmol/g resin loading capacity, respectively. The crude peptides were cleaved from the resin by trifluoroacetic acid/triisopropanolamine/thioanisole/H2O (94:2:2:2) and 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 (200 × 22 mm). The synthesized products were obtained in high yield with purity > 98% as determined by analytical high performance liquid chromatography on a Jupiter RP-18 column and a Vydac 218TP54 column and by liquid chromatography-mass spectrometry. Electrospray mass spectrometry using a Micromass LCT mass spectrometer provided the correct molecular weight for each peptide. All peptides were amamidated at the C terminus.

Sample Preparation—Samples for CD spectroscopic studies were prepared by dissolving a sufficient amount of peptide in 500 μl of one of three solvent systems (440 μl of TFE and 60 μl of H2O, 440 μl of HFIP and 60 μl of H2O, and 200 μl of H2O) to provide a final peptide concentration of 2 mM, unless otherwise stated. NMR samples contained 2 mM peptide in 440 μl of HFIP and 60 μl of H2O. All non-aqueous reagents used for NMR experiments were fully deuterated. The solvents systems were chosen to provide a membrane-mimetic environment and to maximize 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/H2O as described above. The ligand was then added to a peptide concentration of 2 mM.

CD 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 180–280 nm under the following conditions: 0.2-nm resolution, 1.0-nm bandwidth, 100-nm/min scan speed, 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/residue (degrees cm2 dmol−1). Estimates of the helical fraction of the peptide were calculated by the method of Greenfield and Fasman (22) using a value of [θ] at 222 nm.

NMR Spectroscopy—All NMR spectra were acquired on a Bruker Avance-600 spectrometer at 300 K. 3,3,3-Trimethylsilyl propionate was used as an internal reference (0.0 ppm). 1H signals for each peptide were assigned from analysis of the cross-peaks in total correlation spectroscopy (TOCSY) spectra acquired with a 50-ms mixing time and from the sequential connectivities determined from nuclear Overhauser effect (NOE) correlation spectroscopy spectra obtained with a mixing time of 200 ms. Two-dimensional TOCSY and NOE correlation spectroscopy experiments were carried out in the phase-sensitive mode using the States-time-proportional phase incrementation (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 two 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 1 s prior to the first pulse of each free induction decay acquisition.

The amide proton exchange rates in deltorphin II were measured by first incubating a 2 mM solution of peptide in 440 μl of HFIP and 60 μl of D2O for 24 h and then lyophilizing the sample. The lyophilized sample was resuspended in a mixture of 440 μl of HFIP and 60 μl of H2O, and two-dimensional TOCSY spectra were acquired at 15-min intervals immediately following dissolution. After 3 h of incubation, hDOR-(279–299) was added to the HFIP/H2O sample at 2 mM, and spectral monitoring was continued.

A second amide exchange rate experiment involved first incubating the receptor peptide hDOR-(283–297) at 2 mM in HFIP/H2O and then lyophilizing the sample. Samples were dissolved in 440 μl of HFIP and 60 μl of D2O. Disappearance of the amide proton signals was monitored through acquisition of two-dimensional TOCSY spectra every 15 min following sample preparation. The experiment was repeated with deltorphin II also contained in the sample at 2 mM.

RESULTS

CD Spectroscopy

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 was monitored.

Fig. 1. Chemical structures of the opioid receptor ligands used in this study. Deltorphin II is a δ-selective agonist, whereas the endomorphins are selective for the μ-opioid receptor. Dynorphin A(1–13) and U-50488 are κ-selective agonists.
Fluorinated alcohol/water solvents systems have been widely employed for examining peptide conformations in a membrane-like environment. In addition, 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 440 μl of HFIP and 60 μl of H2O using four different peptide concentrations ranging from 2 to 0.1 mM. The CD spectra did not change over the entire concentration range studied. Therefore, the peptide did not aggregate at 2 μM. These controls were repeated for the remaining peptides, leading to the same conclusions.

Conformational Analyses—To assess the overall conformational preference of the hDOR ECLIII peptides, CD spectra were first acquired for the longest peptide, hDOR-(279–299), in a number of solvent systems. As shown in Fig. 3, the CD spectrum of this peptide in HFIP/H2O contained 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/H2O (Fig. 3). However, the percentage helicity marked by the absorbance at 222 nm was more pronounced in this case. In SDS, the shape of the CD curve was typical of a peptide with a β-sheet structure and contained a broad band centered at 216 nm (Fig. 3). The β-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/H2O and compared with 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 α-helical conformation as judged by the observation of two negative peaks at 222 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 with that associated with hDOR-(279–299). Deletion of the N-terminal Ile279-Phe280 motif and the C-terminal Ala298-Ala299 motif in hDOR-(279–299) thus had the effect of moderately destabilizing the secondary structure of the peptide. CD spectra acquired for the remaining medium length peptide, hDOR-(283–299), and smallest analog, hDOR-(283–297), suggested that less helix stability was associated with these two molecules. This result implies that further removal of N-terminal residues Val281 and Ile282 abolishes the helical conformation. Perhaps there is no longer a critical number of amino acids available for establishment of two helical turns along the peptide backbone. However, some conformational preference for the pair was evident from the shoulder at 222 nm and the positive peak at 190 nm in their associated CD spectra (Fig. 4).

α-Opioid Receptor Peptide-Ligand Complexes—Changes in the CD spectrum of hDOR-(279–299) in HFIP/H2O produced by adding opioid receptor ligands (Fig. 1) were investigated. The CD spectrum 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 with that obtained for the receptor peptide alone (Fig. 5a). Interestingly, adding endomorphin-1 and -2 (endogenous ligands of the µ-opioid receptor) to a sample of hDOR-(279–299) also resulted in reduced negative ellipticity at 222 nm (Fig. 5b and c, respectively). Addition of a κ-selective peptide agonist, dynorphin A-(1–13), to a hDOR-(279–299) sample actually increased very marginally the negative absorbance at 222 nm (Fig. 5d), suggesting that helix stabilization occurred. No change was observed in the hDOR-(279–299) CD spectrum when a small molecule κ-selective agonist (U-50488) 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-(281–297) at 202 and 222 nm when deltorphin II was added, again reflecting destabilization of the 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 chem-
ical shift analysis method developed by Wishart et al. (26). As stipulated by this method, observed pronounced upfield chemical shift deviations (>0.1 ppm) from random coil values within a stretch of four or more residues signal the presence of a helical structure. Conversely, a stretch of three or more pronounced downfield resonance shifts defines a region of structure. Analysis of the chemical shifts for the longest loop peptide, hDOR-(279–299), indicated that there is a helix in the Val283–Arg291 region (Fig. 6a). No such feature was observed in any of the other three peptides according to the chemical shift data alone (Fig. 6, b–d). However, a short density of upfield-shifted proton frequencies (>0.1 ppm) was noted in the Asp289–Arg291 region in the case of hDOR-(281–297) (Fig. 6b). It is likely that a nascent helix populates this region of the peptide because 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 transmembrane domain amino acids (Ile279 and Phe280). Therefore, an Ile279-Phe280 sequence may play an important role in stabilizing an N-terminal helix in the hDOR ECLIII. An additional noteworthy observation was the β-conformation formed at the C terminus of 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 Asp288 α-H to Leu295 NH and Pro294 α-H to Val296 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. Because the remaining two peptides, hDOR-(279–299) and hDOR-(283–299), end with an Ala298-Ala299 motif, it may be hypothesized that the presence of these two amino acids destabilizes the β-structure.

When the α-H chemical shift analysis was repeated for the longest peptide, hDOR-(279–299), in the presence of deltorphin II, considerable changes were noted. According to the α-H chemical shift data, a β-structure formed at the C terminus between Asp293 and Val297. Moreover, the binding of deltorphin II destabilized the helix originally present in hDOR-(279–299) (Fig. 7a). Dramatic changes in the hDOR-(279–299) α-H chemical shifts occurred for five receptor amino acids: Trp284, Leu286, Leu295, Val296, and Val297.

Addition of deltorphin II to a sample of hDOR-(281–297) also resulted in an overall destabilization of the helical conformation between Asp288 and Arg291. This is indicated by the shift in α-H chemical shift indices to more positive values and above the 0.1-ppm helix cutoff for amino acids in this region (Fig. 7b). When deltorphin II was added to samples of hDOR-(283–299) and hDOR-(283–297), no changes in their respective α-H 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 deltorphin II, some interesting observations were made. A sample of deltorphin II was first incubated in HFIP/D2O to allow an NH-to-ND exchange among the amide protons. The sample was then freeze-dried and resuspended in HFIP/H2O. 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 min) after dissolving the peptide, all the NH signals reappeared with reasonable intensity, except for the amide proton signal associated with Glu4. Absence of a Glu4 NH signal was still apparent 3 h following dissolution of the peptide in the HFIP/H2O milieu (Fig. 8a). This result suggests that the amide proton of Glu4 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 3-h time point, the Glu4 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 deltorphin II-receptor peptide interaction.

The complex formed between deltorphin II and the hDOR ECLIII surrogate was examined in more detail. hDOR-(281–297) was chosen over hDOR-(279–299) for these experiments because it was easier to work with in terms of sample stability.

FIG. 6. α-Proton chemical shift indices determined for hDOR ECLIII peptides in 440 μl of HFIP and 60 μl of H2O according to the method of Wishart et al. (26). a, hDOR-(279–299); b, hDOR-(281–297); c, hDOR-(283–299); d, hDOR-(283–297). A stretch of residues where index values are more negative than −0.1 ppm for at least four 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 β-conformation. Residue numbers and chemical shift indices (δ ppm) are plotted on the x and y axes, respectively.
Furthermore, hDOR-(281–297) exhibited similar changes in its CD spectrum following an encounter with deltorphin II compared with the longer hDOR ECLIII peptide. A sample of hDOR-(281–297) was first lyophilized from 440 μl of HFIP and 60 μl of H2O and then resuspended in HFIP/D2O at the same solvent ratio. After 1.5 h, all of the amide proton signals had disappeared, except for those belonging to Val287 and Val296 (Fig. 9). When the experiment was repeated with both hDOR-(281–297) and deltorphin II 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 Thr285, Leu286, Val287, Leu295, Val296, and Val297 were still

**Fig. 7.** α-Proton chemical shift indices for hDOR ECLIII peptides in HFIP/H2O following addition of deltorphin II. a, hDOR-(279–299); b, hDOR-(281–297). All peptides are at 2 mM. Residue numbers and chemical shift indices (δ ppm) are plotted on the x and y axes, respectively.

**Fig. 8.** TOCSY spectra acquired for deltorphin II at 2 mM in 440 μl of HFIP and 60 μl of H2O. a, 3 h after sample preparation alone in solution; b, immediately following addition of 2 mM hDOR-(279–299) at the 3-h deltorphin II incubation time point. The intensities of the backbone amide proton cross-peaks were monitored.
visible 1.5 h following sample preparation. In the case of deltorphin II, only the Gly7 amide proton exhibited slow exchange (Fig. 9).

**DISCUSSION**

In this study, the conformations of four synthetic peptides representing the hDOR ECLIII were examined in solution both alone and in the presence of opioid receptor-selective ligands. Each receptor-mimetic peptide involved in this study contained the entire extracellular portion of the hDOR ECLIII and some of the hydrophobic transmembrane amino acids. The purpose of this study was first to determine the propensity of the hDOR ECLIII to form secondary structures and second to gain insight into the mechanism of complex formation between hDOR and deltorphin II. This work focused on three critical hDOR ECLIII amino acids (Trp284, Val296, and Val297) 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 Ile279-Phe280 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 Phe280 and Trp284 is required for stabilizing the helix whose starting point is Val283 (281–297) sample (2 mM) containing deltorphin II in 440 μL of H2O. Residues with visible amide protons signals in the TOCSY spectrum 1.5 h after sample preparation are indicated to the right in boldface.

![Figure 9](https://example.com/f9.png)

*Figure 9. Portion of the TOCSY spectrum acquired for a hDOR-(281–297) sample (2 mM) containing deltorphin II in 440 μL of H2O. Residues with visible amide protons signals in the TOCSY spectrum 1.5 h after sample preparation are indicated to the right in boldface.*

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 Phe2 in addition to the negative charge associated with Glu4 of deltorphin II are critical determinants for hDOR binding. In particular, the acidic function associated with Glu4 is considered a discriminating factor for δ-opioid receptor selectivity (11, 12, 27–31). Upon comparison of the amino acid sequences in the vicinity of the ECLIII for both the δ- and µ-opioid receptors, one notes two positively charged residues in the hDOR sequence (Arg291 and Arg292), whereas the µ-opioid receptor is void of any acidic residues in this region. An intensive site-directed mutagenesis study of hDOR also identified Arg291 as an important residue for the binding of deltorphin II, suggesting that the Glu4 side chain of deltorphin II may interact with Arg291 of the receptor (19). The closest distance of approach between the side chains of Trp284 and Arg291 measured from a crude helical model of hDOR-(279–299) is 4.7 Å (data not shown). It can be hypothesized then that deltorphin II Phe2 and Glu4 interact with hDOR Trp284 and Arg291, respectively. The loss of a helical conformation may arise from the disruption of helix-stabilizing forces, including possibly interruption of a Phe206-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 Thr285, Leu286, and Val297 exhibited a slow exchange with the surrounding aqueous milieu. By contrast, these same protons exchanged more quickly with deuterium when deltorphin II was absent. These data suggest that the amide protons close to Trp284 become more shielded from the solvent in the presence of deltorphin II presumably due to an interaction between deltorphin II and hDOR-(281–297) around Trp284 of the receptor peptide. It should be pointed out that the amide protons of the receptor peptide alone should also be well shielded form the solvent and exhibit a slow NH-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 Glu4 following a deltorphin II-hDOR-(281–297) interaction. Weak Glu4 amide proton signal intensity was consistently found in TOCSY spectra acquired over time for deltorphin II in solution (Fig. 8a). These data reflect a slow ND-TO exchange of the Glu4 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 deltorphin II Glu4 amide proton when encountering the hDOR peptide. A likely explanation for this event is the breaking of an internal hydrogen bond in deltorphin II involving Glu4 NH. Indeed, some groups have reported deltorphin II 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 Val287 has been proposed through NMR and modeling experiments (17). A hydrogen bond involving Glu4 NH would stabilize this type of turn (34). Binding to hDOR could thus open up the deltorphin II U-shaped structure and lead to breaking of the hydrogen bond.

Upon shifting focus to the C-terminal end of the hDOR ECLIII, one notes a number of experimental pieces of evidence that explain events occurring in this region of the receptor following ligand binding. First of all, a β-structure at the C terminus of the hDOR ECLIII peptides was observed when the peptide sequence ended in Val297, but was absent when Ala298 and Ala299 were included. The NOEs observed between Asp293 and Arg291 measured from a crude helical model of hDOR-(279–299) is 4.7 Å (data not shown). It can be hypothesized then that deltorphin II Phe2 and Glu4 interact with hDOR Trp284 and Arg291, respectively. The loss of a helical conformation may arise from the disruption of helix-stabilizing forces, including possibly interruption of a Phe206-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 Thr285, Leu286, and Val297 exhibited a slow exchange with the surrounding aqueous milieu. By contrast, these same protons exchanged more quickly with deuterium when deltorphin II was absent. These data suggest that the amide protons close to Trp284 become more shielded from the solvent in the presence of deltorphin II presumably due to an interaction between deltorphin II and hDOR-(281–297) around Trp284 of the receptor peptide. It should be pointed out that the amide protons of the receptor peptide alone should also be well shielded form the solvent and exhibit a slow NH-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.

**Fig. 9. Portion of the TOCSY spectrum acquired for a hDOR-(281–297) sample (2 mM) containing deltorphin II in 440 μL of H2O. Residues with visible amide protons signals in the TOCSY spectrum 1.5 h after sample preparation are indicated to the right in boldface.**
and Leu$_{295}$ and between Pro$_{284}$ and Val$_{296}$ for hDOR-(281–297) and hDOR-(283–297) suggest that formation of the β-structure is the result of a reverse turn centered on Pro$_{284}$ (35). A Chou-Fasman protein conformational profile was made for different variants of the C-terminal sequence after and including Arg$_{292}$ using the program Peptide Companion (CoshiSoft/PeptiSearch Version 1.25). A high propensity to adopt either a helical conformation or a β-conformation was noted after Pro$_{294}$ when Ala$_{298}$ and Ala$_{299}$ were present, whereas only a β-structure was calculated for this domain when the two 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.

It has been demonstrated that a hydrophobic segment including residues Leu$_{295}$ to Leu$_{300}$ in the intact receptor is an important element for δ-selective ligand binding (21). Much supporting information for this was obtained from the results presented here. According to α-proton chemical shift measurements, hDOR-(279–299) gained a C-terminal β-conformation (Fig. 7a) upon complexation with deltorphin II. In particular, the α-proton chemical shifts for the important residues Val$_{296}$ and Val$_{297}$ moved significantly upfield as a result of deltorphin II binding (Figs. 6 and 7a). 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 deltorphin II was present, whereas only Val$_{296}$ NH appeared shielded from the aqueous environment in the absence of deltorphin II (Fig. 9). Likewise, the amide proton of Gly$_7$ in deltorphin II 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 deltorphin II (deltorphin II Val$_5$–Gly$_7$). Indeed, many groups have reported that the hydrophobicity associated with Val$_5$ and Val$_6$ of deltorphin II was detected from the solvent in the presence of hDOR-(281–297) upon complexation with deltorphin II. In particular, the spectroscopic evidence presented here suggests that Phe$_3$ and Glu$_4$ of deltorphin II interact with Trp$_{284}$ and Arg$_{291}$ of the δ-opioid receptor, respectively, leading to destabilization of the helical structure at the N-terminal end of the receptor ECLIII. Coincidently, the important residues Val$_5$ and Val$_6$ in the address domain of deltorphin II interact with Val$_{296}$ and Val$_{297}$ at the C-terminal end of the hDOR ECLIII in an extended conformation and possibly induce a turn centered on Pro$_{294}$.

In conclusion, one may hypothesize conformational adjustments that accompany deltorphin II binding to the hDOR ECLIII. The spectroscopic evidence presented here suggests that Phe$_3$ and Glu$_4$ of deltorphin II interact with Trp$_{284}$ and Arg$_{291}$ of the δ-opioid receptor, respectively, leading to destabilization of the helical structure at the N-terminal end of the receptor ECLIII. Coincidently, the important residues Val$_5$ and Val$_6$ in the address domain of deltorphin II interact with Val$_{296}$ and Val$_{297}$ at the C-terminal end of the hDOR ECLIII in an extended conformation and possibly induce a turn centered on Pro$_{294}$.

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Exploring Deltorphin II Binding to the Third Extracellular Loop of the δ-Opioid Receptor
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