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Structural biology of human cannabinoid receptor-2 helix 6 in membrane-mimetic environments¹

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Abstract

We detail the structure and dynamics of a synthetic peptide corresponding to transmembrane helix 6 (TMH6) of human cannabinoid receptor 2 (hCB2) in biomembrane mimetic environments. The peptide's NMR structural biology is characterized by two α -helical domains bridged by a flexible, nonhelical hinge region containing a highly-conserved CWFP motif with an environmentally sensitive, *Pro*-based conformational switch. Buried within the peptide's flexible region, W²⁵⁸ may hydrogen-bond with L²⁵⁵ to help stabilize the *Pro*-kinked hCB2 TMH6 structure and position C²⁵⁷ advantageously for interaction with agonist ligands. These characteristics of hCB2 TMH6 are potential structural features of ligand-induced hCB2 activation *in vivo*.

Keywords

agonist ligand; conformational switch; G protein-coupled receptor; high-resolution NMR; peptide helix; proline; spectrofluorometry; structural biology; transmembrane protein; tryptophan

Introduction

G protein-coupled receptors (GPCRs) comprise a superfamily of cell-surface proteins whose iconic structural signature includes seven hydrophobic transmembrane helices (TMHs) [1]. As components of the information-transducing machinery of eukaryotic cells, GPCRs are activated by exogenous agonists whose binding elicits GPCR structural changes that transition the receptor from inactive to active state(s). A "rotamer toggle switch" involving the bend angle or "kink" around a conserved proline in TMH6 and an "ionic lock" involving salt-bridge formation between charged amino acids in TMH3 and TMH6 have been implicated in the structural changes upon ligand-induced GPCR activation, largely from data on those few GPCRs whose high-resolution crystal structures have been solved [2–4].

¹Atomic coordinates and structure factors will be deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank, Rutgers University, Department of Chemistry and Chemical Biology, Piscataway, New Jersey.

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Isolated successes with GPCR crystallization notwithstanding, direct experimental information on GPCR structural biology remains very limited.

Cannabinoid (CB) receptors are class-A, rhodopsin-like GPCRs that are activated by intrinsic lipid mediators (endocannabinoids) and exogenous cannabimimetic agents [5]. The (patho)physiological importance of cannabinergic signaling has prompted the pharmacotherapeutic evaluation of designer ligands (agonists/antagonists) selective for one of the two CB receptor subtypes, CB1 and CB2, which have been cloned, expressed, and sequenced [6]. Because of CB2's largely peripheral localization, CB2-targeted ligands as potential drugs for pain and inflammatory and neurodegenerative disorders have an inherently low risk of inducing centrally-mediated side effects, which mainly reflect CB1 activation [7]. Although CB1 and CB2 have been the focus of mutation and homology-modeling studies, their crystal structures are lacking [8,9]. Consequently, CB-receptor structural properties critical to ligand docking and pharmacological activation remain experimentally ill-defined. Our previous work has implicated a flexible TMH6 CWxP motif in the structural changes critical to agonist-induced CB1 activation [10]. Molecular dynamics simulations suggest the existence of at least two CB1 TMH6 conformations differing in the degree of *Pro*-kink in that flexible-hinge region [11]. Such findings cannot be extrapolated readily to CB2, however, for human CB1 and CB2 (hCB2) differ considerably in their primary sequence, modeled tertiary conformations, and agonist structure-activity profiles [12,13].

The present work is focused on defining experimentally the structural features of CB2 TMH6 and identifying motifs potentially involved in the conformational switching of CB2 TMH6 upon ligand-induced activation. We have utilized a synthetic peptide representing hCB2 TMH6 [9], high-resolution solution nuclear magnetic resonance (NMR) spectroscopy (including 2D-¹H TOCSY and NOESY experiments), and spectrofluometry. This approach is well validated for complete ¹H assignment with small nonlabeled peptides [14,15]. For our NMR experiments, the hCB2 TMH6 peptide was studied in a compatible solvent system that simulates a biomembrane environment [16]. Tryptophan is considered to play a special role in helping anchor and stabilize protein α -helical regions [17]. For our spectrofluometry studies, we have exploited the single hCB2 TMH6 tryptophan residue (W²⁵⁸) modeled as being centrally disposed within the plasma membrane *in situ* [9] to monitor local structural responses of hCB2 TMH6 when the peptide is reconstituted in micelle membrane mimetics [18]. To our knowledge, this study provides the first direct experimental characterization of hCB2 TMH6 structure and implicates a motif around P²⁶⁰ in agonist-induced TMH6 conformational switching.

Materials and methods

Peptide synthesis and purification

The 33-mer [²⁴⁰DVRLAKTLGLVLAVLLICWFPVLALMAHSLATT²⁷²] corresponding to hCB2 TMH6 [9] was synthesized by a standard 9-*H*-fluoren-9-ylmethoxycarbonyl (Fmoc)-polyamide method at the Molecular Biology Core Facility, Dana-Farber Cancer Institute (Boston, MA, USA). The peptide was isolated by reverse-phase LC to >95% purity according to LC and MALDI-TOF mass spectrometry (MS) analyses.

Sample preparation and NMR experiments

For 1-D and 2-D NMR, the hCB2 TMH6 peptide was dissolved (1.0 mM final conc.) in 30% (v/v) aqueous trifluoroethanol-*d*₂ (TFE). 2-Dimethyl-2-silapentane-5-sulfonic acid was added as reference standard. All experiments were conducted at 27 or 37 °C on a 700-MHz NMR spectrometer (Bruker BioSpin, Billerica, MA, USA). TOCSY (with 70, 80 and 90 ms

mixing times) and NOESY (with 200 and 250 ms mixing times) spectra were acquired in phase-sensitive mode. Virtually complete proton chemical shift assignments have been obtained for this TFE/H₂O mixture [16,19].

Circular dichroism (CD) spectropolarimetry

Small micelles composed of either 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (D-6-PC) or dodecylphosphocholine (DPC) were prepared in 10 mM Tris buffer, pH ~ 7.5, containing 0.5 mM EDTA and 10 mM NaCl. The hCB2 TMH6 peptide was dissolved in 30% TFE/H₂O or incorporated into micelles at a 100:1 lipid-to-peptide molar ratio. Analysis of peptide secondary structure was then performed with a nitrogen-flushed J-810 spectropolarimeter controlled by Spectra Manager (version 1.15.00) (Jasco Instruments, Easton, MD, USA) [20]. Spectra were recorded from 280 to 185 nm at 23 °C using a 1-cm cuvette, a scan rate of 1 nm/min, and an average of three scans per sample. Percent α -helical content was calculated as described [21].

Fluorescence spectroscopy

Spectrofluorometry experiments were performed at 30° C using a QuantaMaster QM-1/2005 spectrofluorometer (Photon Technology International, Birmingham, NJ, USA) in a quartz cuvette. The samples were excited at 295 nm, and emission spectra were collected between 300 and 400 nm. The bandwidth for both excitation and emission monochromators was 5 nm. Samples were prepared by mixing D-6-PC or DPC with hCB2 TMH6 peptide in TFE/chloroform at a molar lipid-to-peptide ratio of 100:1. The mixture was dried to a film that was rehydrated with 25 mM Tris buffer, pH 7.4, containing 100 mM NaCl to give a stock solution of 1 mM peptide, which was then suitably diluted with the Tris-NaCl solution to a final peptide concentration of 2.5 μ M in the analyzed sample.

Collisional quenching experiments with acrylamide were performed by adding increasing amounts of a 10 M acrylamide stock solution in 25 mM Tris buffer (pH 7.4) to hCB2 TMH6 peptide samples reconstituted either in 30% TFE/H₂O or in D-6-PC or DPC micelles up to a maximal acrylamide concentration of 5 mM. The difference between the peptide's (i.e., the peptide's sole tryptophan residue, W²⁵⁸) net fluorescence in the presence and absence of a given acrylamide concentration in each respective membrane-mimetic environment was determined. Quenching of the fluorescence intensities at maximum emission was calculated with the Stern-Volmer equation,

$$F_0/F = 1 + K_{sv}[\text{acrylamide}],$$

where: F_0 is the unquenched fluorescence intensity; F is the fluorescence intensity at [acrylamide]; K_{sv} is the Stern-Volmer quenching constant, which was determined as a function of [acrylamide].

Structure determination

Observed nuclear Overhauser effects (NOEs) were classified according to three categories: short, medium, and long range. A total of 830 inter-residue distance restraints were used in the structure calculations. All the spectra were processed with Topspin (Bruker BioSpin) and visualized using CARS software (<http://www.nmr.ch/>). NOE assignments were improved by a KNOE protocol [22]. Structure calculations were performed by Xplor-NIH [23]. Fifteen lowest-energy conformations were subjected to molecular dynamics simulations in explicit water using the Crystallography and NMR System software suite [24–26]. Structures were validated by PROCHECK-NMR and visualized with MOLMOL [27,28].

Results

hCB2 TMH6 solution structure

Superimposition of the fifteen lowest-energy hCB2 TMH6 NMR conformers in membrane-mimetic solvent (30% TFE/H₂O) is depicted in Fig. 1A. The α -helical secondary elements of the averaged hCB2 TMH6 structure are depicted as the solid ribbon in Fig. 1B. The ensemble statistics are presented in Table 1. The solution structure of hCB2 TMH6 is characterized by two helical segments of different lengths separated by a discrete unstructured region with a pronounced *Pro*-based kink. The kink, well defined by numerous NOEs, seems quite rigid in nature and orients the two helical segments at almost 45° degrees with respect to each another. The two hCB2 TMH6 terminal regions, i.e., from the N-terminus to K²⁴⁵ and from A²⁶⁴ to the C-terminus, are dynamically unstructured.

CD structural analysis of hCB2 TMH6

In order to assess the structural responsiveness of hCB2 TMH6 to its immediate environment, we conducted CD studies of the peptide as reconstituted in three biomembrane-mimetic environments: the 30% TFE/H₂O mixture used in the NMR analysis (above) and detergent micelles composed of either D-6-PC or DPC [18]. The shapes and intensities of representative CD spectra (Fig. 2) are indicative of the peptide's having significant α -helical content in all three membrane mimetics. The average α -helical content of hCB2 TMH6 was 44, 48, and 36% in D-6-PC micelles, DPC micelles, and 30% TFE/H₂O, respectively. The convergence (isodichroic) point among the three traces reflects a two-state system associated with environmentally-sensitive changes in hCB2 TMH6 conformation.

Involvement of W²⁵⁸ in hCB2 TMH6 structural dynamics

To determine whether W²⁵⁸ is involved in any local conformational changes of hCB2 TMH6 when the peptide interacts with lipid, we conducted comparative spectrofluorometric studies of that 33-mer reconstituted in D-6-PC or DPC micelles or in 30% TFE/H₂O. As shown in Fig. 3, the fluorescence emission maxima (λ_{max}) of hCB2 TMH6 in D-6-PC, DPC, and TFE/H₂O are 340, 343, and 347 nm, respectively. The 3-nm blue shift between the D-6-PC and DPC samples indicates that the W²⁵⁸ residue resides in a more polar environment when the hCB2 TMH6 peptide is in a D-6-PC *vs.* DPC micelle. These fluorescence data further suggest that TMH6 is flexed such that W²⁵⁸ within the *Pro*-kinked region is better protected when associated with a D-6-PC micelle, D-6-PC hydrocarbon chains being shorter than DPC. The 7-nm blue shift and the 60% increase in the fluorescence intensity at λ_{max} of hCB2 TMH6 in D-6-PC micelles as compared to the peptide in aqueous TFE is indicative of the highly hydrophobic environment of W²⁵⁸ when hCB2 TMH6 is reconstituted in D-6-PC micelles.

W²⁵⁸ is centrally located within the hCB2 TMH6 peptide sequence and is presumed to be buried within the plasma membrane *in situ* [9] where, by analogy with other GPCRs [11,17,29], it may help stabilize the *Pro*-kinked hCB2 structure. Preliminary analysis with 3-D molecular visualization software (DA Visualizer Pro; Accelrys Inc., San Diego, CA, USA) suggested that the steady-state water accessibility of W²⁵⁸ in hCB2 TMH6 reconstituted in 30% TFE/H₂O is limited, W²⁵⁸ being well-protected within the static *Pro*-kinked hCB2 TMH6 structural matrix (data not shown). To gain experimental insight into the degree of dynamic exposure of W²⁵⁸ within the various membrane-mimetic entities studied, fluorescence quenching studies were next conducted [30]. Linear plots of F_0/F *vs.* acrylamide quencher concentration were obtained for all peptide environments examined (data not shown). For peptide reconstituted in D-6-PC or DPC, the respective Stern-Volmer quenching constant (K_{sv}) was $3.3 \pm 0.1 \text{ M}^{-1}$ and $2.3 \pm 0.2 \text{ M}^{-1}$. The K_{sv} for the hCB2 TMH6 peptide in TFE/H₂O was $10.7 \pm 1.2 \text{ M}^{-1}$, a comparatively high value indicating that a

detergent micelle environment restricts the local structural oscillations of hCB2 TMH6 that allow penetration of acrylamide quencher to W²⁵⁸.

Discussion

We have used high-resolution NMR spectroscopy to define experimentally the structural features of a peptide representing hCB2 TMH6 in a membrane-mimetic TFE/H₂O solution. To our knowledge, this is the initial report of an NMR-derived structure for the TMH6 of any GPCR. Contrary to popular conceptualizations of transmembrane receptor domains as ideally linear α -helices, emerging data suggest that such regions may have nonhelical and/or highly flexible components that contribute decisively to receptor structural character and dynamics [31]. The deviation observed in the hCB2 TMH6 from an ideally linear, continuous α -helix implicates its CWFP region as a flexible hinge that could help accommodate hCB2 TMH6 conformational changes associated with, for example, ligand-induced hCB2 activation. This feature of hCB2 TMH6 is functionally reminiscent of the highly-conserved CWxP motif found in other GPCRs [3,32]. Indeed, the NMR solution structure of hCB2 TMH6 proposed herein is generally consistent with the character of the TMH6's of the few GPCRs whose crystal structures are known [33–36].

Similar to the homolog in TMH6 of the rhodopsin and β -adrenergic receptor high-resolution structures [33,34] and in models of some other GPCRs [3,11,37], W²⁵⁸ of the hCB2 TMH6 is localized within the nonhelical *Pro*-kinked region. The spectrofluorometric blue shift of the emission λ_{max} we observe confirms that W²⁵⁸ experiences a more hydrophobic environment in D-6-PC or DPC relative to that in TFE/H₂O. The distinct fluorescence spectral signatures of hCB2 TMH6 when the peptide is in a D-6-PC vs. DPC bicelle can be conceptualized in terms of a dynamic response of W²⁵⁸ to its local environment such that TMH6 assumes an enhanced *Pro*-kinked structure in the shorter-chain D-6-PC detergent. Initial molecular dynamics simulations conducted in our laboratories suggest that the effective hydrophobic length of the shorter-chain D-6-PC vs. DPC micelles induces more α -helical peptide content, perhaps by restricting the flexible hCB2 TMH6 peptide regions so as to avoid hydrophobic mismatch (data not shown). This view of hCB2 TMH6 structural accommodation is supported by demonstrations that biomembrane thickness itself influences protein (including GPCR) TMH conformation and signal transmission [38,39]. It is tempting to speculate that *Pro*-based conformational switching may represent a structural feature of ligand (agonist)-induced hCB2 activation *in situ*.

As schematized in Fig. 4A, we propose that the stability of the *Pro*-kinked hCB2 TMH6 structure derived from our NMR data is controlled by a H-bonding network involving conserved polar residues in the vicinity of TMH6's flexible region. In TFE/H₂O solution, we envision that the side chain of W²⁵⁸ resides in the concave face of the *Pro*-kink, favorably oriented to H-bond with neighboring residues. Our proposed structure features a 2-Å *i* to *i*-3 H-bond between the W²⁵⁸ imino (-NH) and L²⁵⁵ carbonyl (C=O) groups. Another H-bond (4 Å) may exist between W²⁵⁸ and I²⁵⁶ *i* to *i*-2. We have demonstrated that C²⁵⁷ plays a critical role in agonist-induced hCB2 activation [40]. C²⁵⁷ is part of the highly conserved CWxP motif, a putative molecular hinge generally implicated in GPCR agonist recognition [36,37]. It is therefore noteworthy in our NMR structure of hCB2 TMH6 that the C²⁵⁷ side-chain is oriented away from the *Pro*-kinked region toward the aqueous phase, suggesting that H-bonding within the CWFP motif of the *Pro*-kinked structure advantageously positions C²⁵⁷ for accessibility during agonist docking [11,37]. Surface presentation of the atomic charge (Fig. 4B) of our proposed hCB2 TMH6 solution structure shows that the positive and negative charges are oriented toward the hydrophilic medium, whereas the neutral surface (i.e., side chain) is buried within the helical structure.

In summary, this report provides the initial, direct experimental data on the conformation and dynamics of a 33-mer representing TMH6 of hCB2 in membrane-mimetic environments. The peptide is characterized as a discontinuous, *Pro*-kinked α -helix with a hydrophobic core flanked by unstructured nonhelical regions. We have identified in hCB2 TMH6 a *Pro*-based conformational switch and a stabilizing effect of W²⁵⁸ on the structure of the *Pro*-kinked peptide. These characteristics of hCB2 TMH6 are likely structural features of ligand-induced hCB2 activation *in vivo*.

Acknowledgments

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Fig. 1. (A) Fifteen lowest-energy hCB2 TMH6 peptide structures are displayed. Only backbone atoms are shown. In each case, the structures were calculated using NOE and hydrogen bond restraints. (B) The α -helical secondary elements of the mean TMH6 structure are depicted as a solid ribbon.

Fig. 2. CD spectra of hCB2 TMH6 peptide reconstituted in 30% TFE/H₂O or in D-6-PC or DPC micelles.

Fig. 3. Fluorescence emission spectra of the hCB2 TMH6 peptide in TFE/H₂O mixture (dashed line), D-6-PC (dotted line) and DPC (solid line). The lipid-to-peptide molar ratio of TMH6 in D-6-PC or DPC was 100:1.

Fig. 4. (A) The H-bond network between the conserved W²⁵⁸ with the carbonyl (C=O) of L²⁵⁵, which is some 2 Å away. Another H-bond (4 Å distance) between W²⁵⁸ and I²⁵⁶ is depicted. W²⁵⁸ resides in the concave region of the *Pro*-kinked region and contributes to structural stability. (B) Surface presentation of the atomic charge of hCB2 TMH6 peptide. Blue denotes positive charges; red, negative charges; white, neutral surface.

Table 1

Structural statistics for the fifteen best hCB2 TMH6 NMR conformers

Parameter	Ensemble ^a	
Distance restraints		
All	830	
Short range (i-j ≤ 1)	477	
Medium range (1 < i-j < 5)	339	
Long range (i-j ≥ 5)	4	
Violations		
NOE (> 0.5 Å)	0	
r.m.s.d. ^b (residues 6 to 27)		
Average backbone r.m.s.d. to mean	0.46 ± 0.15 ^c	0.64 ± 0.17 ^d
Average heavy atom r.m.s.d. to mean	0.81 ± 0.21 ^c	1.09 ± 0.23 ^d
Van der Waals energy	− 68.41 ± 3.72	
Ramachandran plot ^e		
Residues in most favored regions	55.6% ^c	67.1% ^d
Residues in additional allowed regions	33.6% ^c	27.4% ^d
Residues in generously allowed regions	9.0% ^c	5.5% ^d
Residues in disallowed regions	1.8% ^c	0% ^d
r.m.s.d. from idealized covalent geometry		
Bonds (Å)	0.00318 ± 0.00025	
Angles (°)	0.43285 ± 0.0265	
Impropers (°)	1.21733 ± 0.21031	

^aValues given are means $\pm$ S.E.M., wherever applicable.^bResidues 1 to 5 and 28 to 33 were excluded from r.m.s.d. calculations due to the dynamic disorder in these regions.^cBefore water refinement^dAfter water refinement^eCalculated with PROCHECK-NMR [27]