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FTIR analysis of GPCR activation using azido probes

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We demonstrate the site-directed incorporation of an IR-active amino acid, *p*-azido-L-phenylalanine (azidoF, 1), into the G protein–coupled receptor rhodopsin using amber codon suppression technology. The antisymmetric stretch vibration of the azido group absorbs at ~2,100 cm^{-1} in a clear spectral window and is sensitive to its electrostatic environment. We used FTIR difference spectroscopy to monitor the azido probe and show that the electrostatic environments of specific interhelical networks change during receptor activation.

We have developed a general strategy to probe conformational changes of heptahelical G protein–coupled receptors (GPCRs). GPCRs comprise a superfamily of cell surface proteins that participate in the most important and diverse signaling pathways in nature[1,2]. Understanding the molecular mechanism of ligand recognition, receptor activation and the subsequent interaction of the receptor with G proteins and other cellular signaling components is therefore an important goal in biomedical research. Although recent advances have provided high-resolution crystal structures[3,4] of several GPCRs, understanding the conformational changes associated with receptor activation in lipid membranes remains paramount.

Fourier-transform infrared (FTIR) difference spectroscopy has proven to be an informative tool for analyzing the function, mechanism and dynamics of proteins in membrane enviroments[5,6]. FTIR difference spectroscopy generally relies on intrinsic probes—such as the amide I bond C=O stretching vibration of the protein backbone, the C=O stretch of carboxylic acids involved in proton transfer reactions, and vibrations of water molecules or ligands—to investigate intramolecular changes within a protein 'at work'. The introduction of a unique probe with tailored vibrational properties at a discrete site within a protein offers an attractive solution to identify site-specific environmental changes. Probe moieties such as NO, CN, SCN or N_3 are ideal, as they are comparatively small so that perturbation of the native structure of a protein is minimized. Furthermore, they provide unique vibrational signatures in the spectral range between 1,900 and 2,300 cm^{-1}, which is well separated from intrinsic protein vibrations. But satisfactory methods to introduce site-specific vibrational probes for studies of eukaryotic membrane proteins have not been available.

Amber codon suppression technology involving read-through of an amber stop codon (UAG) in an mRNA by a suppressor tRNA aminoacylated with an unnatural amino acid has been an important recent advance in molecular biology[7]. The development of numerous orthogonal aminoacyl-tRNA synthetase (aaRS)/suppressor tRNA pairs provides in principle a simplified procedure to express any protein of interest containing one of many unnatural amino acids[8,9]. With this approach, investigators have reported incorporation of *p*-cyano-L-phenylalanine (2) (ref. 10), *m*-azido-L-tyrosine (3) (ref. 11) and azidoF

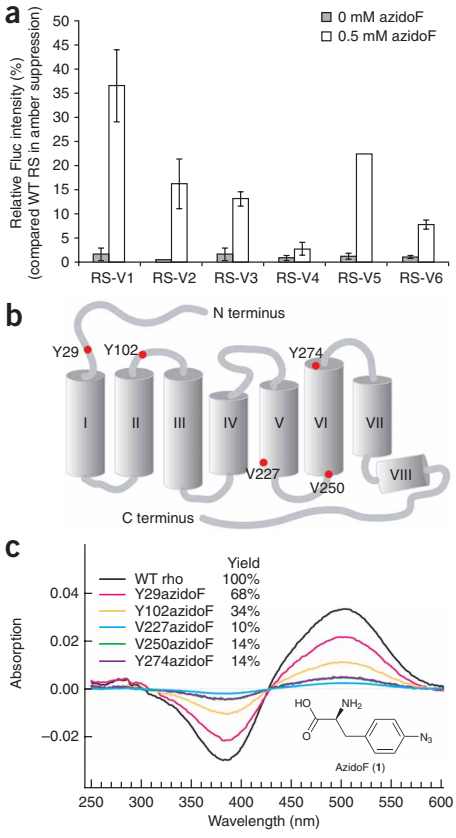

Figure 1 Non-natural amino acid mutagenesis. (**a**) HEK 293T cells were transfected with plasmids carrying the genes for firefly luciferase with an amber mutation (FLuc.Y70am), a suppressor tRNA (*Bst*-Yam), and one of six mutant synthetases (azidoF RS-V1 to RS-V6)[16] at a time. Cells were cultured in the absence and in the presence of azidoF. The average FLuc activities were determined from the chemiluminescence intensities (from three independent experiments). The amber stop codon suppression efficiencies of the mutant synthetases were described as relative FLuc intensities compared to wild-type *E. coli* Tyr-RS in suppression activity (%). Error bars show s.d. (**b**) Positions in rhodopsin subjected to site-specific incorporation of azidoF are indicated as red dots in a schematic secondary structure plot. (**c**) Functional expression and expression yield were determined in light-induced UV-visible difference spectra (dark minus photoproduct) of purified wild-type rhodopsin and azidoF rhodopsin mutants. The structure of azidoF is indicated.

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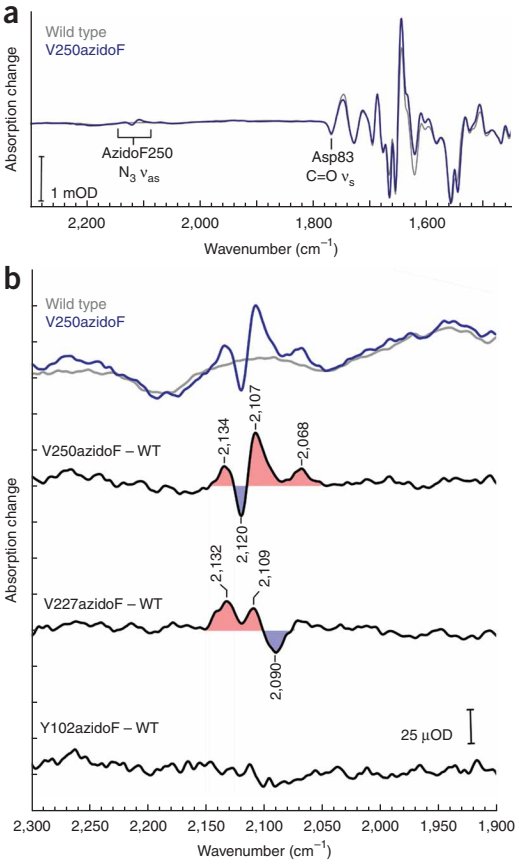

Figure 2 FTIR spectroscopy on azidoF rhodopsin mutants. (**a**) The light-induced FTIR difference spectrum (Meta II photoproduct minus dark state) of Val250azidoF mutant (blue) reveals the characteristic band pattern below 1,800 cm^{-1} that reflects conformational changes of the protein during light-dependent receptor activation, similar to that in wild-type rhodopsin (gray). At 2,100 cm^{-1}, the antisymmetric stretch (ν_{as}) of the azido label is observed (the absorption peak of Asp83 in the dark state is marked for comparison). (**b**) The broad featureless bands underlying the difference spectrum of the wild type were subtracted from the Val250azidoF difference spectrum (top row). The resulting double difference spectrum Val250azidoF minus wild type (second row) reveals in the first approximation a downshift of the azido ν_{as} during activation of Val250azidoF. In the analogous spectrum of the Val227azidoF mutant, on the other hand, a clear upshift is observed, while no substantial changes are observed in the spectrum of the Tyr102azidoF mutant.

(ref. 12) into myoglobin, calmodulin and green fluorescent protein, respectively; however, applications using FTIR analysis were not demonstrated. Recently, we optimized an expression system with human embryonic kidney (HEK) 293T cells using the orthogonal mutant *Escherichia coli* Tyr-RS/*Bacillus stearothermophilus* (*Bst*) Tyr-$tRNA_{CUA}$ pair to incorporate efficiently *p*-acetyl-L-phenylalanine (**4**) and *p*-benzoyl-L-phenylalanine (**5**) into GPCRs site specifically[13]. Here we expand the repertoire of unnatural amino acids with azidoF. The antisymmetric stretching vibration (ν_{as}) of azido results in a strong absorption band at around 2,100 cm^{-1} that is free from other vibrational absorptions typically present in proteins. Furthermore, the position of this band is sensitive to subtle changes of the electric field of its local environment due to the vibrational Stark effect[14,15].

We first screened suppressor tRNA/aaRS pairs that can recognize azidoF using a luciferase reporter assay, testing six previously described mutant synthetases (azidoF RS-V1 to azidoF RS-V6)[16] (see **Supplementary Methods** online). Read-through of the amber codon by the aminoacylated suppressor tRNA leads to the expression of luciferase, which can be conveniently quantified by chemiluminescence measurements. Because the suppressor tRNA can only be aminoacylated by the mutant synthetases, the amount of expressed luciferase corresponds to the aminoacyl activities of the synthetases toward azidoF. Cells cultured in the absence of azidoF showed little luminescence signal (**Supplementary Fig. 1** online), thereby confirming the specificity of the synthetases for azidoF. In the presence of azidoF, different degrees of chemiluminescence were observed, which reflects different levels of synthetase activity (**Supplementary Fig. 1**). Based on the chemiluminescence assay, we selected azidoF RS-V1, which had the highest activity, for further studies (**Fig. 1a**). The tRNA/azidoF RS-V1 pair produced a luciferase yield of ~37% compared with the suppressor tRNA/ wild-type *E. coli* Tyr-RS pair, and the overall suppression yield of the azidoF luciferase mutant was ~13% of the wild-type expressed luciferase.

Using the azidoF RS-V1/pSVB.Yam orthogonal pair, we next studied the incorporation of azidoF into rhodopsin at five different target sites (Tyr29, Tyr102, Val227, Val250 and Tyr274) (**Fig. 1b**). Tyr29azidoF and Tyr102azidoF rhodopsin mutants gave expression yields of 68% and 34% of that of the wild type, respectively (**Fig. 1c**). We also observed a general trend that mutant yields decreased when an amber stop codon was placed more toward the 3′ end of the rhodopsin gene. This might possibly be due to blockage of the translocon systems by partially translated protein after failure of amber stop codon suppression. Overall, the expression data indicate efficient amber suppression at various target sites throughout the entire length of the polypeptide chain such that reasonable amounts of rhodopsin mutants could be generated.

Based on the available crystal structures of rhodopsin and on previous EPR studies[17], we selected Tyr102azidoF, Val227azidoF and Val250azidoF for FTIR difference spectroscopy. Mutant pigments were expressed, purified and reconstituted into lipid membranes. Changes of the stretch vibration of the azido probe during light-induced receptor activation were followed by FTIR difference spectroscopy. Besides the well-characterized spectral pattern below 1,800 cm^{-1} reflecting the vibrational changes due to the transition to the signaling state Meta II (refs. 5,6), wild-type pigment shows in the range around 2,100 cm^{-1} only broad and featureless difference bands that can be subtracted from the mutant spectra to reveal the sharp spectral changes due to the azido label (**Fig. 2**). For the Val250azidoF mutant, the band pattern of the azido ν_{as} consists of negative and positive peaks, reflecting a shift of the absorption of the azido group in the transition from the dark to the activated Meta II photoproduct state. In order to correlate the position of the azido ν_{as} with electrostatic properties of the environment, we recorded absorption spectra of azidoF as a free amino acid in mixtures of polar and nonpolar solvents. By switching from water to isopropanol as solvent (which corresponds to a decrease of the dielectric constant from 78.4 to 19.9), the position of the azido ν_{as} was downshifted by ~13 cm^{-1} from 2,128.6 cm^{-1} to 2,115.5 cm^{-1} (**Supplementary Fig. 2** online).

The azido label used in this study senses in particular the polarity of its environment and the presence of electric fields via the vibrational Stark effect. The latter influence of electric fields on vibrational probes has been characterized previously using Stark spectroscopy on model compounds[14] and by IR spectroscopy on proteins[15]. Our calibration measurement of the position of the azidoF ν_{as} in different solvents indicates an upshift of the peak with increasing polarity of the environment. The site-directed incorporation of azidoF

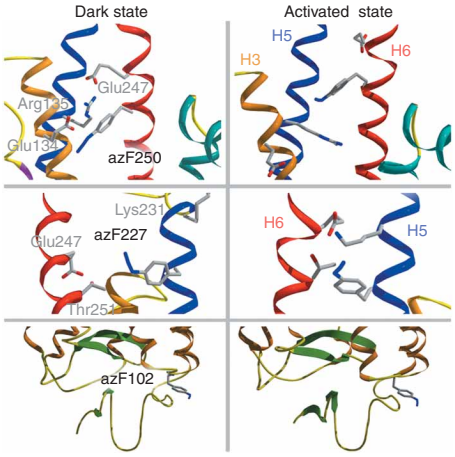

Figure 3 Structural models of rhodopsin activation. Structural models of rhodopsin azidoF mutants derived from the dark state[18] and in an activated conformation of the ligand-free opsin state[19] were obtained by modeling and molecular dynamic equilibration (cytoplasmic side points upwards). Receptor activation leads to rearrangement of a charged cluster at the H3/H6 interface and formation of a new charged cluster at the H5/H6 interface in the vicinity of positions 250 and 227. The environment of position 102, on the other hand, is not hypothesized to be affected by receptor activation. The results of the simulations are consistent with the shifts of the azido stretching bands in the Meta II difference spectra of the three azidoF rhodopsin mutants shown in **Figure 2b**.

into rhodopsin allows us, therefore, to monitor the changes of the electrostatic environment of selected side chains during the conformational transition associated with receptor activation.

To reconcile the spectroscopic data with structural data, we modeled the azidoF side chain into the crystal structures of rhodopsin[18] and an apparently active form of opsin[19] by annealing and energy minimization using NAMD2.6 with harmonic restraints[2]. According to this model, azidoF250 on transmembrane helix 6 (H6) is in contact with charged residues involved in the so-called ionic lock between the cytoplasmic termini of H3 and H6, involving Glu134, Arg135 and Glu247 (**Fig. 3**, upper left panel). The general downshift of the azidoF250 vibration in the transition from the dark state (negative bands) to the active photoproduct state (positive bands) indicates a shift from a very polar to a less polar environment. This is supported by the model shown in the upper right panel of **Figure 3**, which is based on the recently solved crystal structure of opsin at low pH[19] as a template for an activated conformation[4]. This situation is reversed for azidoF227 on H5, which points in the dark state into the nonpolar part of the lipid environment[20]. Upon receptor activation, we observe a substantial upshift of the stretching vibration of azidoF227, which indicates a translocation of this side chain into a polar environment. According to the structural model, such a translocation can be achieved by rotation of H5, which pushes azidoF227 against a charged cluster formed in the active conformation by residues on H6 (Thr251 and Glu247) and H5 (Lys231). Finally, azidoF102 is situated in the extracellular domain of rhodopsin, and the difference spectrum of Tyr102azidoF is essentially flat, which indicates hardly any change during receptor activation.

Our experiments using FTIR difference spectroscopy on azido-labeled rhodopsin extend previous studies directed at rhodopsin activation with fluorescence spectroscopy[21], EPR spectroscopy[17,22] and NMR[23] by adding measurements of the local electrostatic environments. Recently solved structures of adrenergic and adenosine receptors[3,4] underline the conservation of a general packing pattern of transmembrane helices and of key functional components, such as the E(D)RY motif on H3 (refs. 1,2). Also, structural activation patterns were shown to be similar to those of rhodopsin and the β_2 adrenergic receptor[24]. Based on structural data on activated opsin conformations, rearrangement of charged intra- and interhelical networks[19,25], such as in the cytoplasmic domain between H3, H5 and H6, appears crucial for the transition from an inactive to an active conformation. Using the electrostatically sensitive azido probe, we have followed the disruption of a charged cluster around Arg135 of the ERY motif on H3 and the formation of a new charged cluster at the H5/H6 interface.

In conclusion, we developed an expression system that allows *in vivo* synthesis of functionally active proteins containing an IR-active probe. FTIR spectroscopy of this probe allows one to follow the dynamics of the local electrostatic environment at the position of the label during conformational transitions of the GPCR rhodopsin. This general method should prove useful for unnatural amino acid mutagenesis and FTIR analysis of membrane proteins such as GPCRs that notoriously resist efficient functional heterologous expression in bacterial cells.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

S.Y., T.H. and R.V. designed and conducted experiments, analyzed data and wrote the manuscript. T.P.S. designed experiments and wrote the manuscript.

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