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Structural studies of the putative helix 8 in the human β_2 adrenergic receptor: an NMR study

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Abstract

The recently reported crystal structure of bovine rhodopsin revealed a cytoplasmic helix (helix 8) in addition to the seven transmembrane helices. This domain is roughly perpendicular to the transmembrane bundle in the presence of an interface and may be a loop-like structure in the absence of an interface. Several studies carried out on this domain suggested that it might act as a conformational switch between the inactive and activated states of this G-protein coupled receptor (GPCR). These results raised the question whether helix 8 may be an important feature of other GPCRs as well. To explore this question, we determined the structure of a peptide representing the putative helix 8 domain in another receptor that belongs to the rhodopsin family of GPCRs, the human β2 adrenergic receptor (hβ2AR), using two-dimensional ^{1}H nuclear magnetic resonance (NMR). The key results from this structural study are that the putative helix 8 domain is helical in detergent and in DMSO while in water this region is disordered; the conformation is therefore dependent upon the environment. Comparison of data from five GPCRs suggests that these observations may be generally important for GPCR structure and function.

Keywords: Helix 8; β_2 adrenergic receptor; NMR

High-resolution three-dimensional structural information is available for only one G-protein coupled receptor (GPCR), bovine rhodopsin. The structure of rhodopsin, in the inactive state, determined from X-ray crystallography [1] surprisingly showed an eighth helix in addition to the expected bundle of seven transmembrane helices. Because several studies suggested important roles for this region in G-protein activation [2–4], the question arises how general this helical feature is among other GPCRs. It is now widely reported that peptides corresponding to helices [5–17] and turns [18–26] of membrane proteins report on the secondary structure of the native protein with fidelity. Therefore, to approach this question about the H8 domain in other GPCRs of the rhodopsin family in the absence of high-resolution structures from X-ray crystallography, we studied the structure of a peptide corresponding to the putative helix 8 from the human β_2 adrenergic receptor (hβ2AR). The β_2 adrenergic receptor (β_2AR) belongs to the chemoreceptor family of the class A GPCRs. β_2AR are localized in several tissue types and are activated by binding of catecholamine ligands. The molecular mechanism of receptor activation and the structural details of Gs coupling with the receptor are not yet understood for this receptor. We determined the solution structure of a peptide representing H8 of hβ_2AR and found it to be helical under some conditions and disordered in water. The structure of this domain is then examined in the context of current information from other GPCRs.

1. Materials and methods

1.1. Synthesis of peptide

The peptide representing H8 of hβ_2AR was chemically synthesized at the Biotechnology center, University of Connecticut, Storrs, CT using solid phase peptide synthesis with Fmoc chemistry. The resulting peptide was purified on a C18 column using reverse phase column chromatography.

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The sequence used encompassed the H8 domain of hβ_2AR and corresponds to L324–N357 as shown:

$_{324}$LIYCRSPDFRIAFQELLCLRRSSLKAYGNGYSSN$_{357}$
× (H8BAR)

The amino acids underlined belong to the NPXXY sequence that is conserved in the GPCR family. C341 is the palmitoylation site.

Deuterated dodecylphosphocholine (DPC) was obtained from Isotec (Miamisburg, OH).

1.2. Circular dichroism (CD) measurements

CD was employed to obtain information about the secondary structure of H8BAR in DPC micelles and aqueous solvent. Measurements were made in a wavelength range of 260–185 nm employing a 2-mm cuvette in a JASCO J715 CD spectrophotometer. Samples of concentration 10 μM were used for all CD measurements. The resulting spectra were deconvoluted to extract secondary structure information using a neural network program, CDNN, which utilizes a database of proteins whose secondary structure is already known from crystal structures [27]. However, CD of the peptide in DMSO was not obtained as this solvent exhibits strong absorbance in the UV.

1.3. Sample preparation for nuclear magnetic resonance (NMR) measurements of peptides

For NMR studies of H8BAR in DMSO and water, samples were prepared by solubilizing the peptide in the deuterated solvent to a concentration of 2 mM. For detergent micelle samples, the amount of peptide equivalent to 2 mM was solubilized in chloroform and dried to a film. The amount of detergent equivalent to 200 mM of deuterated DPC was solubilized in chloroform in a separate flask and dried to a film. The DPC film was hydrated using 9:1 water/D2O and the resulting solution is added to the peptide film, thus obtaining a dispersion of peptide in DPC micelles. The pH was adjusted to about 4.0.

1.4. NMR spectroscopy

The NMR data were acquired on a Varian 600-MHz NMR spectrometer at the University of Connecticut Health Center, Farmington, CT. For each of the samples, a NOESY and a TOCSY were acquired. NOESY was acquired with a mixing time of 450 ms for the DMSO samples and mixing times of 100 and 150 ms for micelle samples. TOCSY was acquired with a mixing time of 70 ms in the case of both DPC micelles and DMSO. The resulting FIDs were processed using NMRPipe [28]. Processing was done using a Guassian window function for the direct dimension with the parameters lb = – 16 and gc = 0.197, and a 70° shifted squared sinebell in the indirect dimension.

1.5. Structure calculations

Sequence-specific resonance assignments were made using the procedure described by Wüthrich [29]. Resonance assignments were obtained using the program FELIX (Accelrys Inc.). NOE distance constraints were calculated as described previously [30]. Structure calcula-

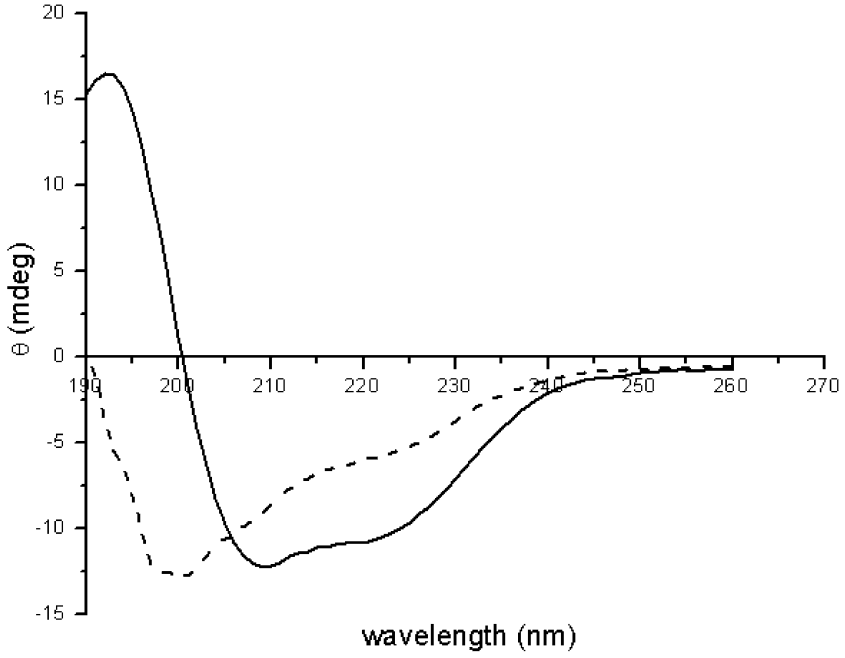

Fig. 1. CD spectra obtained for the peptide H8BAR in aqueous solvent and in the presence of DPC micelles.

tions were performed employing the distance-restrained simulated annealing protocol as described previously [30]. A model peptide was built using the Biopolymer module of the Sybyl (TRIPOS, St. Louis) package and all the distance constraints written on it. The resulting model was used as a starting structure for simulated annealing calculations. The omega torsion angle was restricted to 180° throughout all the structure calculations. The starting structure was heated to 1000 °K for 1000 fs in simulation and cooled to 200 °K for 1500 fs in silico. Ten cycles of simulated annealing were performed and the resulting structures were energy minimized employing the Kollman All Atom force field.

2. Results

2.1. CD studies

Far-UV CD was employed to examine the conformation of H8BAR in DPC micelles and aqueous solvent (CD could not be used to study the secondary structure of H8BAR in DMSO, as DMSO exhibits strong absorbance within the wavelength range used). Fig. 1 shows the CD spectra for H8BAR obtained. As can be seen in the figure, this peptide exhibits CD that is characteristic of a helical conformation in the presence of DPC micelles, while the peptide is disordered in aqueous solvent. As calculated from the CDNN program, H8BAR exhibits no detectable helix in the absence of DPC whereas 31% helix is observed in the peptide in the presence of DPC. This kind of solvent-dependent conformational preference has been observed for peptides from the H8 region of other GPCRs, for example the CB2 receptor [31].

2.2. NMR spectra

To study the effect of solvent on the conformation of H8BAR, we collected NMR data for the peptide in water, DMSO, and DPC micelles. The peptide exhibited a poor dispersion of NOESY peaks in water (data not shown), which is characteristic of a disordered peptide in aqueous solution. In contrast, the peptide showed a good dispersion of NOESY peaks in DPC micelles, consistent with a well-ordered conformation for the peptide. DPC was chosen because it does not have a strong surface charge on the micelle, which as discussed below may influence the peptide structure. NMR experiments were also carried out in DMSO. DMSO has been used as a membrane-mimicking solvent. Structural determinations by NMR of peptides in DMSO representing transmembrane helices of rhodopsin [12,13,30] and bacteriorhodopsin [17] reported with fidelity the secondary structure of the native protein as seen in the X-ray crystal structures. Furthermore, DMSO is not known to induce particular secondary structure and thus is preferable to solvents like trifluoroethanol, which strongly stabilize α-helix. The NOESY spectrum obtained for the peptide in DMSO showed a good dispersion of NOESY peaks as in DPC micelles, indicating that the peptide likely adopts a well-ordered conformation in DMSO. These observations suggest that H8BAR adopts a well-ordered conformation in both DPC micelles and DMSO, whereas a disordered state is likely in aqueous solvent. These observations are in good agreement with those made from the CD studies. Due to the lack of TOCSY or COSY spectra, sequence-specific resonance assignments could not be completed for H8BAR in DPC micelles. Assignments were obtained only for the amino acid sequences 1–5, 10–16, and 28–33 by analyzing the NOESY spectrum (Table 1). Therefore, a complete structure could not be determined for H8BAR in DPC micelles using NMR. However, guided by patterns observed in the NOESY spectrum and the CD studies, a few key features of the structure in the presence of DPC micelles could be obtained (see below).

Table 1
Table showing the proton chemical shifts for the peptide H8BAR in the presence of the DPC micelles

Residue	αH	NH	βH	Others
Leu1	4.17	8.23	–	–
Ile2	4.25	8.81	–	–
Tyr3	–	8.10	–	–
Cys4	–	7.99	–	–
Arg5	4.16	7.87	–	–
Ser6	–	–	–	–
Pro7	–	–	–	–
Asp8	–	–	–	–
Phe9	–	–	–	–
Arg10	4.44	8.11	1.80/1.89	–
Ile11	4.27	8.00	1.95	–
Ala12	4.33	8.03	1.42	–
Phe13	4.45	7.97	3.08/3.24	–
Gln14	4.33	8.28	2.22/2.38	–
Glu15	4.02	8.31	2.33/2.45	–
Leu16	–	7.88	2.01	–
Leu17	–	–	–	–
Cys18	–	–	–	–
Leu19	–	–	–	–
Arg20	–	–	–	–
Arg21	–	–	–	–
Ser22	–	–	–	–
Ser23	–	–	–	–
Leu24	–	–	–	–
Lys25	–	–	–	–
Ala26	–	–	–	–
Tyr27	–	–	–	–
Gly28	4.04	8.28	–	–
Asn29	–	8.31	2.90/2.95	–
Gly30	3.98/4.04	8.46	–	–
Tyr31	–	8.13	3.08/3.18	–
Ser32	–	8.18	3.94/3.97	–
Ser33	–	8.34	4.01	–
Asn34	–	–	–	–

A complete assignment of the chemical shifts was not made as TOCSY and COSY spectra were not obtainable for this peptide in the presence of the DPC micelles.

2.3. Analysis of the low-field region of the NMR spectra

In general, a network of strong sequential NOE resonances, d_{NN} (i, $i+1$) in the low-field region of the NOESY spectrum, suggests regions of ordered structure. Fig. 2 shows the resonances that represent connectivities between amide protons in the H8BAR. A similar pattern is observed for the peptide in both DPC micelles and DMSO. Fig. 2 also shows the assignment of these resonances to the respective amino acids. The assignments indicate that the sequential low-field NOE patterns observed correspond to regions 1–5, 8–18 and 26–34 of the peptide in DMSO and 1–5, 10–16 and 28–33 in DPC micelles. The observations suggest that ordered structure would be expected in one or more of these regions of the peptide in both DPC micelles and DMSO.

2.4. Structure calculations

Table 2 shows proton chemical shifts for all the amino acid residues of H8BAR in DMSO. About 310 NOE peaks were collected from the NOESY spectrum obtained for the peptide in DMSO. Fig. 3 shows the distribution of some of the key NOE connectivities that are involved in folding the peptide into its respective conformation in solution. In particular the prevalence of αβ (i, $i+3$), αN (i, $i+3$), αN (i, $i+4$), NN (i, $i+1$) connectivities spanning the region S6-L16 indicates that this part of the sequence has a tendency to adopt a helical conformation in DMSO. These 11 residues comprise approximately 32% of the peptide in a defined helical conformation, similar to the results from the CD with DPC micelles. This result increases the likelihood that the conformation of the peptide in the DPC micelles is similar to the conformation of the peptide in DMSO. The absence of connectivities other than sequential interactions for the rest of the sequence precluded the determination of a well-ordered structure for the remainder of the peptide.

Distance-restrained simulated annealing was performed as described in Materials and methods. Fig. 4A shows the overlay of the resulting 10 energy-minimized structures. These structures formed a tight cluster in the region S6-C18 with a backbone RMSD of 1.2 Å, indicating that this part of the sequence is well defined in DMSO. Fig. 4B shows an average structure calculated from the 10 conformers obtained from distance-restricted simulated annealing. The average structure is characterized by a helical conformation starting at S6 and ending at L16.

3. Discussion

A number of studies have suggested an important role for H8 in GPCR signaling. For example, peptides of the sequence of H8 have been shown to inhibit the signaling

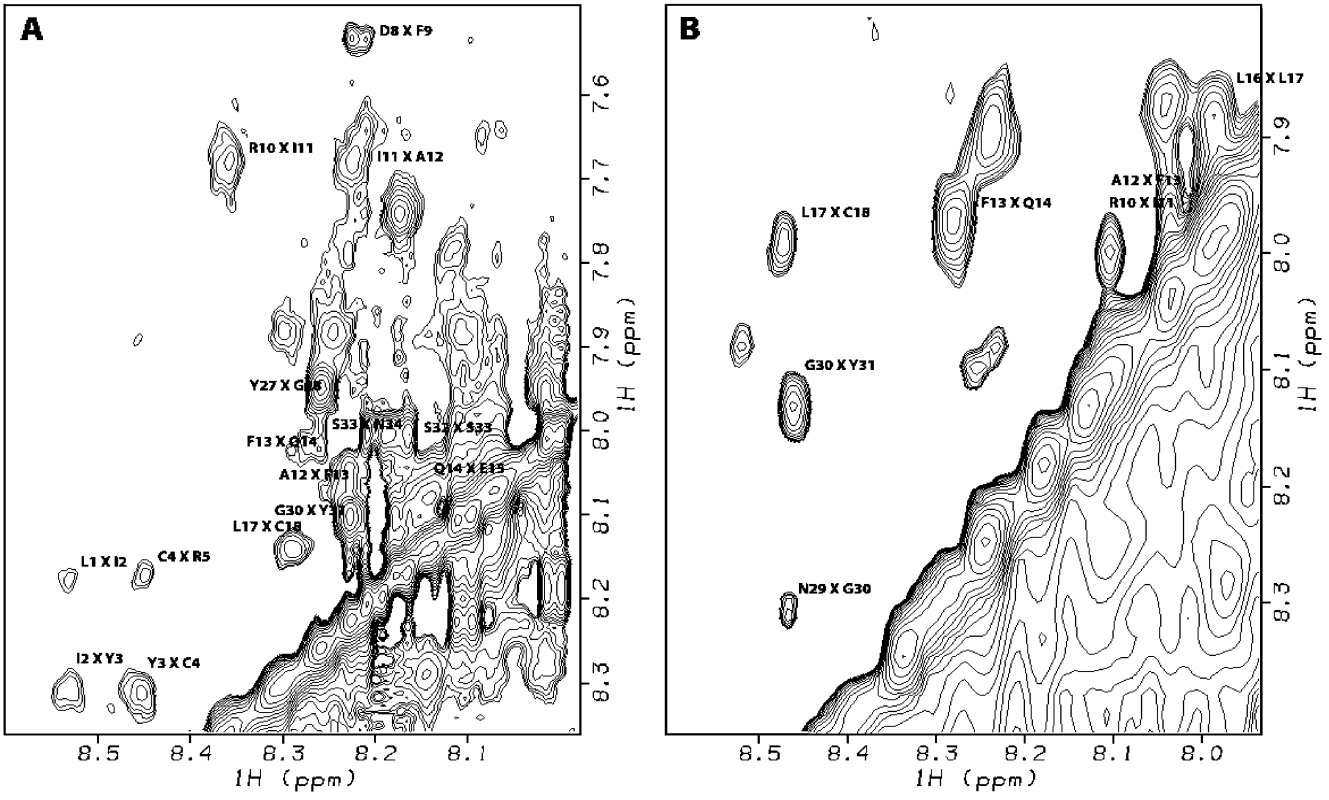

Fig. 2. Spectra showing the low-field region of NOESY spectrum for the peptide H8BAR in (A) DMSO and (B) in the presence of DPC micelles. All the residues are represented by single letter codes and their position in the H8BAR sequence.

Table 2
Table showing the proton chemical shifts obtained for the amino acid residues in the peptide H8BAR in DMSO

Residue	αH	NH	βH	Others
Leu1	3.93	8.17	1.55	1.63/0.91/0.94
Ile2	4.37	8.53	1.75	0.91/0.95/1.12
Tyr3	4.64	8.31	2.78, 2.98	6.70/7.09
Cys4	4.71	8.45	3.02, 3.14	
Arg5		8.17	1.77, 1.58	3.16/7.62
Ser6	4.53	8.23	3.71, 3.66	5.21
Pro7	4.41		2.04, 1.91	1.84/3.71/3.75
Asp8	4.54	8.21	2.48, 2.71	
Phe9	4.63	7.64	2.92	
Arg10	4.34	8.36	1.81, 1.66	
Ile11	4.33	7.68	1.81	1.13/0.88
Ala12	4.28	8.23	1.24	
Phe13	4.48	8.05	2.96, 3.16	
Gln14	4.30	8.06	1.99, 1.89	2.20/6.91/7.40
Glu15	4.36	8.13	1.96, 1.87	2.33
Leu16	4.37	8.08	1.56	0.92/0.96
Leu17		8.14	1.53	1.69/0.91/0.95
Cys18	4.67	8.30	2.99	
Leu19	4.44	7.88	1.53	0.88/0.92
Arg20	4.41	8.25	1.77, 1.61	1.38/3.18/7.64
Arg21	4.27	7.88	1.77, 1.68	
Ser22	4.46	8.10	3.63, 3.71	5.23
Ser23	4.41	8.14	3.68, 3.74	5.15
Leu24	4.36	8.10		0.88/0.92
Lys25	4.29	8.07	1.60, 1.72	1.38/2.84/7.74
Ala26	4.30	8.02	1.26	
Tyr27	4.49	7.95	2.80, 3.00	6.73/7.10
Gly28	3.84	8.26		
Asn29	4.65	8.22	2.57, 2.66	7.07/7.53
Gly30	3.70, 3.74	8.23		
Tyr31	4.57	8.10	2.78, 3.04	6.73/7.13
Ser32	4.46	8.17	3.69, 3.73	5.12
Ser33	4.42	8.00	3.71	4.95
Asn34	4.61	8.20	2.57, 2.64	

of rhodopsin [32], the β_2AR [33], and the CB1 receptor [34], and to activate G_i and G_o proteins [35]. Therefore, H8 constitutes a region of high interest in the structure of GPCRs (Fig. 5).

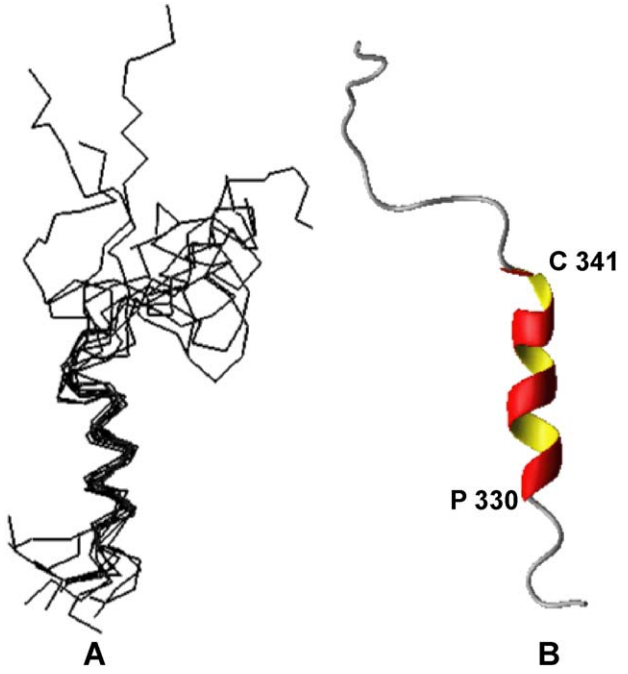

Fig. 4. (A) overlay of the 10 energy-minimized conformers obtained from the simulated annealing calculations. (B) Average structure calculated from the 10 conformers shown in A. The part of the sequence that adopts helical conformation and designated as helix 8 (H8) is shown in red and yellow. The labels represent the residue number in the original sequence of β_2AR.

CD and NMR have been employed to study the conformational preference in different environments of H8 from hβ_2AR. The CD experiments and connectivity patterns in the low-field region of the NOESY spectrum obtained for H8 of hβ_2AR in DPC micelles show that the sequence corresponding to H8 of hβ_2AR has a propensity to adopt a helical conformation in the presence of DPC micelles (Fig. 4A). Previous studies on analogous peptides from adrenoreceptor from turkey erythrocytes [36], angiotensin II receptor [37] and CB2 receptor [31] suggested a

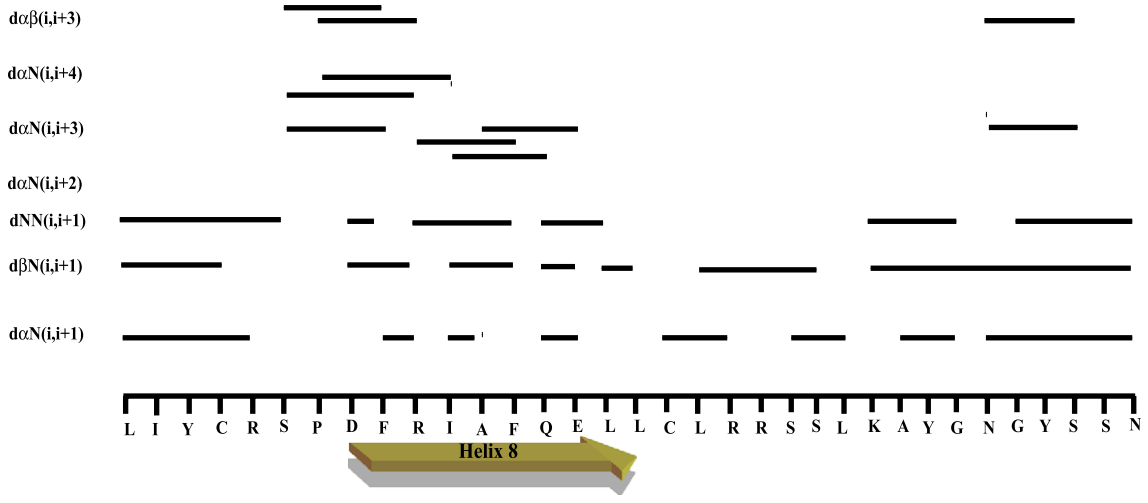

Fig. 3. Connectivity plot showing the NOEs observed for the peptide H8BAR in DMSO.

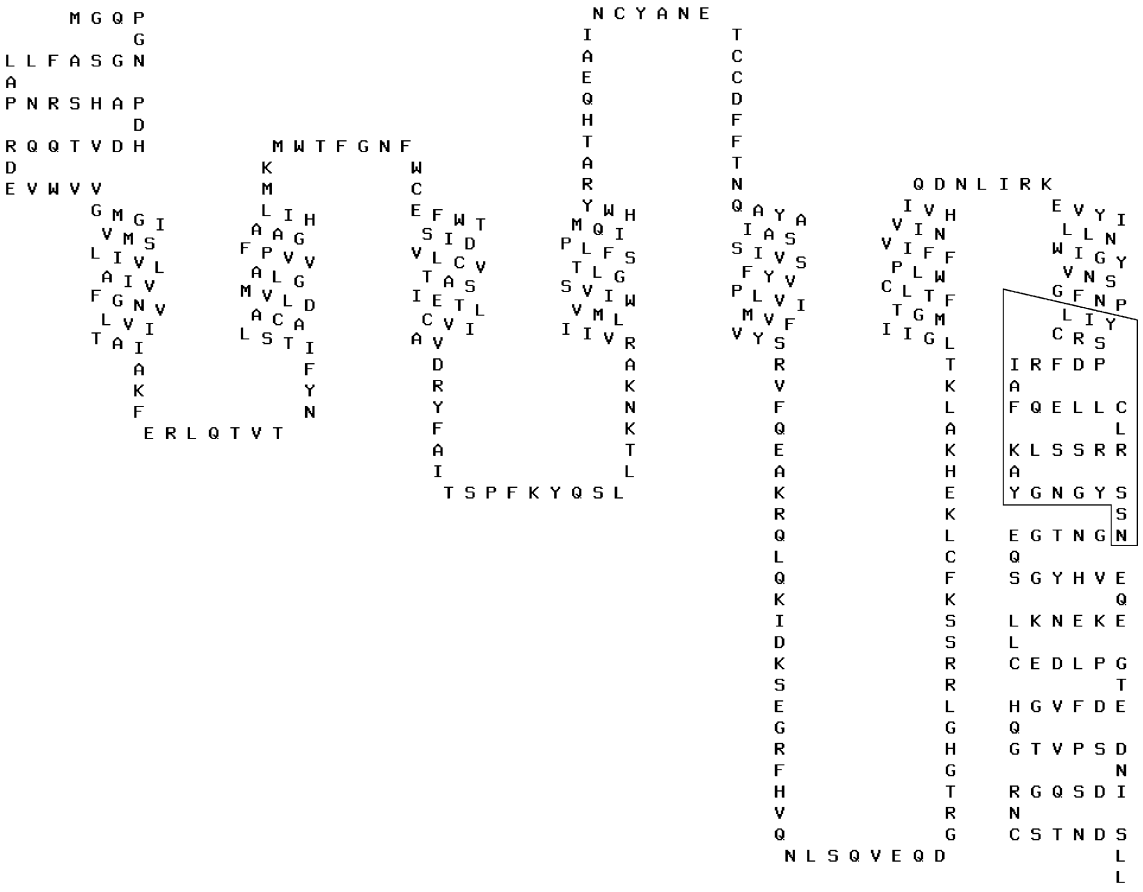

Fig. 5. Schematic representation of the sequence of β_2AR. The sequence of H8 is enclosed in a box. (Figure adapted from Viseur diagram: F. Campagne and B. Maigret, Laboratoire de Chimie Théorique de Nancy, U.A. CNRS 510 B.P. 239-54506, Vandoeuvre-les-Nancy CEDEX France and J.M. Bernassau Sanofi Recherche, 371, Rue du Pr. Blayac, 34184 Montpellier CEDEX 4 France.)

helical conformation for the H8 domain in the presence of detergent micelles.

As complete structural details could not be obtained for H8 of hβ_2AR in DPC micelles, we determined the structure of this peptide in DMSO using NMR. The part of the peptide (P7-L16) that is analogous to H8 in other GPCRs studied also adopted a helical conformation in DMSO (Fig. 4B). An analogous sequence derived from cannabinoid 2 (CB2) receptor also adopted a helical conformation in DMSO [31].

In contrast to the predominantly helical conformation observed in the presence of DPC micelles and in DMSO, CD and NMR studies revealed that this peptide was disordered in water. These data collectively suggest that the sequence P7-L16 in H8BAR adopts conformations dependent upon environment: a helical conformation in DMSO and in the presence of DPC micelles, and a disordered structure in water. By comparison, H8 in rhodopsin forms a helix as part of the whole protein in the presence of detergent [1], while in an aqueous environment H8 of rhodopsin does not form a helix, but rather a loop [38]. The data suggest that the conformational flexibility noted here for H8 is a feature of both hβ_2AR and rhodopsin, two GPCRs of the same family. Work on three other receptors shows that H8 in other GPCRs follows a similar pattern. Protein fragments of CB2 [31], the turkey adrenoreceptor [36], and the angiotensin II AT1A receptor [37], each corresponding to H8, also are disordered in water like H8BAR, but ordered in a helix when bound to the surface of a detergent micelle or in certain nonaqueous solvents.

Fig. 6 schematically summarizes the structural information that is now known about H8 in GPCRs. Five GPCRs have been studied in this regard. H8 from all five receptors is non-helical in aqueous solvent. H8 from all five receptors is helical under some specific conditions. Therefore, even though there is little sequence homology among these five receptors, there is structural homology. Furthermore, H8 is conformationally flexible for all five GPCRs. This conformational flexibility is likely a functionally important feature of the receptor.

Several studies have been carried out to address the role of helix 8 in signaling, mostly with rhodopsin. Site-directed mutagenesis studies carried out by Altenbach et al. [2] and Krishna et al. [3] suggested that this domain in rhodopsin could adopt a loop-like structure when the receptor is activated, in agreement with the structural studies summarized above. Thus, the H8 domain may be acting in part as a conformational switch between inactive and active states of

298IYALRSGEIRSSAHHCLAHWKK319 CB2 receptor
324LIYCRSPDFRIAFQELLCLRRSSLKAYGNGYSSN357 Hβ2AR
345RSPDFRKAFKRLLCF359 turkey adrenoreceptor
302NPVIYIMMNKQFRNCMVTTLCCGKNP327 rhodopsin
300LFYGFLGKKFKKYFLQLLKYI320 angiotensin II AT1A Receptor

Fig. 6. Sequences of GPCR fragments, corresponding approximately to H8, for which structural information has been obtained (CB2 [31], the turkey adrenoreceptor [36], the angiotensin II AT1A receptor [37], Hβ2AR (this work) and rhodopsin [1]). The regions that have been shown to be helical in micelles or in specific nonaqueous solvents are in the shaded boxes. In each case, these same regions are disordered in aqueous solvent (as described in the text). The information for rhodopsin is from a different source. In the shaded box is the region in the crystal structure of the intact receptor identified as H8, obtained in the presence of detergents. This same region is non-helical, though not disordered, in aqueous solvent, as described in the text. The tentative alignment of these sequences is based on the NPxxY sequence, which is highly conserved in GPCRs, and is underlined for rhodopsin.

the receptor. Studies on analogous peptide sequences derived from cannabinoid (CB1) receptors suggested that H8 exists in a non-helical conformation when interacting with the G-protein [34,39], while the available data show that H8 domain is a helix in the inactive state of the receptor. Thus, due to its conformational flexibility and implications in G-protein activation, H8 is a critical domain of the receptor to understand.

The question then arises: what factors may stabilize one conformation over the other, and thus possibly influence the activation of the receptor? The data suggest that H8 in seven TM GPCRs is amphipathic. Fig. 7 shows a helical wheel representation of the sequence of H8 of hβ2AR in the H8BAR sequence. As seen in the figure, one side of the helix is defined by only hydrophobic residues while the other side is dominated by polar residues indicating that H8 is amphipathic. H8 of leukotriene B4 receptor is amphipathic [4] as is H8 of rhodopsin. The amphipathic nature of H8 suggests that this domain would likely bind to the membrane surface, possibly acting as a membrane recognition domain.

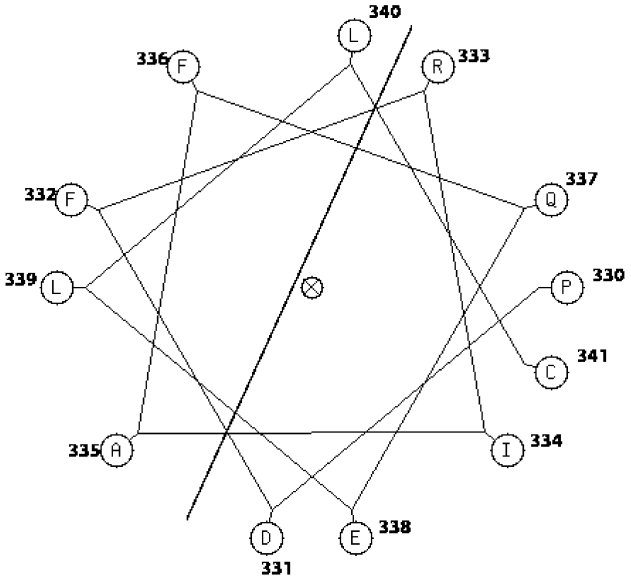

Fig. 7. Helical wheel representation of H8. The line drawn across the wheel approximately divides the hydrophobic face from the hydrophilic face.

An overlay of the H8 of hβ2AR on to the H8 of bovine rhodopsin indicates that the hydrophobic face of both the helices orient in the same direction. Thus, it can be reasonably assumed that H8 of hβ2AR could bind to the membrane surface similarly to H8 of rhodopsin. Beck et al. [40] suggested that rhodopsin exhibits specific lipid binding, which is altered upon illumination of the receptor. These findings are supported by recent studies carried out by Krishna et al. [3], where they showed that the secondary structure characteristics of helix 8 depend on membrane lipid composition, with increasing levels of phosphatidylserine (PS) inducing helical conformation. Studies by Crain et al. [41] showed that some of the PS cannot be labeled in rod outer segment disk membranes possibly because PS is protected from labeling by binding to rhodopsin. Upon activation of the receptor, the binding between PS and H8 was shown to be altered and PS redistributed in the membrane [40]. Hence, a hypothesis can be considered in which PS binds to H8 stabilizing the helix, and the helix converts to a loop-like conformation upon excitation of the receptor.

In addition to the membrane recognition property, the N-terminus of H8 of bovine rhodopsin was previously shown to be involved in initiating transducin binding [32,42]. The involvement of the N-terminus of helix 8 in the binding of the cognate G-protein was shown for other receptors like rat angiotensin II receptor [35,43,44] and the neutrophil n-formyl peptide receptor [45]. Thus, the H8 domain may act as a bifunctional structural motif in the receptor activation.

In summary, H8 of the hβ2AR can exist in two different conformations, one a helix and the other non-helical. The conformation is dependent upon the environment. These data provide a reasonable framework for a model of a conformational change in the GPCR possibly related to receptor activation and influenced by lipid composition of the membrane.

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