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Comparative NMR analysis of an 80-residue G protein-coupled receptor fragment in two membrane mimetic environments

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ABSTRACT

Fragments of integral membrane proteins have been used to study the physical chemical properties of regions of transporters and receptors. Ste2p(G31-T110) is an 80-residue polypeptide which contains a portion of the N-terminal domain, transmembrane domain 1 (TM1), intracellular loop 1, TM2 and part of extracellular loop 1 of the α-factor receptor (Ste2p) from *Saccharomyces cerevisiae*. The structure of this peptide was previously determined to form a helical hairpin in lyso-palmitoylphosphatidyl-glycerol micelles (LPPG) [1]. Herein, we perform a systematic comparison of the structure of this protein fragment in micelles and trifluoroethanol (TFE):water in order to understand whether spectra recorded in organic:aqueous medium can facilitate the structure determination in a micellar environment. Using uniformly labeled peptide and peptide selectively protonated on Ile, Val and Leu methyl groups in a perdeuterated background and a broad set of 3D NMR experiments we assigned 89% of the observable atoms. NOEs and chemical shift analysis were used to define the helical regions of the fragment. Together with constraints from paramagnetic spin labeling, NOEs were used to calculate a transiently folded helical hairpin structure for this peptide in TFE:water. Correlation of chemical shifts was insufficient to transfer assignments from TFE:water to LPPG spectra in the absence of further information.

1. Introduction

Integral membrane proteins (IMPs) are a class of proteins that are difficult to characterize structurally. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have been used to study the high-resolution structures of many proteins and the Protein Data Bank currently has more than 69,000 structures of mainly soluble proteins. The number of unique IMP structures deposited towards the end of 2010 was approximately 270, or less than 0.5% of all the structures in the Data Bank [2,3]. The underrepresentation of IMP structures in the database belies the importance of these proteins. Many membrane proteins are involved in signal transduction, nutrient uptake and various diseases. In fact, 30–60% of the current drugs on the market are targeted to membrane proteins [4–6] and structural information on these proteins is important for the development of more specific drugs that could increase efficacy and decrease side effects.

Crystallization of membrane proteins is complicated by their inherent flexibility and can be hampered by protein–lipid interactions that are required for function. Solution NMR investigations of IMPs require solubilization in a membrane mimetic environment and are hindered by the size of the protein–lipid complex and conformational exchange processes. G protein-coupled receptors (GPCRs) have molecular weights of approximately 50 kDa and size increases in detergent micelles or lipid bicelles result in slow tumbling and short relaxation times with a concomitant loss of signal. Solid state NMR, a technique in which the quality of spectra is less dramatically affected by the size of the protein–lipid complex, has also been used in the structure determination of membrane proteins in lipids [reviewed in 7].

Solution NMR has been very useful when studying protein dynamics and/or protein–ligand interactions. Tools are being developed to better study large protein–lipid complexes in solution [reviewed in 8], but high resolution structures remain elusive [9–11]. Recently, the structure of sensory rhodopsin, a seven transmembrane (TM) GPCR homolog, was determined using a highly deuterated form of the protein which relied on selective introduction of protons into Ile, Leu and Val as well as a protonated protein to determine more methyl group NOEs and aromatic interactions [12,13]. Although this structure represents an important

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milestone in GPCR structural biology, sensory rhodopsin is much smaller than most eukaryotic GPCRs, and it is not clear whether this approach can be generalized to this family of molecules. In the interim we are studying fragments of GPCRs to learn more about the folding of these IMPs, to investigate helix–helix interactions, to develop methodologies that may be useful in the assignment of the NMR spectrum of the entire GPCR, and to gain insights into the secondary and tertiary structures of large but discrete domains of these receptors.

To apply this strategy it is important to work with fragments that are large enough to potentially adopt a tertiary structure and which may also allow testing of ligand binding. The study of GPCR fragments is thought to be valid because these have been shown to reconstitute to form functional proteins both *in vitro* and *in vivo*[14–18]. Furthermore, numerous biological and biochemical studies on Ste2p have been used to elucidate its interaction with the tridecapeptide α-factor ligand and to decipher changes in the structure upon activation of the signal transduction pathway [19–28]. This data will help to place the biophysical analysis of fragments of Ste2p into a biological context.

Polypeptide fragments of Ste2p, including each single TM, loops and the C-terminal tail have been previously studied by solution NMR [29–32] (Bhuiyan unpublished data) and have provided details on the structural tendencies of discrete regions of this GPCR. A high-resolution structure of a peptide containing 2TMs, Ste2p(G31-T110), has been determined in lyso-palmitoylphosphatidyl-glycerol (LPPG) micelles [1]. Based on the fact that a dimer of Ste2p(G31-T110) in micelles is expected to have a fairly long correlation time and that the NMR spectral lines were sharp and of good quality we concluded that the peptide is a monomer [1]. Long-range connectivities were observed between hydrophobic residues of TM1 and TM2 when selective methyl group labeling was used. Comparison of the structures of a GPCR fragment in different membrane mimetic media would be useful to ascertain whether micelles and trifluorethanol (TFE):water stabilize similar structures and whether assignments obtained in organic:aqueous media are relevant to the micellar state. If so, the organic:aqueous medium might be extremely valuable in analyzing membrane proteins of increasing size.

Organic:aqueous media have been used for biophysical analysis of hydrophobic peptides for some time, due to their spectroscopic properties which are ideal for circular dichroism and NMR analyses [33–37]. The correlation time of an IMP fragment in an organic: aqueous solvent is much shorter than when embedded in micelles or bicelles resulting in superior spectra. Media such as TFE:water are not considered to be biologically relevant by some investigators [38,39], although this conclusion has never been systematically evaluated. Indeed, some fragments of both transporters and receptors exhibit highly resolved spectra and some even exhibit long-range contacts in TFE and chloroform:methanol:water and appear to assume folded structures that may be relevant to the biologically active state [31,40–42]. Intrinsically disordered proteins can be studied in solution, micelles and TFE:water. A protein found in human semen that has been shown to enhance HIV viral infection, SEVI, folds into different conformations dependent on the environment [43]. Conversely, structural intermediates in the folding pathway of α-synuclein have been observed both in TFE:water and in micelles [44,45] indicating that the folding pathway may be similar in both environments.

Here we describe our efforts aimed at characterizing the structure of Ste2p(G31-T110; TM1–TM2), an 80-residue two-TM containing GPCR fragment, in organic:aqueous media (TFE:water). The structure of TM1–TM2 was assessed using solution NMR and uniformly labeled peptide and peptide selectively protonated on Ile, Val and Leu methyl groups in an otherwise perdeuterated background. To probe for the presence of interhelical contacts we also utilized an analog of TM1–TM2 in which a nitroxide radical was conjugated to an introduced Cys residue. TM1–TM2 assumes distinct helical regions corresponding to the predicted TM domains of the receptor and also shows a tendency to assume a helix in what would be the N-terminal region of the protein. Spin-labeling data indicated that TM1–TM2 undergoes conformational averaging in which the individual helices form mutual transient contacts. Comparison of our results with those from a previous study in LPPG micelles [1] indicates that lipid–protein interactions within the context of bilayer or detergent micelles may be critical for the formation of stable tertiary structure of this 2TM GPCR fragment.

2. Experimental methods

2.1. NMR sample preparation

NMR samples containing 0.5–4 mg of isotopically labeled TM1–TM2 were prepared in 350 μL TFE-d_2:water (0.1% TFA) (1:1, v:v) or TFE-d_3:D_2O (0.1% TFA-d) (1:1, v:v). The samples were prepared by the addition of 175 μL TFE followed by 175 μL water (0.1% TFA). Sample preparation of amide proton-exchanged peptides were first solubilized in a large excess of TFE:D_2O (0.1% TFA) and incubated at room temperature overnight or at 50 °C for 1 h to exchange a portion of the amide protons to deuterons and then lyophilized. The resulting peptide was solubilized in TFE-d_3:D_2O (0.1% TFA-d) as described above.

2.2. NMR measurements

The NMR experiments were performed on a Varian 600 MHz spectrometer at the College of Staten Island, CUNY, a Bruker Avance 700 MHz spectrometer at the University of Zurich, Switzerland, or a Bruker 900 MHz spectrometer at the New York Structural Biology Center (NYSBC) in New York City. All measurements were conducted at 25 °C or 45 °C using 0.14 mM to1 mM samples.

2.3. NMR experiments

The experimental parameters are defined in Supplementary Table S1. Sample stability and initial [^{15}N,^{1}H]-HSQC fingerprinting analysis was performed at 45 °C, 35 °C, 25 °C and 15 °C [46]. The backbone resonances were assigned by performing HNCO [47–50], HN(CA)CO [51], HNCA [47–50] and HNCACB [49,50,52] experiments at 45 °C and 25 °C. The ^{15}N-resolved NOESY-HSQC [46,53] and the ^{15}N-resolved TOCSY-HSQC [46,53] were performed at 45 °C and 25 °C to assign side chain proton chemical shifts and an HCCH-TOCSY experiment was performed at 25 °C to assign carbon and proton side chain resonances [54,55]. The chemical shifts of methyl protons and carbons of the Ile, Leu and Val residues were deduced from Ile, Leu-(HM)CM(CGCBCA)NH and Val-(HM)CM(CBCA)NH [56] and ct-[^{13}C,^{1}H]-HSQC [57] experiments performed on [^{15}N,^{13}C,^{2}H(^{1}H(methyl)-Ile, Leu, Val)]-TM1–TM2. Further side chain analysis was performed with 3D ^{13}C-edited TOCSY and NOESY experiments at 25 °C. Unlabeled TM1–TM2 was used in 300 or 150 ms NOESY experiments [58] and 60 or 25 ms TOCSY experiments [59,60]. The [^{15}N,^{13}C,^{2}H(^{1}H(methyl)-Ile, Leu, Val)]-TM1-TM2 peptide was also used in NOESY-ct-[^{13}C,^{1}H]-HSQC [46,61,62] and ct-[^{13}C,^{1}H]-HSQC-NOESY-ct-[^{13}C,^{1}H]-HSQC [63] to determine long-range NOEs between the methyl groups of Ile, Leu or Val residues.

Dynamics of [^{15}N]-TM1–TM2 were analyzed by performing [^{15}N,^{1}H]-HSQC versions of CPMG experiments with variable relaxation delays to determine T2 relaxation at 45 °C and 25 °C. A [^{15}N,^{1}H]-HSQC version of the steady-state NOE was measured to determine the heteronuclear NOE [64]. [^{15}N]-TM1–TM2 was solubilized in TFE-d_3:D_2O (0.1% TFA-d) and [^{15}N,^{1}H]-HSQC experiments were performed over time to follow the disappearance of signals.

2.4. Structure calculations

Assignments were determined by spectral analysis using the programs NMRView 5 [65] and CARA [66]. NOESY spectra were automatically assigned based on the assigned chemical shift lists using