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Biosynthesis, Purification and Characterization of A Cannabinoid Receptor 2 Fragment (CB2₂₇₁₋₃₂₆)

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Abstract

Obtaining sufficient amount of purified G-protein coupled receptors (GPCRs) is almost always one of the major challenges for their structural studies. CB2₂₇₁₋₃₂₆, a human cannabinoid receptor 2 (CB2) fragment comprising part of the third extracellular loop (EL3), the seventh transmembrane domain (TM7) and C-terminal juxtamembrane region of the receptor, was over-expressed as a fusion protein into inclusion body (IB) of *Escherichia coli*. The fusion protein was purified by histidine-selected nickel affinity chromatography under denaturing conditions. Then, the fusion protein IBs were solubilized in detergent (Brij58) and the expression fusion leader sequence (TrpLE) was specifically cleaved with tobacco etch virus (TEV) protease. The target fragment, CB2₂₇₁₋₃₂₆, was subsequently purified by reverse-phase HPLC and confirmed by SDS-PAGE and mass spectrometry. This hydrophobic fragment can refold in mild detergents digitonin and Brij58. Circular dichroism (CD) spectroscopy of CB2₂₇₁₋₃₂₆ in digitonin and Brij58 micelles showed that the fragment adopts a more than 75% α -helical structure, with the remainder having β -strand structure. Fluorescence spectroscopy and quenching studies suggested that the C-terminal region lies near the surface of the digitonin micelles and the TM7 region is folded relatively close to the center of the micelles. This study may provide an alternative strategy for the production and structure/functional studies of GPCRs such as CB2 receptor protein produced in the form of IBs.

Keywords

Cannabinoid receptor 2; transmembrane domain; inclusion body; affinity chromatography; HPLC; circular dichroism; fluorescence

Since the discovery of the CB2 receptor in 1993 [1], there has been a growing interest to clarify the importance of this GPCR membrane protein in human physiology, and to investigate it as a possible target for current and future drug development. The CB2 receptor seems to have a significant role in mediating the immunological functions of cannabinoid receptor ligands. Thus, the CB2 receptor is a promising target for drug development that aims at management of neurological pain, inflammation, osteoporosis, and growth of malignant gliomas and tumors

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of immune origin, as well as immunological disorders such as multiple sclerosis [2]. However, structure-function studies and structure-based ligand design have been impeded by the absence of an experimental three-dimensional (3D) structure of the CB2 receptor. Seven transmembrane α -helical domains and the related high hydrophobicity are the major contributing factors that hinder the 3D structural study of CB2 and other GPCRs. Because of its very limited quantities obtainable from natural sources, attempts have been made to heterologously over-express CB2 [3-7]. In general, the *Escherichia coli* expression system is the most attractive strategy due to its advantages of simplicity of use, low cost, easy scale-up, and homogeneous protein production. Though few attempts were promising to express full length CB2 receptor in *E. coli* [3-5], it is still problematic to obtain the entire CB2 tertiary structure due to the difficulties to get enough yield of CB2 protein, to purify it, to entice it to fold properly, and to analyze it by NMR. Based on the above fact, we hypothesized that an alternative strategy, production of large CB2 fragments including up to two TM segments connected by a loop through over-expression in bacteria as fusion proteins, would be tractable. Structural and even functional analysis of bacterially synthesized protein fragments has become a powerful tool in the study of GPCRs and other membrane proteins [8-11].

Our lab has previously over-expressed, purified and structurally characterized CB2 fragments, including CB2₁₈₀₋₂₃₃, CB2₆₅₋₁₀₁, and CB2₂₇₋₁₀₁, which correspond to TM1-IL1-TM2 (from the first transmembrane domain to the second intracellular loop and the second transmembrane domain), TM2 and TM5-IL3 [12-15] respectively. In these publications, the CB2 fragments were expressed as fusion proteins with a modified Trp Δ LE1413 (TrpLE) leader sequence and a 9-histidine tag at the N-terminus. The TrpLE leader sequence facilitates the fusion protein to be highly expressed as IBs, which have the advantages of lower toxicity and higher yield than the strategy of insertion of the protein into the bacterial membrane. The formation of IBs also has an advantage of protecting the recombinant protein from degradation by cytosolic bacterial proteases. Also, the insolubility of IB proteins allows for removal of soluble and membrane proteins by simple centrifugation. The resulting fusion proteins were further purified by immobilized metal-ion affinity chromatography (IMAC) under denaturing conditions (6 M guanidine hydrochloride or 8 M urea). The leader sequence and His-tag were cleaved by either cyanogen bromide (CNBr) under extreme acidic conditions or proteases (e.g. Factor Xa) under mild detergent conditions (e.g. Triton X-100). Finally, the CB2 fragments were purified by RP-HPLC. In the present manuscript, we reported that another CB2 fragment (CB2₂₇₁₋₃₂₆), including part of the extracellular loop 3, TM7, helix 8 and part of the C-terminus based on the predicted 3D structural model of CB2 [16], can be produced according to the established methods generally described above. Some important modifications, as will be detailed below, were made to optimize the protein expression level, IB solubilization, fusion protein cleavage, product purification and refolding. The obtained pure CB2 fragment was characterized by circular dichroism and fluorescence spectroscopy. It has been known from the reported mutation and function studies that Ser₂₈₅, Ser₂₉₂ and Tyr₂₉₉ in TM7 are important for CB2 ligand binding [17,18]. The residue Tyr₂₉₉ in TM7, as well as the conserved cysteins Cys₃₁₃ and Cys₃₂₀ in the C-terminal juxamembrane region were shown to be critical for coupling to adenylate cyclase [17]. The solution NMR structure of CB2₂₇₁₋₃₂₆ fragment and the possible specific interaction of the fragment with G-protein will be examined in our future work. The current study provides useful information for structure/function studies of the fragment as well as the whole CB2 receptor.

Materials and methods

Plasmid construction

A human CB2 cDNA vector pRG/II-hs-MBP-CB2-HF (gift from Dr. Reinhard Grisshammer) is used as a template to construct various CB2 fragment DNA sequences into plasmid pMMHb

(gift from Dr. Peter Kim). pMMHb is a high copy plasmid and is used to express CB2 fragments as IBs in large quantities. The CB2₂₇₁₋₃₂₆ is expressed as a fusion protein consisting of a N-terminal TrpΔLE1413 polypeptide (TrpLE, 13,669.7 Da), followed by a nine-histidine tag, a Tobacco Etch Virus (TEV) protease recognition site, and the CB2 fragment (TrpLE-9His-TEV-CB2₂₇₁₋₃₂₆). The TEV protease was chosen in this study because of its high cleavage specificity and its activity over a broad range of temperature, pH, and different detergents [19,20]. We have frequently observed nonspecific cleavage of the fusion membrane proteins with Factor Xa and enterokinase in the presence of various detergents. To solve this issue, a TEV protease recognition site was integrated between TrpLE encode sequence and CB2 fragment encode sequence on the expression plasmid pMMHb for improving cleavage and specificity efficiency. Recombinant DNA techniques were adopted for the expression plasmid construction, which was similarly detailed elsewhere [12].

Protein expression in *E. coli*

A parallel comparison study was performed to evaluate the expression level of the CB2 fusion protein in *E. coli* strain BL21(DE3)pLysS (purchased from Novagen) and a BL21(DE3) mutant strain C43(DE3) (purchased from Avidis Inc., France). The protein expression level from C43(DE3) was nearly twice higher than BL21(DE3)pLysS. Therefore, C43(DE3) was employed as the host cell for the over-expression of the CB2 fragment in this study. Briefly, a single colony of a freshly transformed C43(DE3) was picked up from a LB-agar plate and a 10 ml of LB medium was inoculated. Both the plate and LB medium contained 100 µg/ml ampicillin. The solution culture was conducted at room temperature, 250 rpm for overnight. 1 L of LB medium was inoculated with the cell pellet from the 10 ml overnight culture and incubated at 37°C, 250 rpm. When the OD₆₀₀ reached 0.8~1.0, the protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated for another 4 hours at 37°C or overnight at room temperature. The cells were harvested by centrifugation (7000 g, 4°C). The cell pellets were washed in 50 mM Tris-HCl (pH 8.0) and centrifuged again. The typical yields were approximately 5 grams wet cells per liter culture. Harvested cells were then stored at -80°C for later use.

Purification, solubilization and cleavage of TrpLE-9His-TEV-CB2₂₇₁₋₃₂₆

TrpLE-9His-TEV-CB2₂₇₁₋₃₂₆ fusion protein was purified by His-selected nickel affinity chromatography. For a typical preparation, 20 grams of frozen C43(DE3) wet cell pellet was thawed at room temperature for 15 min and resuspended in 50 mM Tris buffer (10 ml/per gram cells), pH 8.0. PMSF was added to 1 mM and lysozyme was added to 0.2 mg/ml. After being incubated at room temperature for 30 min, the cell lysis suspension was ice-cooled for 10 min and was sonicated briefly on ice. 5 mM of MgCl₂ and benzonase were added to break bacterial DNA and RNA. IBs were collected by centrifugation at 20,000 g for 30 min at 4°C. The pellet was dissolved in sample buffer (6 M GnHCl, 50 mM Tris, 0.5 M NaCl, 5 mM imidazole, pH 8.0) followed by dilution with 10 times its volumes using cold deionized water. Again, the IBs were collected by centrifugation. The IB pellet was then dissolved in the sample buffer facilitated with mild sonication on ice and incubated 4 hours to overnight to fully dissolve the pellet. The solubilized suspension was centrifuged at 20,000 g for 30 min. The supernatant was loaded on a 20 ml of nickel charged His-bind resin (Novagen) column equilibrated with binding buffer (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris, pH 8.0) containing 5 mM imidazole. The column was then washed in 10 column volumes of the same buffer followed by 5 column volumes of binding buffer containing 30 mM imidazole. The target fusion protein was eluted with 3 column volumes of binding buffer containing 500 mM imidazole. The resulting fusion protein eluate was dialyzed exhaustively against deionized water and then lyophilized.

In order to remove the fusion partners, a two-step solubilization strategy was employed and proved to be very effective in this study. Briefly, the lyophilized IB was suspended in 2 × TEV

cleavage buffer (100 mM Tris-Cl, pH 8.0) containing Brij58 (Sigma-Aldrich). The complete solubility of the IB was achieved by adjusting pH value to ~2.5 with 0.5 M HCl and mild sonication (micro-tip at 20% power output) on a Virsonic Cell Disruptor (Model 16-850, The Virtis Co., New York), then quickly the pH was adjusted back to pH 6-6.5. EDTA, DTT, TEV protease and sterile deionized water were added at this moment to reach the final 0.5 mM EDTA, 1 mM DTT and IB/TEV protease ratio of 1 (mg/mg). After brief centrifugation, the supernatant was incubated at room temperature for 20 hours with shaking.

RP-HPLC purification of CB2₂₇₁₋₃₂₆

After 20 hours of TEV protease cleavage, the reaction was stopped by adding formic acid to 0.1% and the suspension was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was filtered (0.22 µm) and the resulting solution containing the target fragment was separated by a reverse phase C4 column (Vydac 214TP510, 5 µm, 10 mm × 250 mm) on an AKTAbasic chromatography system (GE Healthcare Corp.). The column was eluted with a 3-step gradient of solvent B (isopropanol/acetonitrile/TFA [60:40:0.1]) and the separation of the target fragment from TrpLE and other components in a gradient of solvent B 40%-50% over 100 min at a flow rate of 4 ml/min. The elution was monitored by UV absorbance at 220 nm and 280 nm. The peak fractions were collected and analyzed by MALDI-TOF-MS (Voyage) and SDS-PAGE. The fractions containing CB2₂₇₁₋₃₂₆ from multiple runs were pooled and dried by rotary evaporation.

SDS-PAGE

SDS-PAGE were carried out on 4-12% or 12% NuPAGE Novex Bis-Tris gels (Invitrogen) using NuPAGE MES SDS buffer system (50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3). The samples were prepared with 4 X NuPAGE LDS Sample Buffer (106 mM Tris-HCl, 141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5), 10 X Reducing Agent (500 mM DTT) and incubated at 70°C for 10 min. A brief centrifugation was conducted before the samples were loaded on the gels. Protein bands were visualized by staining with Coomassie brilliant blue R-250.

Circular Dichroism

The CD spectra were the average of eight scans at room temperature recorded by a JASCO J-810 Spectropolarimeter using 1-mm path length cylindrical quartz cuvette. Spectra were recorded from 185 nm to 260 nm in a bandwidth of 0.5 nm, a data pitch of 0.2 nm and a 1-sec average time per point, with a scan speed of 100 nm/min. Background spectra of samples containing detergent without protein were acquired in an identical manner and subtracted from the spectra of protein+detergent. The resulting spectra were used for the analysis of CD spectra.

The solution samples of CB2₂₇₁₋₃₂₆ in detergents were prepared using a modified method of [9]: An aliquot of detergent stock solution in propanol/methanol (50/50, v/v) and an aliquot of CB2₂₇₁₋₃₂₆ stock solution in propanol (containing 18.2% formic acid) were mixed in a test tube and the solvent was evaporated by Speed-Vacuum. The dried peptide-detergent pellet was dissolved in an appropriate volume buffer (10 mM Tris-HCl, pH 7.0, 50 mM NaCl) to give a final protein concentration of ~15 µM and final detergent concentrations of 20 mM Brij58, 4 mM digitonin, or 20 mM SDS. Solutions were passed through a 0.22-µm syringe filter before being used for spectroscopy.

The CB2₂₇₁₋₃₂₆ stock solution in propanol was prepared as following. The CB2₂₇₁₋₃₂₆ peptide dried sample was dissolved in propanol with 18.2% formic acid, mild sonication is needed. After brief centrifugation (13,000 rpm, 5 min), the supernatant was carefully transferred to a new tube and the protein concentration was determined by UV spectrometry using extinction

coefficient $\epsilon_{280}=8295 \text{ M}^{-1}\text{cm}^{-1}$ calculated by the below formula [21] for peptide in solvent propanol:

$$\epsilon_{280}(\text{M}^{-1}\text{cm}^{-1})=(\#\text{Trp})(6075)+(\#\text{Tyr})(1680)+(\#\text{Cys})(135)$$

This calculated concentration was used to convert the CD spectra from CD mdeg to mean residue ellipticity [0]. The resulting spectra were analyzed for the proportions of various secondary structures using the computer program ContinLL supported by an interactive web site DICHROWEB [22,23].

Fluorescence spectroscopy

Fluorescence spectra were recorded in a Cary Eclipse Fluorescence Spectrophotometer (Varian) controlled by a PC computer equipped with Cary Eclipse software. Tryptophan and tyrosine quenching were measured at 23 °C. The excitation wavelength was 295 nm for tryptophan fluorescence spectroscopy and 275 nm for the combination of tryptophan and tyrosine. In peptide folding and denaturing experiment, the fluorescence emission scans were recorded at 305-450 nm for tryptophan and 285-450 nm for tryptophan + tyrosine at 600 nm/min. The excitation slit and emission slit were at 5 nm. The sample preparation was similar as described above for CD spectroscopy, except that the peptide-detergent solutions contained 4 mM detergents and the buffer is 10 mM NaPO₄, pH 7.0, 50 mM NaCl for excitation at 295 nm or 10 mM Tris-HCl, pH 7.0, 50 mM NaCl for excitation at 275 nm.

In fluorescence quenching titrations, the NaI concentrations ranged from 0 to 200 mM, and NaCl concentration ranged from 200 to 0 mM which design to diminish the effect of ionic strength. 0.1 mM sodium thiosulfate was added in all samples to avoid the formation of triiodide (I^{-3}) which may give artifacts. All fluorescence spectroscopic parameters were same as above except the emission scans was 305-400 nm for tryptophan fluorescence and 285-400nm for tryptophan+tyrosine. All quenching experiments were carried out in triplicate.

Results and Discussion

Production of the TrpLE-9His-TEV-CB2₂₇₁₋₃₂₆ fusion protein

The recombinant expression of hydrophobic peptides or transmembrane proteins in IBs as a fusion protein coupled with a leading partner (such as GST, KSI or TrpLE) has been shown to be an effective strategy to achieve high yield [9,12,24-27]. We previously showed high level expression of fusion proteins when TrpLE was fused to the N terminal of other CB2 fragments [12,14,15]. In the current study, the human CB2₂₇₁₋₃₂₆ fragment was fused with TrpLE and the fusion protein was exclusively achieved in the form of IBs in *E. coli* cells. The strategy was modified and optimized for higher yield, specific cleavage and effective peptide purification. Typically, ~10 mg of fusion protein TrpLE-9His-TEV-CB2₂₇₁₋₃₂₆ can be recovered from one liter of LB medium culture in C43(DE3) strain by IMAC, exceeding the yield expressed in BL21(DE3) strain by twofold. The expression level difference observed in the current study suggests that, even though the fusion protein was expressed in its insoluble form, it may still toxic to the host *E. coli* cells, especially at high yield. The C43(DE3) strain is more tolerable to the toxicity of the IB than its parental strain BL21(DE3). This phenomenon is similar to the expression of bovine oxoglutarate-malate transport protein and bovine phosphate carrier reported by Miroex and Walker [28].

As shown by SDS-PAGE (Figure 1) and analysis, there was no detectable expression of the CB2₂₇₁₋₃₂₆ fusion protein without IPTG induction (lane 1). The fusion protein was expressed only after the IPTG induction (lane 2). The IB isolated from cell lysate by IMAC was mainly the fusion protein. Bradford assay (Bio-Rad Protein Assay Kit II, catalog number 500-0002)

showed the IMAC purified fusion protein IB pellet contained roughly 50% (w/w) protein. We assumed that the other 50% of the materials were possibly DNA and/or lipids which hydrophobically interacted with the fusion protein and eluted together from the nickel column.

After cleavage, the target fragment was purified by RP-HPLC on an AKTAbasic Sytem (GE HealthCare Corp.). The cleaved suspension was centrifuged at 15000 g for 30 min and the supernatant was filtered through 0.22 μ m filters. The resulting solution was injected onto HPLC column (VyDAC 214TP series C4 column with particle size 5 μ m). The analyses of the eluted peaks by MALDI-TOF-MS revealed Peak 1 to be mainly DTT and Brij58, which were eluted at the wash step. Peaks 2, 3, and 4 were TEV protease, fusion tag TrpLE and CB271-326; these three components were separated at a shallow gradient and eluted at 40%, 42% and 42.8% of solvent B, respectively (Figure 2). The majority of the fusion tag TrpLE and non-cleaved fusion protein were found in the precipitate of the centrifugation (data not shown). Figure 3 shows the Peak 4 had a mass of 6237.44 Da, which is congruent with the calculated mass of CB271-326, 6237.36 Da, differing by only 13 ppm. This fragment contains an extra Gly residue at its N-terminal, which is from the TEV recognition site (Glu-Asn-Leu-Tyr-Phe-Gln/Gly). The mass spectrum also showed the formation of small amounts of oxidized as well as dimerized CB271-326 (6449.28 Da and 12472.86 Da, correspondingly). The CB271-326 fragment may easily form dimer or polymer due to its primary sequence containing four cysteine residues. The SDS-PAGE and mass spectrometry results did not show any obvious impurities. The yield of the HPLC-purified peptide fragment was determined by the Bradford method (Bio-Rad) showing 1.5~2 mg per liter of *E. coli* culture (Table 1).

Refolding and secondary structure of CB271-326 in different detergents

In order to study if CB271-326 can be properly folded, CD spectra of the peptide in different detergents (20 mM Brij58, 4 mM digitonin and 20 mM SDS) and organic solvent (TFE) were obtained on a JASCO-800 (Figure 4). The spectra of the target fragment in all four environments showed two minima at near 208 nm and 222 nm, typical for α -helical secondary structure. The raw CD data were converted to mean residue molar ellipticity $[\theta]$ and the secondary structure content of the peptide was analyzed by using the program ContinLL on DICHROWEB, an interactive web site allowing the deconvolution of data from circular dichroism spectroscopy experiments [22,23]. The results showed that CB271-326 adopts high α -helical structure in the all of the environments. The average α -helical contents of CB271-326 in different solvent environments were calculated in percentage as TFE>digitonin>Brij58>SDS (Table 2). TFE has long been used as an excellent solvent to stabilize α -helices in hydrophobic peptides, and it also is known to induce α -helical structure even in parts of proteins that normally do not adopt this structure [9,29,30]. This could account for the higher proportion of α -helix in this solvent in comparing with the other three detergents. SDS is a strong detergent and often used to solubilize GPCR membrane proteins. The CD analysis showed CB271-326 can retain a significant amount of α -helical structure but it may not be in the native tertiary structure in SDS. In the current CD study, the α -helix content in this harsh detergent was slightly less than in the mild detergents Brij58 and digitonin, which may be the result of either the denaturing of a small amount of α -helical stretches of CB271-326 that are not stable, or the non-native folding of CB271-326 in SDS. Brij58 is an efficient detergent to keep CB271-326 solubilized as described earlier in this context. Digitonin is the most viable choice because it has been shown to keep many GPCRs in their native states [31] and, more importantly, Kiefer et al. [25] showed that OR5 olfactory receptor is not only properly folded but also exhibits functional ligand binding under reconstitution in digitonin. Therefore, the structure in the mild detergents digitonin and Brij58 likely represents the native secondary structure of CB271-326. However, digitonin, compared to Brij58, may keep CB271-326 in its more natural state. Furthermore, CB271-326 fragment monomer clarified on the lane 8 in Figure 1(B) was achieved by solubilizing CB271-326 fragment in digitonin,

whereas dimer or even polymer always appeared when Brij58 was used for this purpose though with 50 mM DTT added (data not shown). Therefore, digitonin might be a better detergent for the *in vitro* refolding of GPCR fragments from IBs. The shortcoming of using digitonin, however, is that digitonin sometimes may precipitate in resuspension solutions during the sample preparation for CD or fluorescence studies as described in the method section.

Fluorescence spectroscopy of CB2₂₇₁₋₃₂₆ in folding and unfolding conditions

The intrinsic fluorescence of proteins arises primarily from the side chains of tyrosine and tryptophan residues. Tryptophans within a hydrophobic core display emission maxima at 330 nm or less, whereas those localized in more hydrophilic surroundings have an emission peak around 350 nm [32,33]. Chemical denaturation of folded proteins can cause a dramatic shift in both the amount and the peak wavelength of their intrinsic tryptophan fluorescence as these parameters are subject to the microenvironments of each tryptophan [33].

CB2₂₇₁₋₃₂₆ has a single tryptophan (Trp₃₁₇) in its C-terminal region close to the cytoplasmic helix 8 domain of CB2 receptor. In our current CB2₂₇₁₋₃₂₆ fluorescence experiment, the tryptophan (Trp₃₁₇) in detergent micelle environment of 4 mM digitonin or Brij58 has maximum fluorescence intensity at 328 nm, but the maximum fluorescence intensity was red-shifted to about 353 nm upon denaturation in 8 M of urea (Figure 5). The red-shift in emission maximum is attributed to the micelle-folded protein fragment being denatured in the “polar” environment of urea. The tryptophan fluorescence intensity of the peptide in digitonin is 31% higher than in Brij58 and urea after solvent baseline was subtracted. The higher fluorescence intensity of CB2₂₇₁₋₃₂₆ in digitonin than in Brij58 might be the result of the following two reasons. One possibility of the more severe quenching of tryptophan in Brij58 than in digitonin is because the peptide molecule is surrounded by more Brij58 molecules than digitonin. Brij58 has an aggregation number of 70 in one micelle while digitonin has a value of 60 (Sigma-Aldrich data). Another possibility is that CB2₂₇₁₋₃₂₆ may form more oligomers when solubilized in Brij58 than in digitonin; this is supported in part by SDS-PAGE data (not shown). Both the oligomers and bigger detergent micelles contributed to the shielding of the tryptophan side chain and therefore caused the more severe quenching in Brij58 than that in digitonin. Regardless of mechanism, the results suggested that digitonin is a better choice to solubilize this particular set of hydrophobic peptides and membrane proteins for further structure/function study.

Stern-Volmer quenching

The presence of Tyr₂₉₉ on TM7 and Trp₃₁₇ on Helix8 offers an opportunity to characterize the helical fragment by intrinsic fluorescence. The solvent exposure and accessibility of the tryptophan and the tyrosine on peptide fragment CB2₂₇₁₋₃₂₆ to the collisional quencher iodide was evaluated by Stern-Volmer analysis. Iodide is an ionic quencher and therefore will act only on fluorophores situated on the outer surface of proteins, and has been used for the quenching of proteins or peptides [9,32,34,35]. Stern-Volmer quenching for the tryptophan and the tyrosine on CB2₂₇₁₋₃₂₆ in iodide concentrations ranging from 0 to 200 mM was examined. When excited at 295 nm (for tryptophan alone), the fluorescence emission spectra of CB2₂₇₁₋₃₂₆ exhibited a maximum emission wavelength at 328 nm, a typical characteristic of a tryptophan residue. These results also suggest that the tryptophan residue may be buried in a nonpolar environment, such as a detergent-based micelle. When excited at 275 nm (where both tyrosine and tryptophan residues absorb), the maximum emission wavelength was at 322 nm, which is an intermediate of the tryptophan (330 nm) and tyrosine (303 nm) residues. The steady-state fluorescence quenching data were analyzed using the Stern-Volmer equation [34]:

$$F_0/F = 1 + K_{sv}[Q]$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quencher (Q) respectively, and the K_{sv} is the Stern-Volmer quenching constant, obtained from the slope of the plot F_0/F versus $[Q]$. Figure 6 shows that the addition of sodium iodide significantly reduced the fluorescence intensity of tryptophan alone (A) and tryptophan + tyrosine (B) in a concentration-dependent manner. The fluorescence of the CB2 protein fragment follows the Stern-Volmer rule as judged by the linearity of the plot in Figure 6 (C). The quenching constant (K_{sv}) for tryptophan alone was 1.122 M^{-1} , while that for tryptophan + tyrosine was 1.561 M^{-1} . The observed K_{sv} enhancement for tryptophan + tyrosine is assumed to be attributed to the presence of Tyr₂₉₉. Therefore, the quenching constant (K_{sv}) for tyrosine alone can be calculated by subtracting K_{sv} for tryptophan alone from the combination: $1.561 - 1.122 = 0.439 \text{ M}^{-1}$. Based on the magnitudes of the quenching constants, we predict that both tryptophan and tyrosine residues are partially shielded from iodide by their local peptide structure in the detergent, but that Trp₃₁₇ located in the cytoplasmic domain of CB2 is more exposed to the surface of a detergent micelle. Compared to Trp₃₁₇, the relative inaccessibility of iodide to Tyr₂₉₉ residue in TM7 domain is most likely the result of this fluorophore being buried in the detergent micelles and folded close to the peptide hydrophobic core.

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Abbreviations

CB2	cannabinoid receptor 2
CD	circular dichroism
CNBr	cyanogen bromide
3D	three-dimensional
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EL	extracellular loop
GnHCl	guanidine hydrochloride
GPCR	G protein-coupled receptor
IB	inclusion body
IL	intracellular loop
IMAC	immobilized metal-ion affinity chromatography
IPTG	isopropyl-beta-D-thiogalactopyranoside
K_{sv}	Stern-Volmer quenching constant
LB	

Luria Bertani

LDS

lithium dodecyl sulfate

MALDI-TOF-MS

matrix-assisted laser desorption ionization time of flight mass spectrometry

NMR

nuclear magnetic resonance

RP-HPLC

reverse-phase high performance liquid chromatograph

SDS-PAGE

sodium dodecyl sulfate – polyacrylamide gel electrophoresis

TEV

tobacco etch virus protease

TFA

trifluoroacetic acid

TFE

trifluoroethanol

TM

transmembrane

TrpLE

TrpΔLE1413 leader sequence

UV

ultraviolet

Figure 1. SDS-PAGE gels. (A) 1, cell lysate (not induced); 2, cell lysate (induced); 3, inclusion body; 4, fusion protein purified from nickel column; 5, suspension of TEV protease cleavage; 6, precipitate of cleavage; 7, supernatant of cleavage. Bands from top to bottom are TEV protease, uncleaved fusion protein, TrpLE tag and CB₂₇₁₋₃₂₆. (B) 8, purified CB₂₇₁₋₃₂₆ from RP-HPLC (right) and protein marker: P7708 (New England Biolabs) (left).

Figure 2. C4 RP-HPLC (VyDAC 214TP) purification of CB2₂₇₁₋₃₂₆ from the supernatant of TEV protease cleaved TrpLE-9His-TEV- CB2₂₇₁₋₃₂₆ fusion protein. Peaks: 1, DTT and Brij58; 2, TEV protease; 3, TrpLE tag; 4, CB2₂₇₁₋₃₂₆.

Figure 3.
MALDI-TOF-MS (Voyager, ABI) spectrometry analysis of HPLC purified CB₂₇₁₋₃₂₆.
CB₂₇₁₋₃₂₆ sequence:
GTTLS₂₇₁DQVKKAF₂₇₂FC₂₇₃SM₂₇₄L₂₇₅CL₂₇₆IN₂₇₇SM₂₇₈V₂₇₉NP₂₈₀VI₂₈₁YAL₂₈₂RS₂₈₃GE₂₈₄IR₂₈₅SS₂₈₆AH₂₈₇H₂₈₈CL₂₈₉AH₂₉₀W₂₉₁KK₂₉₂CV₂₉₃R₂₉₄GL₂₉₅G₂₉₆
S (the N-terminal Gly residue is from the TEV protease recognition site).

Figure 4.

CD spectra of CB271-326. All samples contained $\sim 15 \mu\text{M}$ CB271-326. Samples were solubilized either in Brij58, digitonin, or SDS containing 10 mM Tris-HCl, pH 7.0, 50 mM NaCl, or in TFE.

Figure 5.

Intrinsic tryptophan fluorescence spectrum of $\sim 15 \mu\text{M}$ CB₂₇₁₋₃₂₆ in 4 mM Brij58, 4 mM digitonin and 8 M urea containing 10 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl. The spectra shown were baseline subtracted.

Figure 6.

Fluorescence spectra and fluorescence quenching of $\sim 15 \mu\text{M}$ CB₂₂₇₁₋₃₂₆ corresponds to the increasing concentrations of iodide: (A) quenching of tryptophan alone; (B) quenching of tryptophan + tyrosine; (C) Stern-Volmer plot of the effect of iodide on maximum fluorescence intensity of tryptophan (o) and tryptophan+tyrosine(▲). Each point is an average of triplicate determinations. The peptide was solubilized in 4 mM digitonin containing 10 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl.

Table 1Purification summary of the recombinant CB2₂₇₁₋₃₂₆ fragment

Step	Total protein (mg) [*]	Percentage of total protein	Purity (%)
Whole cell lysate	458.6	100	—
Fusion protein	9.52-12.4	2.4	44-57
Target protein fragment	1.5-2	0.38	>95

^{*} From 1 liter of cell culture (4~6 grams of wet cell).

Table 2 CB2271-326 secondary structure calculation from CD data and peptide sequence*

Solvent	α -helix	β -strand	β -turn	Unordered	Total
Brij58	0.768	0.091	0.141	0	1
Digitonin	0.786	0.075	0.138	0	1
SDS	0.731	0.091	0.178	0	1
TFE	0.825	0.025	0.059	0.092	1

* The CD spectra taken in four different environments were analyzed by the program ContinLL [22,23].