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ARTICLE

Isotope labeling of mammalian GPCRs in HEK293 cells and characterization of the C-terminus of bovine rhodopsin by high resolution liquid NMR spectroscopy

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Abstract High amino acid coverage labeling of the mammalian G protein coupled receptors (GPCR) rhodopsin was established with ^{15}N and $^{15}N/^{13}C$ isotopes. Rhodopsin was expressed at preparative scale in HEK293S cells and studied in full-length by NMR spectroscopy in detergent micelle solution. This resulted in the assignment and detailed study of the dynamic properties of the C-terminus of rhodopsin. The rhodopsin C-terminus is immobilized until Ala333, after which it becomes unstructured.

Keywords NMR spectroscopy · Mammalian GPCR · Rhodopsin · Isotope labeling

Introduction

G protein coupled receptors represent a large superfamily of signaling proteins which respond to a variety of external signals including hormones, neurotransmitters, odorants, and light (Pierce et al. 2002). Rhodopsin, the best studied GPCR, is responsible for the transduction of light signals in photoreceptor cells. Capture of a photon isomerizes the covalently bound chromophore 11-*cis*-retinal and induces conformational changes in the protein. This activated form of rhodopsin is capable of binding the G protein transducin and mediating an amplification cascade which leads to a neural impulse. The lifetime of activated rhodopsin is determined by a two-step inactivation process. Rhodopsin is first deactivated by phosphorylation through the rhodopsin kinase GRK1 at up to seven amino acids at the C-terminus (Maeda et al. 2003), preferably serines at positions 334, 338, and 343 (Kennedy et al. 2001; Lee et al. 2002). Following phosphorylation, the protein arrestin binds to rhodopsin in the second step of deactivation in a complex multi-step mechanism (Gurevich and Gurevich 2004). NMR studies of completely phosphorylated peptides representing the rhodopsin C-terminus suggest an induction of secondary structure elements upon binding to arrestin (Kisselev et al. 2004).

Generally, it is believed that the C-terminus in intact rhodopsin is flexible and dynamic as determined by NMR spectroscopy (Klein-Seetharaman et al. 2002), EPR spectroscopy (Langen et al. 1999), and X-ray crystallography (Palczewski et al. 2000; Okada et al. 2004). However, in the most recent crystal structure of bovine rhodopsin, which resolves the protein backbone (Okada et al. 2004), the C-terminus is proposed to be in close proximity to helix 8 and the first cytoplasmic loop (Fig. 1a), the latter being one of the preferred interaction sites for arrestin upon activation (Raman et al. 1999, 2003). This raises the question whether this proximity induces previously not detected structural restraints or perturbations in the C-terminal domain. To investigate this question, isotope

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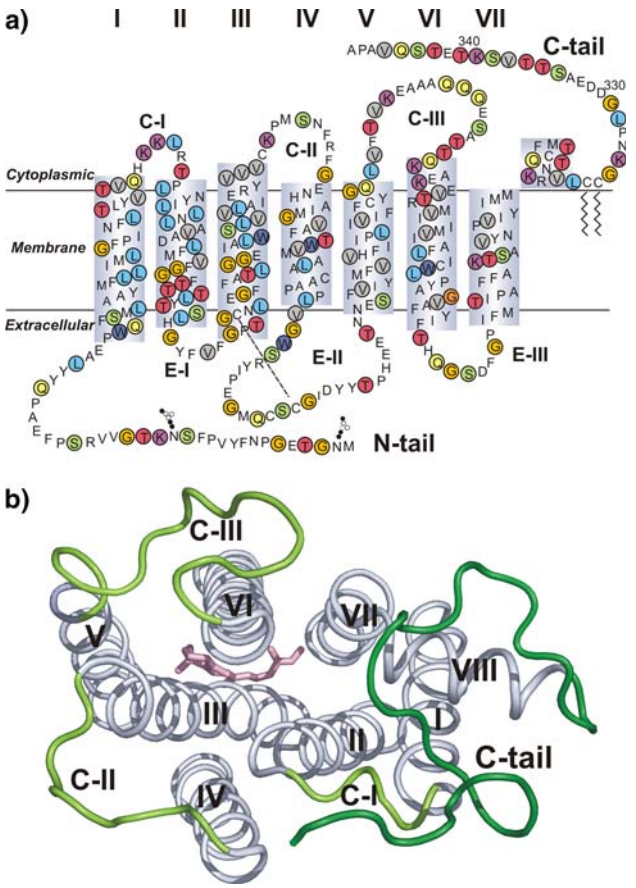

Fig. 1 (**a**) The three-dimensional structure model of the transmembrane and cytoplasmic domains of bovine rhodopsin based on the crystal structure (Palczewski et al. 2000). The cytoplasmic loops are shown in bright green and the C-terminus in dark green; 11-*cis*-retinal is highlighted in pink. (**b**) Secondary structure model of bovine rhodopsin. The isotope labeled amino acids Gly (orange), Gln (yellow), Leu (light blue), Lys (purple), Ser (green), Thr (red), Trp (dark blue), and Val (grey) are highlighted

labeling of significant parts of the C-terminus are a prerequisite for solution NMR.

Using solution NMR techniques to solve the structure of full-length membrane proteins is still rare. Typically, only helical domain fragments have been studied for most membrane proteins investigated by NMR. Partial or nearly complete backbone assignments exist for full-length bacteriorhodopsin (Schubert et al. 2002), KcsA (Chill et al. 2006), TehA (Trbovic et al. 2005), and diacylglycerol kinase (Oxenoid et al. 2004). The first assignments on a mammalian GPCR were obtained on selectively labeled rhodopsin, recombinantly expressed in mammalian HEK293 cells (Klein-Seetharaman et al. 2002; Werner et al. 2007). Isotope labeling of multiple amino acids has been achieved for other eukaryotic proteins obtained from yeast (Wood et al. 2000), insect cells (Strauss et al. 2005), and mammalian CHO cells (Wyss et al. 1997), but so far, no investigations on a uniformly labeled GPCR have been reported. Here, we describe the preparation of ^{15}N and $^{15}N/^{13}C$ isotope labeled wild-type rhodopsin samples in HEK293S cells, with simultaneous addition of the amino acids Gly, Gln, Leu, Lys, Ser, Thr, Trp, and Val in isotope labeled form. This labeling scheme covers 49% of all amino acids in the protein based on its sequence and enables a detailed characterization of the C-terminal domain (Fig. 1b). No difference in the expression yield was observed when comparing labeled media with conventional unlabeled media (see Supporting Information). With the samples prepared, we demonstrate the ability of solution NMR spectroscopy to provide detailed insight into the dynamic properties of mobile regions of a large, full-length macromolecule in a membrane-mimetic environment.

Materials and methods

Rhodopsin was expressed in a tetracycline-inducible HEK293S cell line stably transfected with the wild-type opsin gene. Labeled media was obtained from CIL, unlabeled media was prepared from individual components as described (Reeves et al. 2002). For determining the viable cell count, 1 ml of pelleted cells was washed with buffer A (137 mM NaCl, 2.7 mM KCl, 1.5 mM K_2HPO_4, pH 7.2), repelleted and then incubated 1 min with 100 µl of trypsin (0.05%). The trypsonized cells were resuspended in 1 ml complete DMEM (Dulbecco's modified Eagle's medium high glucose) media, diluted further 2–5 times, mixed with trypan blue and counted with a hemacytometer. A doubling time of approximately 24 h for the cell growth was observed during the first 3 days of expression. The maximum viable cell count reached usually on day 4 is approximately 2.2×10^6 cells/ml. No significant differences in cell growth are observed between labeled and unlabeled media. After purification 5.1 and 5.3 mg fully functional rhodopsin was obtained from 2.5 l ^{15}N GKLQSTVW and $^{15}N/^{13}C$ GKLQSTV(W) labeled media, respectively, which is identical with the amount of protein obtained previously from unlabeled media or selectively isotope labeled media.

NMR experiments for resonance assignment were carried out on Bruker spectrometers, operating at proton frequencies of 800 or 900 MHz and equipped with 5 mm HCN cryogenic probes and *z*-axis gradients. Spectra were recorded at a temperature of 310 K using a WATERGATE (Piotto et al. 2002) pulse sequence for water suppression, except for a TOCSY experiment, where solvent suppression was achieved by excitation sculpting. The NMR data were processed using TOPSPIN version 1.3 (Bruker Biospin) and CARA (Keller 2004). (For more information on the assignment and the determination of the relaxation rates see Supporting Information).

Results and discussion

Membrane proteins represent a particular challenge for solution NMR spectroscopy. It is difficult to obtain high quality NMR spectra due to the slow tumbling of the micelle/protein complex and the poor signal dispersion of helical structure elements. In Fig. 2a, a conventional ^{1}H, ^{15}N-HSQC spectra of ^{15}N GKLQSTVW labeled rhodopsin is shown. ^{1}H, ^{15}N-TROSY spectra do not provide any improvement (data not shown). The seven helical GPCR rhodopsin is stabilized in solution in dodecyl maltoside (DM) micelles with a total complex size of 100 kD, therefore broad and overlapping signals are observed in the backbone region.

However, the indole NHs of the five tryptophans are separated from the backbone resonances and well resolved (indicated with an arrow in Fig. 2a). At an increased threshold level (Fig. 2b) around 20 signals are found to display a considerably higher signal/noise ratio and narrower line widths as compared with the rest. Very likely, these signals arise from flexible regions of the protein which would include the termini and the loops. To verify this assumption, a peptide was synthesized representing the C-terminal 19 amino acids (D330–A348) of bovine rhodopsin. A first overlay of the 19mer natural abundance ^{1}H, ^{15}N-HSQC with the ^{1}H, ^{15}N-HSQC spectra from Fig. 2b of full-length rhodopsin showed already a good chemical shift agreement for numerous peaks. Therefore, the 19mer was further assigned by standard NMR methods (see SI). No significant long-range NOESY cross peaks could be detected, indicating a completely unstructured peptide. In parallel, the assignment of the intense resonances in full-length rhodopsin was initiated. By analysis of triple resonance HNCO and HNCACB spectra run on the double labeled sample, about 2/3 of the resonances in Fig. 2b could be linked by a sequential walk.

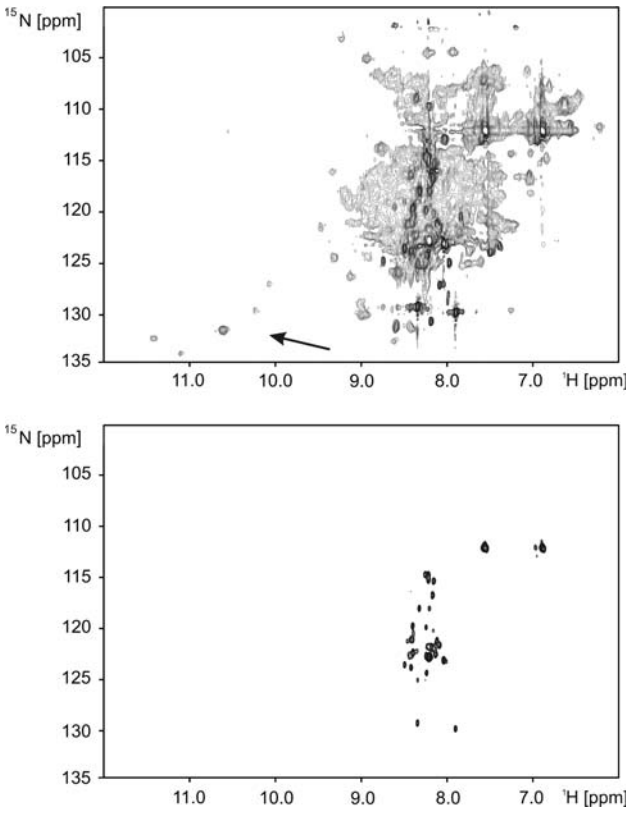

Fig. 2 ^{1}H, ^{15}N-HSQC NMR spectra of a 0.5 mM ^{15}N GKLQSTVW labeled rhodopsin sample in d25-DM at 310 K represented at a low (**a**) and a high (**b**) threshold level. The tryptophan indole signals are indicated with an arrow

As shown in the strip plot of residue E332–A346 (Fig. 3) the Cα resonances are continuously connected, but the Cβ resonances of some of the signals are absent. It is noteworthy that signals are observed also for the nonlabeled amino acids E332 and E341, A333, A346, and A348 (the latter is not shown in the strip plot). A333 shows only a very weak Cα resonance and the dotted lines connecting A333 and S334 refer to the missing Cαi-1 signal. The appearance of signals from unlabeled amino acids can be attributed to the high flexibility of the C-terminus or to scrambling effects during protein expression. In Fig. 3b and c the individually obtained assignments for the rhodopsin C-terminus and for the 19mer are brought together. For better comparison the full-length rhodopsin numbering is maintained for the 19mer. The amide proton assignment of D330 and D331 is not available in both cases and cannot be compared. A general chemical shift agreement is observed between 19mer and C-terminus except for the residues E332 and A333. This good agreement strongly suggests an unstructured C-terminus in the context of full-length rhodopsin. This finding is further supported by the fact that only small deviations from random coil chemical shifts and therefore no significant secondary structure elements could be identified (see SI). The discrepancy for E332 and A333 on the other hand might mark in rhodopsin the beginning influence of the detergent micelle and/or interactions with helix 8 considering the arrangement of this part of the C-terminus in the crystal structure. This is confirmed by heteronuclear ^{15}N longitudinal (R_1) and transverse (R_2) relaxation rates measured on the C-terminus of full-length rhodopsin. Relaxation rates reflect on backbone motion and flexibility on the subnanosecond timescale. In Fig. 4, the relaxation rates of the residues for which an assignment was possible are shown. A clear dependence from the relative position in the primary sequence is seen. Displaying increased relaxation rates, E332 and A333 are found to be conformationally restricted when compared with residues further down the sequence. This is at the same time a reliable proof that the C-terminus

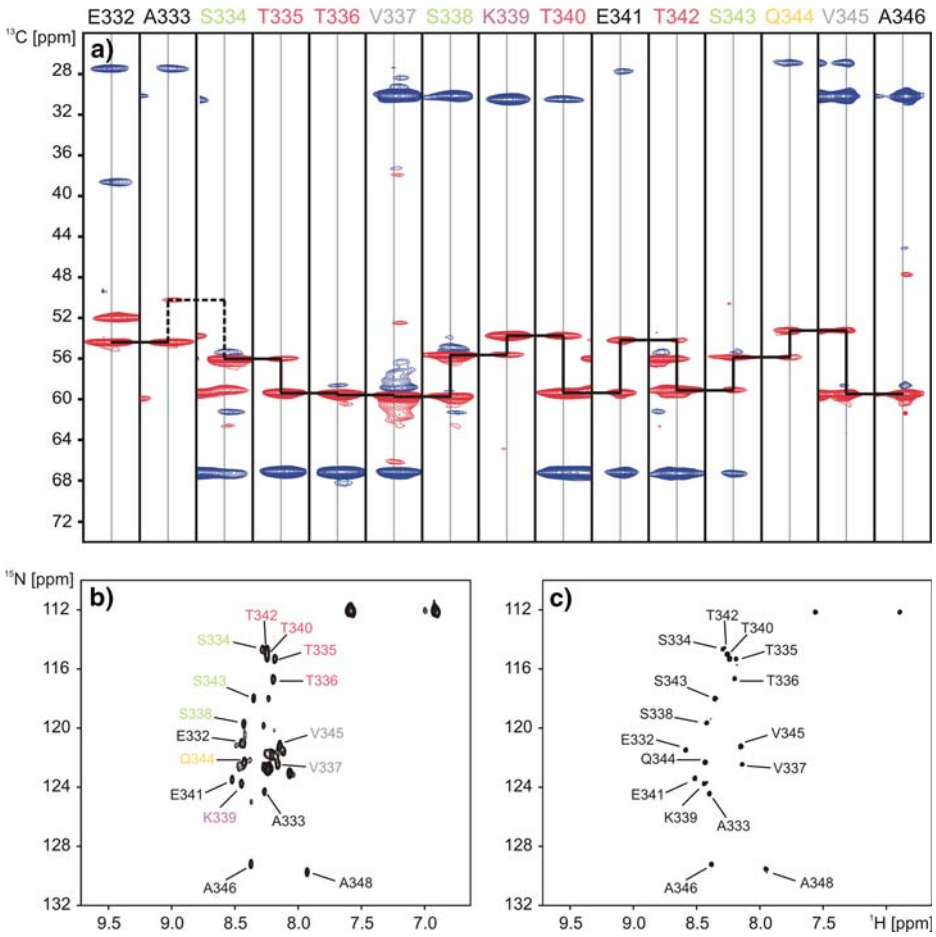

Fig. 3 (**a**) Strip plot representation of residue E332–A346 extracted from an HNCACB spectrum of $^{13}C/^{15}N$ GKLQSTV(W) labeled rhodopsin. Each strip shows resonances for the Cα and the Cβ of the respecting residue and the preceding residue. Connected are only the Cα resonances. Dashed lines indicate an interrupted sequential walk because of not present isotope labeling in Ala. (**b**) and (**c**) Comparison of ^{1}H, ^{15}N-HSQC of full-length rhodopsin (high threshold level) and 19mer. The individually obtained assignments are integrated in the figures

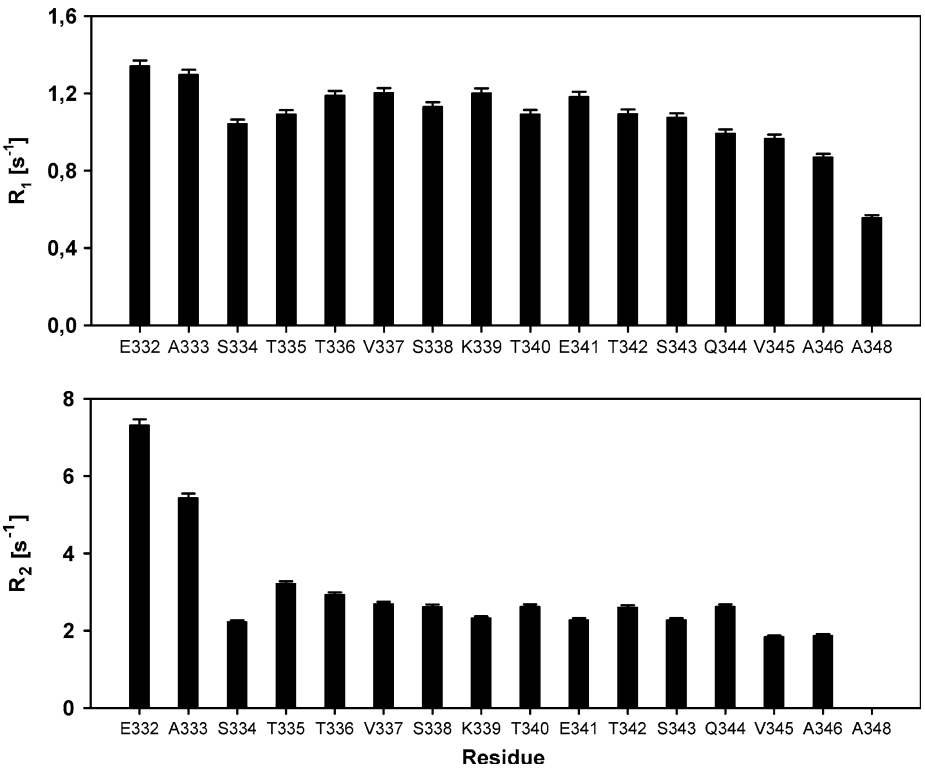

Fig. 4 ^{15}N-spin relaxation properties of full-length rhodopsin C-terminus. (**a**) R_1 and (**b**) R_2 relaxation rates measured at 310 K. The R_2 value of A348 could not be determined

is not degraded but still part of the protein. Generally, a decrease in R_1 and R_2 values going towards the end of the C-terminus is observed. The R_1 and R_2 values are in the range observed for a soluble protein, which is surprising considering the absolute size of the protein/micelle complex. Is should be mentioned that relaxation rates measured on the five tryptophan residues which are located in the transmembrane helices and at the protein/lipid interface are at least by one order of magnitude larger than the values obtained for the C-terminus (data not shown).

Conclusion

To permit a highly detailed analysis of the structural and dynamic properties of the rhodopsin C-terminus a media composition was used that ensured the labeling of almost 50% of all amino acids, which is the highest labeling ratio so far successfully known for a mammalian GPCR. The majority of the signals in the high threshold ^{1}H, ^{15}N-HSQC were related to the C-terminus and could be assigned unambiguously. Relaxation measurements suggest that the motion and dynamic behavior of the extreme C-terminus (334–348) is essentially independent from the slow tumbling rest of the protein. Conformational restriction in motion is only seen from position A333 and lower.

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