Protein Structures

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The Structure of the Neuropeptide Bradykinin Bound to the Human G-Protein Coupled Receptor Bradykinin B2 as Determined by Solid-State NMR Spectroscopy**

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G-protein coupled receptors (GPCRs) are responsible for a large number of physiological processes, such as sensory transduction, mediation of hormonal activity, and cell-to-cell communication.[1,2] GPCRs are membrane proteins with seven transmembrane helices and are the target of some 50% of modern drugs. They constitute the largest known protein family and have had more than 800 species identified in the search for medical solutions to human illnesses.[3,4] However, owing to the lack of three-dimensional structures, structure-based drug design has not been possible. To date, the structures of only two GPCRs have been solved.[5,6] Pharmacological research aimed at GPCRs is therefore restricted to mostly computational and experimental trial-and-error approaches.[7] This limitation could be potentially overcome by determining the structures of bound agonists, which activate GPCRs, and using these as structural templates for drug design. To do so, the availability of GPCRs needs to be increased.[7–9] Herein, we describe the backbone structure of the agonist bradykinin bound to the human bradykinin B2 receptor, which was determined by solid-state NMR spectroscopy. This is only the second detailed investigation of its kind.[10]

Bradykinin (BK, $R_1P_2P_3G_4F_5S_6P_7F_8R_9$) is a hormone which, once released into the bloodstream, binds to both bradykinin receptor subtypes B_1R (ca. 37 kDa) and B_2R (ca. 44 kDa). These receptors are involved in a variety of pathophysiological responses, such as inflammation, blood vessel dilation, pain,[11,12] and higher brain function.[13] Studies indicate that targeting B_2R could lead to the development of cures for chronic epilepsy.[14,15] The pharmacological importance of BK has elicited considerable interest in the design of new antagonists for both receptors.[16–18] A plethora of structural studies using circular dichroism (CD) as well as fluorescence, EPR, and solution-state NMR spectroscopy has been carried out on the peptide alone.[19–31] The only structural models of receptor-bound BK were derived from liquid-state NMR spectroscopy experiments using a receptor-mimicking antibody fragment[28,32] and from an extensive computational study which simulated the docking of BK to a homology model of rat B_2R.[25] Despite promising results, the authors of the latter state that "much of the model is speculative", owing to lack of experimental data.[25] To date, a structural investigation of B_2R-bound BK was prevented by the lack of 1) B_2R availability at a preparative scale and 2) a method for obtaining structural data of high-affinity ligands bound to large membrane proteins. Recently, B_2R could be overexpressed in mammalian cells and insect cells.[33–35] Furthermore, solid-state NMR spectroscopy has progressed, and data of high-affinity ligands bound to large membrane proteins in a noncrystalline state have been reported.[10,36,37]

These advances have enabled us to carry out solid-state NMR spectroscopy experiments on B_2R in complex with ^{13}C-labeled bradykinin. Purified B_2R was solubilized in DDM (dodecyl maltoside) micelles. We refrained from experiments with B_2R embedded within lipid bilayers, because the limited amount of receptor did not allow reconstitution screens. Moreover, DDM offers a membrane-mimicking environment suitable for the purpose of this study, as the peptide binding affinity is identical to that of B_2R in native membranes (see the Experimental Section). Furthermore, lipid-reconstituted B_2R would have only half of the binding sites exposed to the ligand, as approximately half of the receptor molecules would orient themselves facing the outside (and inside) of the liposome. Two different labeling schemes were applied to bradykinin: 1) uniform ^{13}C labeling of residues P_2, P_3, G_4, F_5, S_6, P_7, F_8 (BK7) and 2) uniform ^{13}C labeling of residues F_8 and R_9 (BK2). Both samples for NMR spectroscopy contained approximately 1.5 mg B_2R and 25 μg BK2 or BK7. The large ^{13}C natural abundance background stemming from B_2R and DDM, and the small number of spins from BK, poses a challenge for signal detection and resonance assignment.

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These problems can be addressed by dipolar INADEQUATE-type double-quantum/single-quantum correlation (DQSQ)[38] experiments carried out at low temperature under magic-angle spinning (MAS) conditions. Characteristic ^{13}C DQSQ spectra of BK7, both bound to B_2R and in DDM alone, are shown in Figure 1a,b (for further spectra, see the Supporting Information). "Assignment walks", indicated by horizontal and vertical lines in Figure 1, allowed chemical shift (CS) measurements of all carbon atoms in the BK backbone. Amino acid types were identified by their characteristic patterns. Owing to the restricted number of amino acids of the peptide ligand, sequential assignment was not necessary. The same chemical shifts were obtained for residues of the same type.

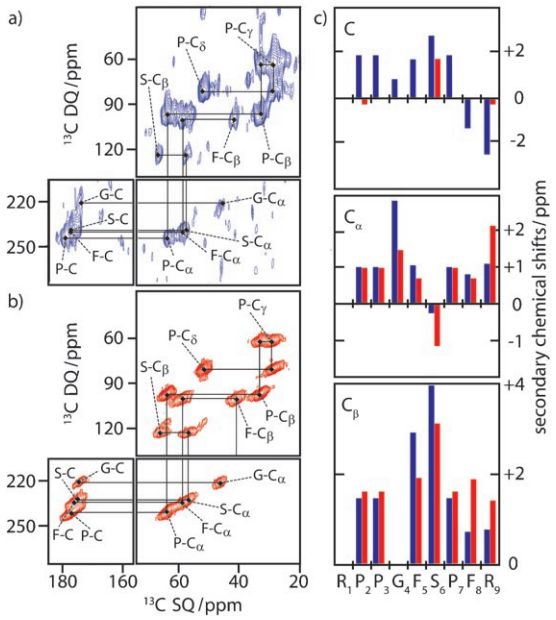

Figure 1. 2D DQSQ spectra of BK7 bound to a) B_2R and b) DDM. INADEQUATE "assignment walks" are indicated by horizontal and vertical lines. Secondary backbone CS $\Delta\delta$ are plotted in (c). Discrepancies in secondary CS values point to distinct backbone conformations of BK in B_2R (blue) and in DDM (red). Please see the Supporting Information for further details.

Information about the BK backbone torsion angles ϕ, ψ is contained in the "secondary chemical shift" $\Delta\delta$, which describes the deviation of CS values of a folded protein from random-coil CS values.[39–41] Figure 1c shows a bar plot depicting $\Delta\delta$ for the backbone atoms C, C_α, and C_β, for BK in DDM (red) and bound to B_2R (blue). A comparison of $\Delta\delta$ for the receptor-bound and the micelle-dissolved states clearly indicates two different backbone structures. Using the program TALOS,[42] torsion-angle restraints were derived from $\Delta\delta$ and used for structure calculations with the help of CYANA.[43] Backbone structures of BK bound to B_2R are depicted in Figure 2a. The 500 lowest-energy structures converge with an average backbone root-mean-square deviation (RMSD) of 0.5 ± 0.2 Å. The structure of BK is elongated along the center with a C-terminal β turn ($S_6P_7F_8R_9$) and an N-terminal α-helical bend ($R_1P_2P_3G_4$). Residues P_2, P_3, and P_7 are found in their all-*trans* conformations, as indicated by differences of approximately 5 ppm in their C_β and C_γ chemical shifts.[44] The most coherent superposition of the structures of BK was found for amino acids $S_6P_7F_8$, indicating high structural homogeneity at the C-terminal end and less order at the N terminus. This result is consistent with immunology assays, which have shown the C-terminal end and especially R_9 to be essential for receptor binding.[45]

It is worth noting that the CS values recalculated from the 500 lowest-energy structures exhibit a spread which lies within the line width of the experimental spectra (see Figure S3 in the Supporting Information for the SHIFTX analysis). This finding indicates that the line widths in our spectra are caused by structural flexibility of the ligand trapped during freezing of the sample, which agrees with the line widths reported by others for similar cases.[10,36] We have also predicted a large number of backbone torsion angles based on sets of chemical shifts, which were randomly sampled within the line widths observed in our spectra. With the exception of some outliers, similar torsion-angle ranges are obtained which are comparable to the set of CS values obtained from the peak maxima along the assignment walk (see Figure S4 in the Supporting Information).

Kyle et al.[25] have used a rat B_2R homology model for docking simulations of BK to derive a structural model. The BK C-terminal β turn is thought to be buried in B2 just below the extracellular boundary of the cell membrane, and the N terminus is thought to interact with negatively charged residues D268 and D286 in the B2 coils.[25] This interpretation

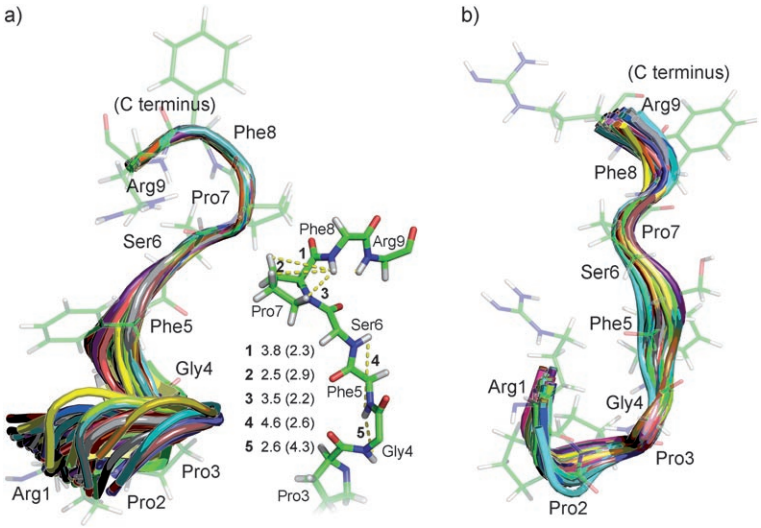

Figure 2. Backbone structure models of BK a) bound to human B_2R in DDM and b) in DDM alone. In both cases, 100 structures of lowest energy were superimposed. Relevant distances (see text) for BK bound to B2 are indicated using the stick model in (a).

would be supported by the higher structural order we find at the C terminus and the less well defined N terminus.

In a solution NMR spectroscopy study using a receptor mimic, Ottleben et al.[28,32] derived structural features for BK which partly contradict the model put forth by Kyle et al. On the basis of NOE peaks, distances between amide proton pairs along the ligand backbone were estimated (indicated in Figure 2a). We list the estimates by Ottleben et al. (and by Kyle et al., in brackets), in Å: amide proton distances for G_4–F_5 2.6 (4.3) and F_5–S_6 4.6 (2.6). For distances between the F_8 amide proton and P_7 ring protons H_β, H_γ, and H_δ, the model of Kyle et al. predicts distances of 2.3, 2.9, and 2.2 Å. Ottleben et al., however, detect no NOE cross-peaks for these proton pairs, which is in agreement with the distance values we derive: 3.8, 2.5, and 3.5 Å. As indicated in Figure 2a, our structure, while exhibiting distances which account comprehensively for the experimental findings of Ottleben et al., also agrees with the overall description of Kyle's model (C-terminal β turn, "twisted S" structure).

The result of backbone structure calculations of BK in the presence of DDM is shown in Figure 2b. A convergence of the resulting models is indicated by an average backbone RMSD of 0.4 ± 0.1 Å. BK assumes a defined structure in the presence of DDM, with a β turn at the C terminus and an elongated backbone.

The conformations of BK have been studied extensively, using CD, fluorescence resonance energy transfer (FRET), EPR, $^1H/^{13}C$ NMR spectroscopy, and molecular dynamics simulations.[19–23,26,27,29–31,46–49] In water, BK possesses a large degree of conformational flexibility and does not adopt a single preferred stable conformation. In an environment with apolar features, or in aqueous mixtures with amphiphilic molecules, BK exhibits a β-type turn made up of the four C-terminal amino acids $S_6P_7F_8R_9$. These studies show a general agreement with the DDM structure pictured in Figure 2b.

It is of interest to note that BK assumes an ordered structure, distinct from the GPCR-bound form, in the presence of micelles. This could imply that, in the mechanism leading to the recognition and binding of the peptide, a certain preorganization in the membrane might take place. Further research, however, would best entail a detailed study using native lipids.

In summary, the backbone structure of BK bound to human B_2R has been determined and shows a distinct double-S-shaped structure. This is the first time that the structure of ligand bound to a human GPCR has been obtained. Our data offer a template for potential drug design. This study and the recent results obtained elsewhere[8,10] highlight the fact that solid-state NMR spectroscopy has become a valuable option for ligand-receptor studies.

Experimental Section

Preparation of bradykinin (BK): Two variants of BK ($R_1P_2P_3G_4F_5S_6P_7F_8R_9$) were synthesized: BK7 (U-^{13}C labeling of all residues except R_1 and R_9) and BK2 (U-^{13}C labeling of F_8, R_9) using standard 9-fluorenylmethyloxycarbonyl (FMOC) solid-state procedures. Peptide purity was higher than 95% as determined by HPLC and electrospray mass spectroscopy.

Preparation of human GPCR B_2R: B_2R was produced in Sf9 cells using the baculovirus system. Expression constructs and optimized production conditions were reported earlier.[33–35] Large-scale cultures (25 L) were grown using a Wave bioreactor (SYSTEM20/50, WAVE BIOTECH). Membrane preparation and [^{3}H] BK binding experiments were performed as described in references [33–35]. K_d values of 0.4 ± 0.1 nM were obtained and compare favorably to those reported for human decidual cells (0.85 nM).[50] To solubilize the receptors, membranes (5 mg mL^{-1}) were resuspended in buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 100 mM NaCl, and protease inhibitors) containing DDM and 0.2% cholesterol hemisuccinate. The suspension was incubated for 2 h with gentle mixing at 4 °C and ultracentrifuged at 100 000 *g* for 1–2 h. The supernatant containing solubilized B_2R was used for purification. Solubilizate (ca. 200 mL), was filtered through 0.65-μm filters and incubated with pre-equilibrated (10–15 mL) Ni-NTA (NTA = nitrilotriacetic acid) superflow resin (Quiagen) for 2 h at 4 °C. The mixture was packed into a column and washed with 15–20 column volumes of buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% DDM, protease inhibitors, 40–60 mM imidazole). B_2R was eluted with 5–10 column volumes of buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% DDM, protease inhibitors, 200 mM imidazole). Purified protein was concentrated using Vivaspin protein concentrators (MW cutoff 50 kDa) until the desired volume was achieved. B_2R was further purified using gel filtration chromatography. Concentrated eluate from the Ni-NTA column (50–100 μL at 5–10 mg mL^{-1}) was applied to a pre-equilibrated Superose 6 PC 3.2/30 column (Pharmacia). The sample was resolved using gel filtration buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% DDM, 2 mM ethylenediaminetetraacetic acid (EDTA)) at a flow rate of 50 μL min^{-1}. On the basis of total protein determination (using BCA protein assay kit, Peirce) and [^{3}H] BK binding analysis, a specific activity of 9–11 nmol mg^{-1} B_2R was calculated. A K_d value of 1.0 ± 0.2 nM was obtained.

For NMR spectroscopy samples, purified B_2R (0.8–1.0 mg) was incubated with BK (ca. 220 μg) at 4 °C for 2 h (BK/B2 molar ratio of ca. 10). To remove unbound BK, repeated dilution (up to 1:100) and concentration of B2–BK was performed with a final volume of 70 μL (at 14–17 mg mL^{-1}). Then the concentrated sample was transferred to a 4-mm MAS rotor and flash frozen in liquid nitrogen. Two samples were available for NMR spectroscopy analysis, each with 1.5 mg B_2R in complex with 25 μg BK.

Solid-state NMR spectroscopy and data analysis: NMR spectroscopy experiments were carried out on a Bruker Avance 600 spectrometer with a widebore magnet (Bruker Biospin, Rheinstetten, Germany) using a triple-resonance (^{1}H, ^{13}C, ^{15}N), 4-mm MAS DVT probe head modified for low-temperature experiments. Experiments were performed at 200 K. DQSQ spectra of BK in DDM and bound to B_2R typically took approximately 2 and 10 days of measuring time. All spectra were referenced to tetramethylsilane (0 ppm, at 10 kHz spin rate, 250 K). MAS spinning rates of 10 kHz were used with SPINAL64[51] decoupling (55 kHz) during signal detection. For ^{13}C cross polarization, a ^{1}H 90° pulse of 3.25 μs was used, and a ramped 40-kHz ^{1}H and 50-kHz ^{13}C spin lock field with a contact time of 750 μs was applied. DQF experiments were carried out with the POST-C7[52] dipolar recoupling scheme with symmetric dipolar excitation and reconversion durations of 457 μs. Processing and assignments were carried out with Topspin 1.3 (Bruker Biospin) and Sparky.[53] Assignments were aided using "SpectrumViewer", an interactive Python tool written in house.

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