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NMR solution structure of human cannabinoid receptor-1 helix 7/8 peptide: candidate electrostatic interactions and microdomain formation

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Abstract

We report the NMR solution structure of a synthetic 40-mer (T³⁷⁷-E⁴¹⁶) that encompasses human cannabinoid receptor-1 (hCB1) transmembrane helix 7 (TMH7) and helix 8 (H8) [hCB1(TMH7/H8)] in 30% trifluoroethanol/H₂O. Structural features include, from the peptide's amino terminus, a hydrophobic α -helix (TMH7); a loop-like, eleven-residue segment featuring a pronounced *Pro*-kink within the conserved NPxxY motif; a short amphipathic α -helix (H8) orthogonal to TMH7 with cationic and hydrophobic amino-acid clusters; and an unstructured C-terminal end. The hCB1(TMH7/H8) NMR solution structure suggests multiple electrostatic amino-acid interactions, including an intrahelical H8 salt bridge and a hydrogen-bond network involving the peptide's loop-like region. Potential cation- π and cation-phenolic OH interactions between Y³⁹⁷ in the TMH7 NPxxY motif and R⁴⁰⁵ in H8 are identified as candidate structural forces promoting interhelical microdomain formation. This microdomain may function as a flexible molecular hinge during ligand-induced hCB1-receptor conformer transitions.

Keywords

Cannabinergic ligand; G protein-coupled receptor; interhelical microdomain; proline kink; signal transduction; structural biology; transmembrane protein

Introduction

In humans, G protein-coupled receptors (GPCRs) are ubiquitous signal-transduction elements and therapeutically important drug targets [1]. GPCRs continue to garner intense research and translational interest aimed at defining GPCR activation dynamics, optimizing the design of drugs that modulate the function of specific GPCRs, and improving extant GPCR-targeted therapies [2,3]. Traditional GPCRs exhibit three hallmark architectural

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features: an extracellular amino terminus, an intracellular carboxyl terminus, and seven interconnected transmembrane helices (TMHs) [1,3]. The heptahelical, integral-membrane character of GPCRs presents formidable challenges to their purification in quantities sufficient for high-resolution structural analysis [3]. Alternatively, homology models of some therapeutically interesting GPCRs have been constructed, mainly by using refined, rhodopsin crystal structures as templates [3,4]. The limited global sequence homology among class-A GPCRs and the ligand-independent nature of rhodopsin activation compromise the accuracy of rhodopsin-based GPCR computational modeling and its utility as a drug-discovery tool [2,4]. Consequently, experimental data are avidly sought on the structural features and functionally-relevant conformational dynamics of specific GPCRs. In this regard, interactions between the highly-conserved NPxxY motif in transmembrane helix 7 (TMH7) and its cytoplasmic extension, helix 8 (H8), appear to participate in the conformer transitions underlying the activation of class-A GPCRs, including rhodopsin and the serotonin 2C (5-HT_{2C}) and insulin-like growth factor-1 receptors [1,5–7]. The intracellular H8 domain is believed to assist in establishing productive interactions between GPCRs and soluble cytoplasmic G-proteins requisite for signal transmission [1,3,8].

A component of the endogenous cannabinoid (endocannabinoid) signaling system, human cannabinoid receptor-1 (hCB1) is a class-A, rhodopsin-like GPCR activatable by intrinsically-produced lipid ligands and exogenous cannabinergic agents [9]. Its roles in diverse (patho)physiological processes make hCB1 a prominent “druggable” GPCR to which designer ligands are being targeted as potential treatments for common health problems including cardiometabolic disorders, substance abuse/drug addiction, mood disturbances, and pain management [10]. The current unavailability of a high-resolution hCB1 crystal structure undermines hCB1 therapeutic exploitation [9,10]. Mutational, pharmacological, and rhodopsin-based homology-modeling data for hCB1 have allowed inferences that TMH7 resides within the plasma membrane and H8 is juxtaposed to the plasma membrane’s inner aspect as likely participants in ligand-dependent hCB1 activation [11–13]. In contrast to other GPCRs [3,5–7], however, the potential for short-distance, electrostatic interactions within and between hCB1 domains and the putative relationship of such interactions to hCB1 structure and activation have not been characterized. Notably, hCB1 lacks the H8 phenylalanine F³¹³ residue that contributes to an activating molecular switch in rhodopsin through aromatic interactions with TMH7 Y³⁰⁶ [12]. For this reason, a structurally identical activation mechanism for both hCB1 and rhodopsin cannot be postulated.

The present work is focused on determining the solution structure of the hCB1 TMH7/H8 region and identifying structural features that might participate in hCB1’s conformational response to activation. For this purpose, we have studied by nuclear magnetic resonance (NMR) spectroscopy a synthetic peptide 40-mer (T³⁷⁷-E⁴¹⁶) [hCB1(TMH7/H8)], encompassing the conjoined hCB1 TMH7 and H8 regions, in trifluoroethanol (TFE)/H₂O [14,15]. Peptides representative of defined polytopic GPCR segments have proven useful for interrogating GPCR structure and inferring structure-function relationships [16]. Our data define the overall structural signature of hCB1(TMH7/H8) in solution and allow information to be extracted regarding favorable electrostatic interactions having potential importance to hCB1 regional architecture and overall function.

Materials and methods

Peptide synthesis and purification

The numbering system of Bramblett et al. is employed [11]. The 40-mer [T³⁷⁷TVFAFCMSMLALLNSTVNPIIYALRSKDLRHAFRSMFPSAE⁴¹⁶] peptide, hCB1(TMH7/H8), was synthesized by a standard 9-*H*-fluoren-9-ylmethoxycarbonyl (Fmoc)–

polyamide method at the Molecular Biology Core Facility, GenScript Corporation (Scotch Plains, NJ, USA). To optimize spectral resolution and preclude confounding disulfide formation/peptide dimerization, two conservative substitutions (C³⁸⁶A and C⁴¹⁵A) were made, as indicated in the above sequence. The peptide was isolated by reverse-phase LC to >95% purity according to LC and MALDI-TOF mass spectrometry analyses. The procedure yielded 23 mg of purified hCB1(TM7/H8) peptide with a molecular mass of ~ 4.5 kDa.

Sample preparation and NMR experiments

For 1-D and 2-D NMR, hCB1(TM7/H8) (1.0 mM final conc.) was dissolved in 30% (v/v) aqueous TFE-*d*₂. 2-Dimethyl-2-silapentane-5-sulfonic acid was added as reference standard. All ¹H-NMR spectra were recorded at 37 °C on a 700-MHz Bruker AVANCE II NMR spectrometer. Complete sequential assignments were made using 2D TOCSY and NOESY experiments. For spin system identification, TOCSY spectra with mixing times of 70, 80, and 90 msec and a decoupling in the presence of scalar interactions (DIPSI) composite pulse sequence for mixing were obtained. NOESY spectra with 150, 200 and 250 msec mixing times were acquired in phase-sensitive mode. TOCSY and NOESY analyses incorporated the WATERGATE 3-9-19 pulse sequence with gradients for water suppression.

hCB1(TM7/H8) NMR structural analysis

All NMR spectra were processed using Topspin 2.1 (Bruker BioSpin) and visualized using CARA [17] and CCPN [18] software suites. Nuclear Overhauser effect (NOE) assignments were improved by a KNOWNOE protocol [19]. All resonances were assigned by using interactive interpretation of standard phase-sensitive TOCSY and NOESY NMR spectra [20]. A total of 1224 inter-residue NOEs were classified as short, medium, and long range (Table 1). Structure calculations were performed by Xplor-NIH [21]. The eighteen lowest-energy conformations in explicit water were obtained by molecular dynamics simulation refinement using the Crystallography and NMR System software suite [22,23]. Structures were validated by the PSVS Protein Structure software suite (<http://psvs-13.nesg.org/>) and visualized with MOLMOL [24] and Discovery Studio Visualizer (Accelrys, Inc., San Diego, CA, USA). Atomic coordinates have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (pdb id: _____). The NMR data have been deposited in BioMagResBank (accession number _____).

Results

hCB1(TM7/H8) NMR solution structure

hCB1(TM7/H8) dissolved in 30% TFE/H₂O yielded 2D TOCSY and NOESY spectra of excellent quality, allowing almost complete sequence-specific assignment. There was no evidence of polypeptide precipitation or change in the NMR spectra during data acquisition. The assignment for each individual residue and the NH- α , β , γ connectivity for each assigned residue are shown in Fig. 1A. The NH-NH region of the NOESY spectrum with sequential NOE connectivities is shown in Fig. 1B. Identification of only one set of signals is indicative of hCB1(TM7/H8) structural homogeneity in TFE/H₂O. Sequential assignment was completed by analyzing cross-peak patterns in the fingerprint region of the NOESY spectrum. NOEs calculated from the NOESY spectrum, as well as H α chemical shift indices (CSIs), are shown in Fig. 2. The strong sequential NH_{*i*} – NH_{*i*+1} connectivities in the V³⁷⁸-T³⁹¹ region and the many medium-range α H_{*i*} – NH_{*i*+3} NOEs, together with a α H_{*i*} – NH_{*i*+4} and several α H_{*i*} – β H_{*i*+3} NOEs, are indicative of an α -helical region spanning these residues. Evidence for the α -helical nature of V³⁷⁸-T³⁹¹ is strengthened by the long continuous bend of negative CSI values therein. A series of strong sequential NH_{*i*} – NH_{*i*+1} NOEs and the presence of several α H_{*i*} – NH_{*i*+4} and α H_{*i*} – β H_{*i*+3} NOEs indicate that D⁴⁰³-R⁴⁰⁹ is also α -helical. These α -helices represent footprints for the predicted hCB1 TM7

and H8 regions, respectively [11–13]. The initial N-terminal residue (T³⁷⁷), the eleven residues from V³⁹² to K⁴⁰², and seven residues at the peptide's C-terminal end (S⁴¹⁰-E⁴¹⁶) are nonhelical, as evidenced by the low number of inter-residue NOEs in these regions.

The NMR results were used to analyze hCB1(TM7/H8) structural details. Superimposition of the eighteen lowest-energy conformers is shown in Fig. 3A, and the ensemble statistics are summarized in Table 1. In our structure, eight residues predicted [11–13] to be helical in the hCB1 TM7 α -helix (V³⁹²-L³⁹⁹) and the initial three residues predicted [11–13] to be helical in the H8 α -helix (R⁴⁰⁰-K⁴⁰²) are nonhelical, even in the helix-inducing [14] TFE/H₂O solvent. The NPxxY motif is a component of this eleven-amino-acid, loop-like hCB1(TM7/H8) region, and the proline residue induces a kink. The two hCB1(TM7/H8) α -helices are oriented approximately orthogonally to each other at either side of the interposed loop-like region. The ribbon representation of the mean hCB1(TM7/H8) solution structure (Fig. 3B) depicts the peptide's major structural domains: the TM7 and H8 α -helices; a loop-like segment between the two α -helices with a pronounced *Pro*-kink; and an unstructured C-terminal end. The orthogonality between TM7 and H8 suggests the presence of interhelical amino-acid interactions.

Noncovalent interactions in hCB1(TM7/H8)

The hCB1(TM7/H8) NMR solution structure was examined to evaluate potential, short-distance amino-acid interactions residing therein. The R⁴⁰⁰-E⁴¹⁶ hCB1(TM7/H8) region containing H8 features five hydrophilic residues (R⁴⁰⁰, K⁴⁰², R⁴⁰⁵, H⁴⁰⁶, and R⁴⁰⁹) oriented to form a compact cationic cluster, contralateral to which is a hydrophobic “face” of clustered nonpolar residues (L⁴⁰⁴, A⁴⁰⁷, F⁴⁰⁸, M⁴¹¹, F⁴¹², and P⁴¹³) (Fig. 4A). These cationic and hydrophobic clusters lend an amphipathic character to both H8 and the entire hCB1(TM7/H8) C-terminus. NOE interactions between the negatively-charged D⁴⁰³ (HN, H α , H β 1, H β 2) and positively-charged H⁴⁰⁶ (H δ 2) side chains are indicative of a salt bridge between these two proximal (2.6 Å) H8 amino-acid residues (Fig. 4B).

The hCB1(TM7/H8) NMR solution structure suggests hydrogen-bonding involving the peptide's loop-like region. Specifically, hydrogen bonds between the NPxxY Y³⁹⁷ backbone carbonyl and the backbone amides of both L³⁹⁹ and R⁴⁰⁰ as well as the side-chain N³⁹³ amino group would help position Y³⁹⁷ for electrostatic interaction between its hydroxyl moiety and the cationic -NH₂⁺ group of R⁴⁰⁵ in H8, thereby establishing a hydrogen-bond network (Fig. 4C). An additional hydrogen bond may exist between the N³⁸⁹-NH₂ and the N³⁹³ carbonyl oxygen (not shown).

The hCB1(TM7/H8) NMR solution structure is also suggestive of cooperative interhelical cation- π and cation-phenolic OH interactions which may define a microdomain (Fig. 4C). The close proximity (< 4.5 Å) of the π -electron cloud of the Y³⁹⁷ aromatic ring within the TM7 NPxxY motif to the side-chain cationic amino group of H8 R⁴⁰⁵ implies a possible cation- π interaction [25] between these residues. Existence of this cation- π binding force is substantiated experimentally by the NOESY cross peaks between Y³⁹⁷ (H β 2 and H ϵ) and R⁴⁰⁵ (H δ 2 and H α), in that order. The hCB1(TM7/H8) NMR solution structure further suggests a cation-phenolic OH hydrogen bond between Y³⁹⁷ and R⁴⁰⁵. Solvent exposure of R⁴⁰⁵ is considerable, whereas Y³⁹⁷ is both relatively shielded (~20% maximum solvent-accessible surface) and conformationally restricted by the hydrophobic V³⁹², I³⁹⁵, and I³⁹⁶ side chains. Van der Waals contacts of I³⁹⁵ and I³⁹⁶ with Y³⁹⁷ tend to orient the Y³⁹⁷ phenol moiety toward the R⁴⁰⁵ guanidinium moiety for cation- π interaction between Y³⁹⁷ and R⁴⁰⁵. Guanidinium groups in amino-acid residues have a propensity for hydrogen-bond donation to electronegative atoms such as a tyrosine phenolic oxygen [26]. Hydrogen-bonding between Y³⁹⁷ and R⁴⁰⁵ would also help stabilize the orientation of the Y³⁹⁷ aromatic ring and simultaneously assist in positioning the R⁴⁰⁵ guanidinium group perpendicularly to it. In

turn, the electron-deficient R⁴⁰⁵-CH₂ directly adjacent to the R⁴⁰⁵ guanidinium group would reside over the center of the Y³⁹⁷ aromatic ring, distinguishing the cation- π interaction between Y³⁹⁷ and R⁴⁰⁵ in hCB1(TM7/H8) from the typical cation- π interaction that centers the arginine guanidinium moiety over the aromatic ring [25].

Discussion

Important roles in hCB1 activation and hCB1-G protein coupling have been attributed to the hCB1 TMH7/H8 region in the absence of high-resolution, experimental characterization of this GPCR's structure [1,9,11–13]. We have determined the NMR solution structure in TFE/H₂O of hCB1(TM7/H8), a synthetic polypeptide encompassing the hCB1 segment that includes TMH7 and a conjoined cytoplasmic extension containing H8. Whereas the general importance of electrostatic amino-acid interactions to protein secondary structure, substrate/ligand recognition, and catalysis is well recognized [27], their potential occurrence within hCB1 as intrinsic determinants of hCB1 conformation/function is not known. The structural insights garnered with our model system have also allowed information to be extracted regarding potential electrostatic forces within hCB1(TM7/H8).

Several features of the overall hCB1(TM7/H8) NMR solution structure have been identified: a lengthy hydrophobic α -helical segment (TMH7); a short amphipathic α -helix (H8) oriented orthogonally to TMH7 and containing an intrahelical salt bridge and both cationic and hydrophobic amino-acid clusters; a loop-like segment of eleven residues interposed between the two α -helices that features a pronounced *Pro*-based kink and includes the conserved [1,5–7] NPxxY motif and a hydrogen-bonding network; and an unstructured C-terminal end. The TMH7 NPxxY motif in bovine rhodopsin and the human β_2 adrenergic receptor exhibits a *Pro*-kink and a non-canonical distortion and has been linked to the activating structural responses of these two GPCRs [1,3,28–30]. The NMR solution structure of hCB1(TM7/H8) provides evidence for a more extensive, loop-like segment that includes a P³⁹⁴-induced kink and involves up to eleven amino acid residues conjoining the TMH7 and H8 α -helices. Unlike the comparatively circumscribed molecular motion afforded by NPxxY-associated *Pro*-kinks considered typical of GPCRs [1,28,29], the additional flexibility afforded by this hCB1 loop may be important to the purported involvement of the NPxxY motif and nearby residues in the conformational dynamics associated with hCB1 activation [11–13]. Hydrogen-bonding potential within the hCB1(TM7/H8) loop-like region appears extensive enough to act as a local structural force that may help coordinate ligand-induced hCB1 protein motion with the docking of cytoplasmic G-protein subunits. A critical role in rhodopsin's conformational response to activation has been attributed to a hydrogen-bonding network in that GPCR [31].

Prior NMR structural investigations of hCB1 C-terminal polypeptides have focused specifically on its cytoplasmic tail including H8 [32–35]. We demonstrate herein that hCB1(TM7/H8) H8 adopts an α -helical secondary solution structure congruent with its conformation in other class-A GPCRs [1,3,8] and similar to the NMR conformation of a short, synthetic C-terminal rat CB1 peptide reconstituted into phospholipid micelles [33]. The salt bridge between D⁴⁰⁴ and H⁴⁰⁷ in that rat CB1 peptide [33] is also reminiscent of the H8 salt bridge between D⁴⁰³ and H⁴⁰⁶ in a corresponding hCB1(TM7/H8) region. Other features of rat CB1 C-terminal peptides reconstituted in phospholipid or detergent environments [34,35] are shared by the H8 region of hCB1(TM7/H8) in TFE/H₂O: i.e., significant helicity, a cationic amino-acid cluster, and amphipathicity. Conservation of key CB1 H8 characteristics in diverse environments and the almost complete lack of secondary structure within a hCB1 H8 peptide in water [32] may be indicative of this region's general importance as an activation domain for transmitting receptor-based structural information to intracellular G proteins, perhaps through hydrogen-bonding associations with specific G-

protein subunits [12,36]. In this manner, the H8 ionic clustering evident in the hCB1(TMh7/H8) solution structure may help define the specificity of hCB1-G protein interactions [12].

In models of class-A GPCRs including hCB1, H8 has been oriented approximately orthogonally to the hydrophobic TMh7 domain, juxtaposed parallel to the inner (i.e., cytoplasmic) face of the plasma membrane [1,3,12,32]. We have previously shown that the two hCB1(TMh7/H8) α -helical segments are similarly disposed when the peptide is reconstituted and mechanically oriented in phospholipid bilayers [15]. As demonstrated herein, orthogonality between TMh7 and H8 is also characteristic of the hCB1(TMh7/H8) NMR solution structure, suggesting that intrinsic hCB1 properties independent of the plasma membrane help define the receptor's structure (and, by inference, functional responses). In turn, as we have shown previously for peptides representing helical regions of both principal cannabinoid GPCRs [37,38], conformational determinants encoded within the hCB1 primary structure will likely be modulated by a local phospholipid matrix.

π - π interactions between TMh7 NPxxY tyrosine and a H8 phenylalanine establish an interhelical microdomain that helps control activating structural changes in rhodopsin and the 5-HT2C receptor [5,6]. An identical π - π structural force is precluded in hCB1, for the hCB1 NPxxY(X)_{5,6}L motif possesses a leucine instead of a phenylalanine residue [12]. The present work describes an extended interhelical microdomain in hCB1(TMh7/H8) involving electrostatic forces that pair the NPxxY tyrosine (Y³⁹⁷) with an arginine in H8 (R⁴⁰⁵) through cooperative cation- π and cation-phenolic OH interactions. Identification of such helix-helix interactions has recently been highlighted [16] as essential to an understanding of polytopic GPCR structure/function. By analogy to rhodopsin and the 5-HT2C receptor [5,6], it is tempting to hypothesize that reversible changes in this hCB1 interhelical microdomain may represent an important feature of hCB1 activation, contributing to ligand-induced transitions among distinct hCB1 conformers.

In summary, the NMR solution structure of hCB1(TMh7/H8), a 40-mer that encompasses hCB1 TMh7 and H8, has been characterized. In solution, the peptide evidences a hydrophobic α -helix (TMh7) oriented approximately orthogonally to a shorter, amphipathic α -helix (H8); an interposed, loop-like segment that features a *Pro*-kink and includes the conserved NPxxY motif; and an unstructured C-terminal end. The hCB1(TMh7/H8) NMR solution structure suggests electrostatic amino-acid interactions including an intrahelical salt bridge in H8 and a hydrogen-bonding network involving the peptide's loop-like region. Potential cation- π and cation-phenolic OH interactions between Y³⁹⁷ in TMh7 NPxxY and H8 R⁴⁰⁵ would promote formation of a stable interhelical microdomain which, by analogy to other GPCRs [5,6], could participate in hCB1 conformer transitions. These data provide the basis for mutational studies directed at specific hCB1(TMh7/H8) amino-acid residues to probe the potential impact of their electrostatic interactions on hCB1 function [31].

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Fig. 1.

(A) The amide region of the TOCSY spectrum for hCB1(TM7/H8) in 30% (v/v) aqueous TFE-*d*₂ (mixing time, 70 msec). The NH – α, β, γ connectivity and assignment of each residue is labeled and color-coded. Residues 2 to 25 (V³⁷⁸ to S⁴⁰¹) corresponding to the TMH7 transmembrane region are blue. Residues from 26 to 35 (K⁴⁰² to M⁴¹¹) corresponding to the cytoplasmic region are red. (B) The amide region of the 2D NOESY spectrum for hCB1(TM7/H8) in aqueous TFE-*d*₂ (mixing time, 250 msec). The lines map the sequential assignments of the amide N-H resonances starting at V2 (8.63 ppm). The assignments of inter-residue NHⁱ – NHⁱ⁺¹ crosspeaks are labeled and color-coded. Connectivity from 2 to 25 (V³⁷⁸ to S⁴⁰¹) is colored blue, and from 26 to 35 (K⁴⁰² to M⁴¹¹), red.

Fig. 2.

Summary of NOE connectivities observed for hCB1(TM7/H8). Sequential, midrange, and long-range NOE connectivities are linked by line segments. Strength is indicated by box height and line thickness. The chemical shift indices for H α protons are also shown. Negative values indicate a helical conformation.

Fig. 3. (A) Eighteen lowest-energy hCB1(TMH7/H8) structures are displayed. Only backbone atoms are shown. (B) Ribbon depiction of the mean hCB1(TMH7/H8) structure.

Fig. 4.

(A) Clustering of cationic and hydrophobic residues in the hCB1(TMH7/H8) cytoplasmic region. Cationic residues are blue; hydrophobic residues, yellow; anionic residues, red; polar residues, purple. (B) The H8 salt bridge between D⁴⁰³ and H⁴⁰⁶. (C) An interhelical microdomain (circled and expanded) reflects potential cooperative cation- π and cation-phenolic OH interactions (- - - -) between proximal (< 4.5 Å) TMH7 Y³⁹⁷ in the NPxxY motif and H8 R⁴⁰⁵. Potential hydrogen-bonding interactions of Y³⁹⁷ with L³⁹⁹, R⁴⁰⁰, N³⁹³ are also indicated (/////////).

Table 1

Structural statistics for the eighteen hCB1(TMh7/H8) NMR conformers

Parameter	Ensemble ^a
<i>Distance restraints</i>	
All	1224
Short range ($ i-j \leq 1$)	574
Medium range ($1 < i-j < 5$)	624
Long range ($ i-j \geq 5$)	26
<i>Violations</i>	
NOE ($> 0.5 \text{ \AA}$)	0
r.m.s.d. ^b (residues 2–14, 16–21, 26–38)	
Average backbone r.m.s.d. to mean	1.1 $\text{\AA}$
Average heavy atom r.m.s.d. to mean	1.5 $\text{\AA}$
Van der Waals energy	-92.74 ± 8.67
<i>Ramachandran plot</i> ^c	
Residues in most favored regions	65.2 %
Residues in additional allowed regions	30.2%
Residues in generously allowed regions	4.3%
Residues in disallowed regions	0.4%
<i>r.m.s.d. from idealized covalent geometry</i>	
Bonds ($\text{\AA}$)	0.011 ± 0.00012
Angles ($^{\circ}$)	0.76 ± 0.021
Impropers ($^{\circ}$)	1.18 ± 0.17

^aValues given are means $\pm$ SEM, wherever applicable.^bResidues 1, 15, 22 to 25, and 39, 40 were excluded from r.m.s.d. calculations due to the dynamic disorder in these regions.^cCalculated by the PSVS Protein Structure software suite (<http://psvs-13.nesg.org/>).