Published in final edited form as:

Methods Mol Biol. 2012 ; 914: 127–153. doi:10.1007/978-1-62703-023-6_8.

UV-Visible and Infrared Methods for Investigating Lipid-Rhodopsin Membrane Interactions

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Summary

Experimental UV-visible and Fourier transform infrared (FTIR) spectroscopic methods are described for characterizing lipid-protein interactions for the example of rhodopsin in a membrane bilayer environment. The combined use of FTIR and UV-visible difference spectroscopy monitors the structural and functional changes during rhodopsin activation. Such studies investigate how membrane lipids stabilize the various rhodopsin photoproducts, analogous to mutating the protein. Interpretation of the results entails a non-specific flexible surface model for explaining the role of membrane lipid-protein interactions in biological functions.

Keywords

flexible surface model; G protein-coupled receptor; hydrophobic mismatch; infrared spectroscopy; lateral pressure; lipids; membrane curvature; protein-lipid interactions; rhodopsin; vision

1. Introduction

Rhodopsin is a G protein-coupled receptor (GPCR) that is present in the disk membranes of the retinal rod cells and mediates the process of visual signaling. Experimental studies of rhodopsin allow one to test the role of membrane lipids in governing key biological activities of membrane proteins, including GPCRs, transporters, and ion channels (1). Membrane lipid-protein interactions significantly affect rhodopsin function within the membrane lipid bilayer (2–7). Notably, rhodopsin activation in native and synthetic membranes can be experimentally investigated by the combined use of UV-visible and FTIR spectroscopy. Absorption of light by retinal yields 11-*cis* to *trans* isomerization of the ligand bound to rhodopsin within the lipid bilayer. Subsequent deprotonation of the protonated Schiff base

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⁴The A_{500} absorption is due to retinal and is a specific marker for rhodopsin. However, the A_{280} absorption includes contributions from aromatic amino acids and is not specific to rhodopsin. It follows that the A_{280}/A_{500} provides a simple criterion of the rhodopsin purity.

¹⁹Lipids with small head groups (DOPE) or increasing acyl chain unsaturation (DOPC versus POPC, Figs. 4C,D) forward shift the metarhodopsin equilibrium towards Meta II. Lipids with larger head groups (e.g. DOPC versus DOPE (21, 33)) and less acyl unsaturation (3, 5) back shift the equilibrium towards Meta I. In general, small head groups such as phosphoethanolamine (PE), bulky polyunsaturated DHA chains, or greater bilayer thickness favor the activated Meta II photoproduct. By contrast larger head groups such as phosphocholine (PC), a decrease in acyl chain unsaturation, or smaller bilayer thickness favor the inactive Meta I state.

(PSB) of retinal and activating conformational changes of the protein are probed with UV-visible and FTIR spectroscopy (8–10). The complementarity of these methods with X-ray crystallography is brought out by the crystal structures of rhodopsin in the dark state (11–13) and ligand-free opsin (14). In interpreting spectroscopic measurements, the reader will find it useful to examine X-ray structures with molecular visualization software (15). Further analysis of UV-visible and FTIR results for rhodopsin in a native-like bilayer environment entails lipid-membrane protein interactions as described by a flexible surface model (FSM). A chemically non-specific curvature stress field due to the membrane lipids governs the energetics of membrane proteins. Lipid influences on vision and other key biological functions of GPCRs, transporters, and ion channels are explainable by a simple continuum picture.

2. Materials

Experimental protocols are briefly described below for the preparation and characterization of retinal disk membranes (RDM), and recombinant lipid membranes containing rhodopsin. Native hypotonically washed disk membranes are prepared from bovine retinas following standard procedures (16). Characterization of the photochemical activity of rhodopsin involves bleaching and regeneration of opsin with 11-*cis*-retinal. Membranes with a defined lipid composition are prepared by purifying a detergent extract of rhodopsin by column chromatography on hydroxyapatite. Dialysis in the presence of phospholipids forms membrane bilayers that are harvested by ultracentrifugation.

2.1. Buffers, Detergents, Lipids

1. **Phospholipids:** Highly pure phospholipids are readily procured from commercial sources (e.g. Avanti Polar Lipids, Alabaster, AL, USA). Alternatively phospholipids are synthesized as described (17, 18), starting from egg yolk phosphatidylcholine prepared from locally obtained hen eggs and commercially available fatty acids (Nu-Chek Prep, Inc., Elysian MN, USA). For further details see Refs. (17, 18). Representative phospholipids include 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE).
2. **Detergents:** Ammonyx LO (Stepan Co., Northfield, IL, USA), dodecyltrimethylammonium bromide (DTAB) (Sigma, St. Louis, MO, USA), lauryldodecylamine oxide (LDAO) (Sigma, St. Louis, MO, USA) (*see* Note 1).
3. **Buffers and solutions:** *Homogenizing solution* - 30% sucrose (w/w), 5 mM Tris-acetate pH 7.4, 65 mM NaCl, 2 mM MgCl₂, and 2 mM EDTA. *Dilution buffer* - 10 mM Tris-acetate pH 7.4. *Stock detergent buffer* - 30% (v/v) Ammonyx LO or 30% (v/v) LDAO containing 100 mM NH₂OH (hydroxylamine) and 10 mM Na phosphate buffer pH 7.0. *Dialysis buffer* - 5 mM HEPES, 1 mM EDTA, and 1

¹Detergents used for rhodopsin solubilization include Ammonyx LO, sodium cholate, digitonin, *n*-dodecyl β -D-maltoside (DDM), dodecyltrimethylammonium bromide (DTAB), Emulphogene, lauryldodecylamine oxide (LDAO), octaethylene glycol *n*-dodecyl monoether (C₁₂E₈), octyl β -D-glucopyranoside (OG), and Tween 80. Bleached rhodopsin (i.e. opsin) can be regenerated with 11-*cis*-retinal in the detergents cholate, digitonin, OG, or DDM.

mM DTT (dithiothreitol) (add fresh). *Chromatography buffer* - 15 mM Na phosphate pH 6.8 containing 0.02% (w/w) NaN₃ (sodium azide) and 1 mM DTT (add fresh). *Chromatography detergent buffer* - 100 mM DTAB plus chromatography buffer.

4. Density gradient solutions: 1.10, 1.11, 1.13, 1.15 g/mL sucrose (23.8, 25.9, 30.3, and 34.0 % (w/w), respectively) containing Tris-acetate pH 7.4 and 1 mM EDTA.
5. Column packing materials: Hydroxyapatite (Bio-Gel HTP Gel; Bio-Rad Laboratories, Hercules, CA, USA).
6. Isomerically pure 11-*cis*-retinal can be obtained via the U.S. National Eye Institute (Bethesda, MD, USA).

2.2. Purification of retinal disk membranes containing rhodopsin

1. Retinas: Frozen bovine retinas are procured from commercial sources (Lawson, Omaha, NE, USA). Alternatively, fresh bovine eyes are obtained from a local abattoir and are transferred to an ice chest in the dark. Eyes are dissected under dim red light (15 W bulb with Kodak safelight filter 1A) within ≈ 4 hr postmortem by excising the front part of the eye with a sharp scalpel or razor blade, followed by inverting the eye cup, removal of the lens and vitreous humor, and gently separating the retina from the pigment epithelium with a blunt scapula. The retina is cut at the optic nerve with a sharp scissors and dropped into a glass jar containing homogenizing solution (30% (w/w) sucrose) on ice. Retinas are stored in lightproof glass containers under nitrogen or argon at -80°C until use.
2. Preparation of retinal disk membranes: All procedures are done in a cold room (4°C) or using an ice bucket under dim red light (see above). Thawed frozen bovine retinas or alternatively freshly obtained retinas are used (see above). Gently separate the retinas and centrifuge at $2600 \times g$ for 20 min at 4°C . Transfer the pelleted retinas to a *loose* fitting Teflon homogenizer (machined). Add 30 mL of homogenizing solution (30% sucrose) per 50 retinas under a gentle stream of argon gas. (Do not add excess because that will change sucrose density for subsequent steps.) Homogenize the retinas by applying about 10 strokes slowly under a gentle argon gas stream. Centrifuge at $2600 \times g$ for 20 min at 4°C . Remove the supernatant with a spring-loaded syringe or by decanting, and transfer to a *tight* fitting Teflon homogenizer (as obtained from the manufacturer). Add an equal volume of homogenizing solution and apply about 6–8 strokes under gentle argon gas stream. Centrifuge at $2600 \times g$ for 20 min at 4°C and combine the supernatants from the two centrifugation steps.
3. Add two volumes of dilution buffer (10 mM Tris-acetate pH 7.4) to the retinal homogenate and centrifuge at $8000 \times g$ for 50 min at 4°C . (If necessary increase the time of centrifugation but do not increase speed above $8000 \times g$ as this will lead to additional contaminating material in the pellet.) Re-suspend the pellet in a small volume (< 25 mL) of 1.10 g/mL sucrose density gradient solution. Prepare

discontinuous sucrose step gradients on ice or in the cold room (1.11, 1.13, and 1.15 g/mL; volumes of 10, 10, and 8 mL, respectively) in polyallomer centrifuge tubes (four tubes for 50 retinas or six tubes for 100 retinas). Pull up the pellet with an 18-gauge needle and then it expel through a 21-gauge needle to fragment the crude rod outer segments. Layer onto each of the density gradient tubes and top off with additional 1.10 g/mL sucrose density gradient solution. Centrifuge at $113000 \times g$ in a swinging bucket rotor for 1 hr at 4 °C. Collect the carpet (band) at the 1.11/1.13 g/mL interface with a spring-loaded syringe having a cut-off 18-gauge tip (total volume ≈ 75 mL).

4. Combine the collected bands and dilute two-fold with water, add argon gas, and centrifuge at $48000 \times g$ for 30 min at 4 °C. Repeat 1–2 times to ensure removal of sucrose. Note that water washing (hypotonic) produces membrane fragments due to the osmotic shock. Re-suspend the retinal disk membranes (RDM) in either 10 mM or 67 mM Na phosphate buffer pH 7.0 (≈ 10 mL) and transfer to Eppendorf vials. Overlay with argon gas and store at -80 °C until use. For additional details see Ref. (16) (*see* Note 2).

2.3. Characterization of rhodopsin by spectrophotometry

1. All procedures are done under dim red light (15 W bulb, Kodak safelight filter 1A). A scanning spectrophotometer (dual beam; *see* below) is used to acquire UV-visible absorption spectrum of rhodopsin by solubilizing a small aliquot of membranes in stock detergent buffer (30% (v/v) Ammonx LO containing 100 mM NH_2OH) at room temperature. First, record a baseline of 10:1 dilution of detergent buffer (3%) (blank sample) versus detergent buffer (3%) (reference) from 700 to 250 nm using 100 μL quartz microcuvettes with a 10 mm path length (*see* Note 3).
2. Next dissolve 0.1 mL of rhodopsin-containing membrane sample in 0.9 mL of stock detergent buffer at room temperature. Invert the cuvette to mix the contents thoroughly and immediately record an absorption spectrum of the dissolved membranes containing rhodopsin from 700 to 250 nm. Scan several times to ensure reproducibility and to establish that no time-dependent changes are occurring (*see* Note 3).
3. Fully bleach the sample with ≈ 6 flashes from a photoflash lamp (Sunpak auto 555 or equivalent) fitted with a high-pass OG515 filter (Schott, city, state, USA), and re-scan to record the fully-bleached spectrum with scattered light. Measure both the 500-nm absorption and the 280-nm absorption versus the bleached spectrum and calculate the A_{280}/A_{500} absorption ratio. Note that highly pure

²Store retinas in lightproof glass containers (wrapped with aluminum foil or electrical tape) under argon. It is recommended to use retinas harvested between the months of October through March due to seasonal variability. Retinas can be stored at -80 °C for several years without adverse effects on biophysical and functional parameters.

³Note that hydroxylamine, NH_2OH , is present in stock detergent buffer, but does not react with rhodopsin in the dark state. After bleaching NH_2OH reacts with retinal bound to rhodopsin at the Meta II stage. The reaction is completely forward shifted by le Châtelier's Principle to give the final products opsin + retinaloxime.

samples of native disk membranes typically have A_{280}/A_{500} ratios (unregenerated; see below) of $\approx 2.4 \pm 1$ (see Note 3).

4. Quantify the molar concentration of rhodopsin using $\epsilon_{500} = 40600 \text{ M}^{-1} \text{ cm}^{-1}$ for the molar extinction coefficient/absorptivity. Alternatively use optical density units (OD or A_{500} units). One OD unit is defined as 1 mL of a solution with absorption $A_{500} = 1$ and affords a convenient measure of the equivalents of retinal. The correspondence to the mass of rhodopsin is $1.00 \text{ OD} = 2.46 \text{ nmol} = 0.958 \text{ mg rhodopsin}$ (assuming $M_r = 39000$).

2.4. Regeneration assay for rhodopsin photochemical functionality

1. Conduct regeneration assays of the rhodopsin-containing membranes by adding 11-*cis*-retinal to obtain the percent regenerability, percent of bleached rhodopsin (i.e. opsin) in the original sample, and the regenerated A_{280}/A_{500} ratio. Note that in general the purified RDM may be partially bleached (i.e. containing both rhodopsin and opsin). The % of bleached rhodopsin as well as the % of the opsin that can be regenerated from rhodopsin is found by incubating the RDM preparation with added 11-*cis*-retinal.
2. Prepare three 0.2 mL samples of RDM designated as: control, regenerated, and bleached + regenerated. The bleached sample is exposed to light from a photoflash unit as described above. Add at least as many equivalents (ODs) of 11-*cis*-retinal as rhodopsin (e.g. 1.5–2 equivalents, corresponding to 0.5–1-fold molar excess) to the regenerated and bleached + regenerated samples. Incubate all three samples for 1.5 h at 37 °C. Record the UV-visible absorption spectra as described above.
3. Note that to obtain the % bleached and % regenerability it is necessary to correct for partial regeneration of opsin. Assume the fraction of rhodopsin that is unregenerable when bleached to opsin is identical to the fraction of unregenerable opsin present in the original RDM sample (partially bleached). In what follows, all quantities refer to the absorbance at 500 nm measured as described above. (It is helpful for the reader to sketch the corresponding absorption spectra.) Let A be the amount of regenerable opsin in the regenerated sample, i.e. before adding 11-*cis*-retinal. B is the total amount of opsin (i.e. regenerable plus unregenerable) in the regenerated sample before adding 11-*cis*-retinal. C is the total absorbance that would be measured if all the opsin in the sample regenerated. D is the amount of regenerable opsin in the bleached + regenerated sample. E is the total amount of rhodopsin in each of the samples (i.e. prior to bleaching or 11-*cis*-retinal addition). F is the unknown absorbance that would be produced if all the unregenerable opsin could be regenerated. Last, G is the amount of rhodopsin in the regenerated sample after incubation with 11-*cis*-retinal. Note the experimentally observed values are D (bleached + regenerated sample), E (control sample), and G (regenerated sample).
4. Assume that the fraction of regenerable opsin is the same before and after bleaching so that $A/B = D/C$. The unknown quantity is then $C = ED/(D - G + E)$

which is the total amount of the visual protein (rhodopsin + opsin) present in the samples. The % bleached (opsin) in the original sample = $100B/C$ ($\approx 100A/G$ = $100(1-E/G)$ assuming 100% regenerability). The % regenerability = $100D/C$ ($\approx 100D/G$). Last, the regenerated A_{280}/A_{500} ratio is $A_{280}(\text{control})/A_{500}(\text{regenerated})$. Typical results in our laboratory are: 93% regenerability; 2% bleached; 2.1 post-regeneration A_{280}/A_{500} ratio. For further details see Ref. (19).

2.5. Purification of rhodopsin by hydroxyapatite chromatography

1. Rhodopsin is extracted by solubilizing the native disk membranes at 4 °C with the cationic detergent dodecyltrimethylammonium bromide (DTAB). Centrifuge the purified RDM (see above) at $48000 \times g$ for 20 min at 4 °C. Remove the supernatant and dissolve the pellet in 10–15 mL of chromatography detergent buffer (100 mM DTAB containing 15 mM Na phosphate pH 6.8 with 0.02% NaN_3 and 1 mM DTT). Incubate on ice for 1 hr and then centrifuge at $163000 \times g$ for 20 min at 4 °C.
2. Purify the detergent-solubilized rhodopsin (50 mg) by column chromatography (column size 2.5×6.5 cm) on hydroxyapatite. Equilibrate the column with chromatography detergent buffer (100 mM DTAB, 15 mM Na phosphate pH 6.8, containing 0.02% (w/w) NaN_3 (sodium azide) and 1 mM DTT) at 4 °C. Apply the sample onto the hydroxyapatite column and elute with a linear gradient of 0–0.5 M NaCl in chromatography detergent buffer with a flow rate of 0.6 mL/min. Identify the rhodopsin-containing fractions by their A_{500} absorption. Note that rhodopsin is typically eluted beginning at ≈ 4 h in the 100–150 mL fractions, and the yield is typically $\approx 70\%$. The A_{280}/A_{500} ratio of the pure rhodopsin fractions is typically 1.6–1.8. For specifics see Refs. (20, 21) (*see Note 5*).

2.6. Preparation of rhodopsin/lipid recombinant membranes

1. Combine the eluted rhodopsin fractions from hydroxyapatite column chromatography (typically ≈ 50 mL) and always maintain rhodopsin in DTAB at 4 °C to avoid denaturation (*see Note 5*). Dissolve the phospholipid(s) in 3–4 mL of 300 mM DTAB plus chromatography buffer. Note that rhodopsin is typically recombined with phospholipids (Avanti Polar Lipids, Alabaster, AL, USA) at a 1:100 molar ratio of rhodopsin/lipid, although ratios from 1:50 to 1:400 are possible. Combine rhodopsin with the solubilized phospholipids at 4 °C and adjust the DTAB concentration to 300 mM plus chromatography buffer. Add the rhodopsin plus solubilized phospholipids to a dialysis sack (≈ 2.0 mL solution/cm of dialysis tubing) prepared by pre-boiling the dialysis tubing in distilled water (M_r cutoff = 12000–14000) (Spectra/Por; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA).

⁵Warning: rhodopsin is unstable in DTAB and thermally bleaches if the sample temperature rises above 4 °C. All procedures with DTAB should be done on ice or in the cold room. When in doubt, record a UV-visible absorption spectrum in 3% Ammonyx LO as described above to measure the A_{280}/A_{500} ratio.

2. Recombinant proteolipid bilayer membranes are formed by dialysis of the detergent in a cold room at 4 °C in darkness with a continuous stream of nitrogen gas (use a gas dispersion tube). Exchange the dialysis buffer eight or more times every 6–8 hr. Note that a constant rate of dialysis with regular exchange intervals is desirable for relatively homogeneous vesicle formation. The total volume of dialysis buffer used should be ≈ 900 mL per mL of DTAB solution added to the dialysis sack.
3. Collect the recombinant proteolipid membranes by centrifuging the contents of the dialysis sack at $105000 \times g$ for 1 hr at 4 °C. Finally, re-suspend the pellet in 67 mM Na phosphate buffer pH 7.0 or 100 mM HEPES buffer pH 7.0 containing 1 mM EDTA, and store in sealed Eppendorf vials under argon at -80 °C (stable for several years). For further details see Refs. (20, 21).

2.7. Characterization of rhodopsin/lipid membranes by density gradient centrifugation

1. Investigate the homogeneity of proteolipid bilayer membranes with respect to lipid/rhodopsin ratio by isopycnic density gradient centrifugation. Note that proteins are denser than lipids, so the position of the proteolipid band depends on the protein/lipid ratio. Use a gradient maker (commercial or fabricated locally) to pour a linear 0 to 50% (w/w) sucrose density gradient containing 10 mM Tris-acetate pH 7.4 and 1mM EDTA in a polyallomer centrifuge tube.
2. Centrifuge at $113000 \times g$ for 6–12 hr at 4 °C. Note that homogenous samples yield a single tight lipid/rhodopsin band, whereas polydispersity is indicated by diffuse multiple bands (not typically observed using DTAB for membrane recombination). If desired, pour a shallower gradient with a smaller range of sucrose density (higher resolution) and repeat the procedure to test for the presence of a single band (see Note 6).
3. Determine the lipid/rhodopsin ratio using phosphorus analysis (22) to quantify the phospholipid content and the A_{500} absorbance for the amount of rhodopsin in the fractions. (The centrifuge tube can be punctured at the bottom to collect the drops corresponding to the fractions.) Use the molar absorptivity of $\epsilon_{500} = 40600$ $\text{M}^{-1} \text{cm}^{-1}$ to quantify the amount of rhodopsin (see Note 6).

3. Methods

UV-visible spectrophotometry and FTIR spectroscopy employ either freely suspended membranes or sandwich-type samples of membranes deposited on planar substrates (9, 23, 24). Absorption of light by rhodopsin leads to 11-*cis* to *trans* isomerization of retinal (see above), followed by the reactions: $\text{Meta I} \rightleftharpoons \text{Meta II}_a \rightleftharpoons \text{Meta II}_b \rightleftharpoons \text{Meta II}_b\text{H}^+$, e.g. as described by an activated ensemble mechanism (25, 26) (Fig. 1B). Alternatively, a modified square reaction scheme is applicable in which a deprotonated early intermediate (Meta I₃₈₀) is observed prior to forming the Meta II substates of the extended sequential scheme (27).

⁶Because the lipid density is less than protein density, the proteolipid band migrates to a position intermediate between the top and bottom of the centrifuge tube. Typically the lipid/rhodopsin ratio determined in this manner is $\approx$ same as the stoichiometric ratio before dialysis. However for careful work this should not be assumed.

3.1. Preparation of freely suspended membrane samples

1. Membranes are centrifuged and the pellet re-suspended in buffer at the desired pH and used immediately. The pH is controlled by equilibration of the sample with various buffers with overlapping pH ranges, e.g. 200 mM sodium citrate (pH<6.0), sodium MES (2-(*N*-morpholino)ethanesulfonic acid) (pH 6.0 to 7.0), or sodium BTP (1,3-bis(tris(hydroxymethyl)methylamino)propane) (pH>7.0) buffers.
2. Alternatively the pH is adjusted by titration with 0.1 M HCl or NaOH, e.g. at the extremes of pH where time-dependent changes may be occurring. All pH values are measured at the same temperature as the spectra.
3. To reduce the considerable light scattering, the samples can be lightly sonicated in an ice bath under a gentle argon gas stream (3 min with a 50% duty cycle), using a microtip sonifier (Sonics & Materials, Newtown, CT, USA). Another alternative is to expel the freely suspended membrane suspension through an extruder (Avanti Polar Lipids, Alabaster, AL, USA).

3.2. Preparation of sandwich-type membrane samples

1. Prepare the membrane films by drying under nitrogen ≈ 0.4 – 0.5 nmol of rhodopsin in native disk or recombinant lipid membranes in water or very low ionic strength buffer on 20-mm CaF₂ FTIR windows (note that BaF₂ is more fragile) (28). Gently overlay the films with 80 μ L of 200 mM buffer, e.g. citrate, MES, MOPS, or BTP. Let sit for 3 min to ensure adequate hydration and equilibration of the salt concentration and pH of the membrane stacks. Water content in the samples is typically $\approx 90\%$ (v/v) as estimated from the water OH stretch band used as an internal marker in FTIR absorption spectra (*see* Note 7).
2. All pH values should be measured at the same temperature as the spectra. If necessary, correct the nominal pH values for the temperature dependence of the buffer pK_a .
3. Alternatively it may be desirable to avoid drying of the samples (29). Membranes in 50 mM sodium phosphate or sodium BTP buffers are centrifuged at $100000 \times g$ for 30 min at 4 °C. If necessary the pH is adjusted with 0.1M HCl or NaOH. The pellet is then transferred to CaF₂ or BaF₂ FTIR windows with a 3- μ M polytetrafluoroethylene spacer and sealed.

3.3. Experimental conditions for actinic light exposure

1. Representative conditions for exposure of rhodopsin to actinic light (bleaching) used in UV-visible and/or FTIR studies (8–10) are described below. Use either of the following two procedures.

⁷In control experiments, membranes are washed with a large volume of 1 mM BTP buffer adjusted to the appropriate pH value prior to preparation of the sandwich samples. Typically the same results are obtained as with subsequent pH adjustment, thus verifying the reliability of the pH procedure.

2. Method (1): Samples are photolyzed by exposure for 20 s to a 150-W tungsten-halogen light source fitted with a high pass filter ($\lambda > 500$ nm) using a fiber optics light guide (Schott-Fostec ACE light source; Fostec, Auburn, NY, USA). This procedure bleaches $\approx 100\%$ of the rhodopsin.
3. Method (2): Photolyze samples by exposure to an array of 6 ultra-bright green ($\lambda_{\text{max}}=520$ nm) 5-mm light-emitting diodes (LEDs) (Nichia, Tokushima, Japan) having nominal 16 candela at 20-mA current and 15° emission half angle (9). For rapid-scan experiments (FTIR or UV-visible with diode array) a 100-ms actinic flash is delivered at a LED current of 100 mA, which bleaches $\approx 70\%$ of the rhodopsin. For conventional FTIR or UV-visible experiments, the sample is illuminated for 2 s at a LED current of 20 mA to completely bleach rhodopsin (*see* Note 8).

3.4. Experimental protocols for UV-visible spectrophotometry

UV-visible spectrophotometry is used to establish the protonation state of the retinylidene Schiff base (SB) and to quantify the fractional concentrations of the Meta I and Meta II photoproducts of rhodopsin (30). Either a scanning spectrophotometer or a diode-array spectrophotometer can be employed (*see* Note 9).

1. A scanning spectrophotometer (double beam) (e.g. Perkin-Elmer Lambda 19, Waltham, MA, USA; Shimadzu 2500, Columbia, MD, USA; Cary 50 or Cary 4000, Santa Clara, CA, USA, or equivalent) with thermostatted sample holder is used to record absorption spectra over the wavelength range from 700 nm to 250 nm (or 650 nm to 300 nm) (acquisition time of ≈ 45 s for Shimadzu 2500 or Cary 4000). (In the case of highly scattering suspended membranes the spectrophotometer can be equipped with an integrating sphere to more efficiently collect the scattered light.) Alternatively, employ a diode-array spectrophotometer (single beam) (e.g. Hewlett Packard 8453, or equivalent) (acquisition time of 200 ms). Use either freely suspended or sandwich-type membrane samples with actinic illumination conditions as described above.
2. For experiments with a scanning UV-visible spectrophotometer, first record a baseline of buffer blank versus buffer blank. Either sandwich samples or freely suspended membrane are used. The advantage of sandwich samples prepared by dehydration is the very low light scattering in the visible range (28). Both types of preparation can be employed and checked relative to one another. For sandwich samples, the baseline is subtracted and the absolute spectra are recorded directly (due to the reduced light scattering). Measurements with freely suspended membranes utilize an identical bleached sample (containing 10 mM hydroxylamine) as the reference to compensate for the high degree of light

⁸The contribution of isorhodopsin to the photoproduct equilibria is found to be small under these conditions by using its FTIR fingerprint bands as spectral markers ($<10\%$ of Meta I) (10). Spectra are not typically corrected for the isorhodopsin contribution.

⁹A scanning spectrophotometer has the advantage in that sample is exposed to only a narrow band of wavelengths at any one time, and thus exposure to the monitoring light beam is minimal. The disadvantage is that measuring the spectrum is relatively time consuming. A diode array system has the advantage that all the light is passed through the sample prior to wavelength dispersal and detection, so the spectrum is acquired rapidly. The disadvantage is that the sample is exposed to all of the light simultaneously, which may initiate undesired photochemical processes.

scattering. In either case, the sample is illuminated by exposure to actinic light as described above. The light and dark absolute spectra are then subtracted to obtain the difference spectra. Multiple dark spectra are recorded to establish the temporal evolution of the spectral drift, and to correct the offset of the light minus dark difference spectra. All spectral corrections involve difference spectra rather than absolute spectra.

3. In UV-visible experiments employing a diode-array spectrophotometer, use a neutral density filter (e.g. 10% transmission; Thorlabs, Newton, NJ, USA filter ND10B) to minimize sample bleaching caused by the measuring beam. Record multiple dark spectra with a relatively long acquisition time (e.g. 1 s) and average 2–4 spectra to reduce the signal/noise. (Note that the dark spectral noise is propagated in the difference spectra.) Photolyze the sample by exposure to actinic light as described above. For light spectra recorded at lower temperatures (10 and 20 °C), use a spectral acquisition time of 1 s. At higher temperatures (30 and 37 °C) an acquisition time of 200 ms is employed (due to the faster changes). Acquire replicate scans to follow the temporal evolution of the spectra and to check for photoproduct stability. Average multiple light spectra for which the photoproduct is fully stable to increase the signal/noise (typically 2–4 spectra). Subtract the dark reference spectrum and smooth the resulting difference spectrum using the spectrophotometer software. Experiments are done at least in duplicate and typically the results of four experiments are averaged. Under unstable conditions at least four independent runs are carried out.
4. Establish conditions of (T , pH) needed to stabilize rhodopsin in the Meta II or Meta I states following actinic light absorption, i.e. for the membrane samples to be studied. Results for freely suspended or sandwich-type samples are similar (28). The Meta II photoproduct has a deprotonated SB that absorbs at 380 nm, whereas the Meta I photoproduct has a PSB absorbing at 480 nm. The Meta I state is stabilized at 10 °C and pH 9.5 in disk membranes (Fig. 2A). Meta II is typically stabilized at 20 °C and pH 5.0 in natural disk membranes and corresponds to the Meta II_bH⁺ substate (Fig. 2B).
5. Measure the UV-visible spectra of rhodopsin-containing membranes from 700 nm to 250 nm as a function of pH at several different temperatures (Figs. 2C,D). Note that at higher temperatures (20 to 37 °C) the 380-nm absorption due to Meta II persists even at very alkaline pH (Fig. 2D); hence the equilibrium is not fully shifted to Meta I. Record the spectra as a function of time to investigate the stability of the photoproducts (*see* Note 10).
6. Evaluate the Meta II fraction $\theta_{\text{UV-vis}}$ with a deprotonated SB by decomposing the photoproduct minus dark state difference spectra (Figs. 2C,D) into a linear combination of Meta I and Meta II spectra (8, 10). Alternatively, calculate the

¹⁰Because such experiments involve a combination of elevated temperature and alkaline pH values, photoproduct stability needs to be monitored carefully (9). Thermal stability of the photoproduct states is confirmed by obtaining a series of replicate post-illumination spectra as a function of time. Near room temperature (> 20 °C) at even mildly alkaline pH values (> 8) the photoproducts change on the time scale of a few seconds, giving a loose bundle state for an α -helical membrane protein (38) that is the analogue of a molten globule state for water-soluble proteins.

fraction of rhodopsin with a deprotonated Schiff base (θ , fractional Meta II) from the absorption difference, e.g. at 380 or 480 nm (use Eq. 1 of Section 4) (8, 10) (Fig. 2E).

7. Fit the experimental plots of $\theta_{\text{UV-vis}}$ versus bulk solution pH to theoretical functions for the binding isotherms for hydronium ions (Section 4) (Fig. 2E). If necessary, correct the nominal pH values for the temperature dependence of the buffer pK_a . Typically, at relatively low temperature (10 °C) a simple titration equilibrium is seen. However, at higher temperatures (20–37 °C) the alkaline endpoint $\theta_{\text{UV-vis}}^{\text{alk}}$ does not reach zero, suggesting a manifold of Meta II substates (Fig. 2E).

3.5. Experimental protocols for Fourier transform infrared (FTIR) spectroscopy

- 1 Apply FTIR spectroscopy to investigate structural changes occurring in the Meta I–Meta II equilibrium of rhodopsin and the mechanism of the reaction. Prepare sandwich-type samples containing ≈ 0.4 nmol of rhodopsin³³ in buffers with overlapping pH ranges, e.g. 200 mM citrate for pH < 6.0, MES for pH range from 6.0 to 7.0, and BTP for pH range from 6.0 to 9.5. Measure the buffer pH at the temperature of the FTIR measurements. As an alternative, if necessary correct the nominal pH values for the temperature coefficient of the buffer pK_a values.
- 2 Employ a Fourier transform infrared (FTIR) spectrometer (e.g. Bruker Vertex 70 spectrometer or equivalent) with a liquid nitrogen-cooled mercury-cadmium-telluride (MCT) detector) and thermostatted sample holder to acquire spectra as a function of pH over the temperature range 0–37 °C for sandwich samples of rhodopsin-containing membranes (9, 10).
- 3 Use actinic flash illumination protocol as described above. Scale the pre- and post-illumination spectra to the same height of the intense fingerprint mode of retinal near 1237-cm^{-1} in the dark state. Calculate the difference of the photoproduct minus the pre-illumination dark state using FTIR software (9) (Fig. 3A). Contributions of overlapping buffer bands are corrected as described (15). Average multiple spectra to improve the signal-to-noise (see above). Flatten the baseline of the FTIR spectra using standard FTIR software.
- 4 Utilize conventional FTIR spectroscopy (24) at lower temperatures (e.g. 10 and 20 °C), typically with 4 cm^{-1} resolution and acquisition times of 12 s for pre-illumination spectra and between 1.5 and 12 s for post-illumination spectra, depending on temperature and stability of the photoproduct states. Employ rapid-scan FTIR spectroscopy at the higher temperatures (30 and 37 °C) due to faster spectral changes (9, 31). For rapid-scan FTIR the spectra are recorded with a resolution of 4 cm^{-1} and an acquisition time of 6 s for the pre-illumination spectrum, and 120 or 240 ms for single post-illumination spectra (9, 10) (see Note 11).

- 5 Acquire FTIR spectra for sandwich samples of membranes containing rhodopsin in either the Meta I or Meta II_bH⁺ states; normalize and subtract the pre-illumination spectrum from the post-illumination spectrum as described above (Fig. 3A). Note for the native disk membranes the Meta II_bH⁺ and Meta I states are stabilized at 20 °C and pH 5.0 (acid end point) and at 10 °C and pH 9.5 (alkaline endpoint), respectively (see UV-visible spectra in Figs. 2A,B). Note also that the Meta II_b and Meta II_bH⁺ substates share a similar active state FTIR signature (Fig. 3B). Use these results as reference (basis) spectra for decomposing the experimental FTIR difference spectra in the conformation-sensitive region from 1800 cm⁻¹ to 1600 cm⁻¹ into Meta I and Meta II contributions (*see* Note 12).
- 6 For native retinal disk membranes, record difference FTIR spectra (post-illumination minus pre-illumination) in the pH range from 5–10 at a series of temperatures from 10 to 37 °C. Use sandwich samples of retinal disk membranes containing rhodopsin. Note the disk membrane phospholipids include a mixture of phosphocholine (PC), phosphoethanolamine (PE), and phosphoserine (PS) head groups, and various acyl groups including ≈45–50% polyunsaturated docosahexaenoic acid (DHA; C22:6ω3) chains.
- 7 Fit the experimental FTIR difference spectra in the conformation-sensitive region between 1800 cm⁻¹ and 1600 cm⁻¹ as a linear combination (weighted sum) of the Meta I and Meta II_bH⁺ basis (reference) spectra (Fig. 3A). The reference spectra correspond to inactive and active receptor states, respectively. Calculate the Meta II fraction from FTIR spectroscopy (θ_{FTIR}) using the proportions (coefficients) of the weighted sum (linear combination) (Section 4). Alternatively use the amide I band at 1644 cm⁻¹ as a marker for Meta II (Section 4) (*see* Note 13).
- 8 Plot the Meta II fraction θ_{FTIR} as a function of pH and fit the results to simple theoretical functions for the binding equilibrium of hydronium ions (H₃O⁺) (Figs. 3C,D) (Section 4). Typically the pH titration curves follow a regular Henderson–Hasselbalch function at 10 °C and below. However, at higher temperatures the alkaline endpoint value $\theta_{\text{FTIR}}^{\text{alk}}$ does not fall to zero (Fig. 3B), as also seen for UV-visible data (Fig. 2E).
- 9 Compare the results for the Meta II fraction θ_{FTIR} (Fig 3C) to the Meta II fraction $\theta_{\text{UV-vis}}$ as a function of pH from UV-visible spectrophotometry (Fig.

¹¹Difference spectra obtained by conventional FTIR spectroscopy and by rapid-scan FTIR spectroscopy utilize pre-illumination spectra that are acquired with the separate protocols.

¹²Notably the reference FTIR spectra for Meta I at the alkaline endpoint become more like Meta II as the temperature is raised. By contrast, the Meta I reference FTIR spectra are less sensitive to temperature. Assuming the PSB photoproduct has a single Meta I difference spectrum and that the SB photoproduct has a single Meta II difference spectrum may represent a simplifying approximation (9).

¹³Such a decomposition into two basis (reference) spectra assumes the spectral signatures of Meta II_b and Meta II_bH⁺ are similar, and that the population of the additional Meta II_a state in the equilibrium mixture is small (9). Differences are seen for the Asp⁸³/retinal SB bands compared to the Glu¹²²/amide I marker bands that moreover depend on the lipid composition (10). Consequently, use of a basis set comprising just two reference spectra (Meta I and Meta II_bH⁺) (Fig. 3A) may be an oversimplification. Further investigation may be required, e.g. using singular value decomposition.

2E). Typically at 30 and 37 °C the alkaline endpoint value $\theta_{\text{FTIR}}^{\text{alk}}$ is less than the $\theta_{\text{UV-vis}}^{\text{alk}}$ value (Fig. 3C). Interpret the difference between $\theta_{\text{UV-vis}}^{\text{alk}}$ and $\theta_{\text{FTIR}}^{\text{alk}}$ by a contribution from the Meta II_a photoproduct to the equilibrium at the alkaline endpoint, amounting to only $\approx 10\%$ at 37 °C and less at lower temperatures (Fig. 3C). Explain the combined results by an extended reaction scheme with an ensemble of Meta II states (e.g. Meta II_a, Meta II_b, Meta II_bH⁺).

- 10 For rhodopsin/POPC membranes (1:100 protein/lipid molar ratio), conduct analogous FTIR difference studies over the pH range 5–10 at a series of temperatures between 10 °C and 37 °C (see Note 14). Calculate the Meta II fraction θ_{FTIR} from a linear combination of reference (basis) spectra for Meta I and Meta II_bH⁺ (Fig. 3A), or use the amide I band at 1644 cm⁻¹ as a marker for Meta II (see below).
- 11 Fit the results versus pH and compare the parameters ($\text{p}K_a$ and alkaline endpoint) for the rhodopsin/POPC membranes to the native disk membranes, which have a mix of head groups and polyunsaturated DHA chains. Typically for rhodopsin/POPC membranes, the alkaline endpoint values (Fig. 3D) are less than for the native RDM (Fig. 3C), amounting to shifts of the Meta I–Meta II_b equilibrium constant by < 2 . However, the apparent $\text{p}K_a$ values of the titration curves are decreased by ≈ 1.5 units, due to a reduction of the equilibrium constant for H₃O⁺ uptake by a factor of ≈ 30 (9).
- 12 Conduct more detailed FTIR studies of the individual marker bands in the conformation-sensitive region between 1800 cm⁻¹ and 1600 cm⁻¹ for rhodopsin in different membrane lipid environments. The various marker bands are sensitive to conformational changes that occur during receptor activation (9). E.g. compare sandwich samples of native RDM (as described above) to recombinant membranes containing rhodopsin with either POPC or DOPC lipids (10).
- 13 Explore whether the FTIR difference spectra can be represented by a linear combination of just two reference (basis) spectra due to Meta I and Meta II states (9) (Fig. 3A). Note that the individual marker bands (Fig. 4A) encompass the amide I vibrations of the protein backbone at 1644 cm⁻¹ (+) and the C=O stretch due to protonated Glu¹²² at 1745 cm⁻¹ (+) and 1727 cm⁻¹ (–) and Asp⁸³ at 1768 cm⁻¹ (–). Specifically Glu¹²² monitors the hydrogen-bonded network between helices H3/H5, and Asp⁸³ is a marker for the hydrogen-bonded network among helices H1/H2/H7 (10).
- 15 For retinal disk membranes, plot the Meta II fraction obtained from both the FTIR marker bands (amide I, Glu¹²², Asp⁸³) and UV-visible spectrophotometry (retinal SB) as a function of bulk solution pH (Fig. 4B) (Section 4). If the pH

¹⁴1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) is a natural phospholipid having a phosphocholine head group at the glycerol *sn*-3 position, an unsaturated oleoyl (18:1 ω 9) chain at the glycerol *sn*-2 position, and a fully saturated palmitoyl (16:0) chain at the glycerol *sn*-1 position. It is distinct from the natural retinal disk membrane lipids in that a single head group type is present and in the lack of polyunsaturated DHA (22:6 ω 3) chains.

titration curves for the different marker bands are superimposable then just two spectral forms contribute, Meta I and Meta II (i.e. Meta II_a, Meta II_b, Meta II_bH⁺ share a similar spectral signature). In this case, the structural changes are concerted, and the coefficients are θ ($\equiv \theta_{\text{FTIR}}$ or $\theta_{\text{UV-vis}}$) for the Meta II fraction and $(1-\theta)$ for the Meta I fraction (9). However, different photoproducts typically contribute, and the curves for the various marker bands do not superimpose (Fig. 4B). Such nonequivalence implies multiple photoproducts are present, e.g. due to an ensemble of states (Fig. 1B).

- 16 Fit the intensities of the marker bands versus pH to the modified Henderson-Hasselbalch function with a non-zero alkaline endpoint (use Eq. 3 of Section 4). Typically, a non-zero alkaline endpoint is evident for both the FTIR and UV-visible marker bands, indicating the process is not a simple two-state equilibrium (Figs. 3C, 4B). Moreover, the alkaline endpoint obtained from UV-visible spectra ($\theta_{\text{UV-vis}}^{\text{alk}}$) is usually higher than the endpoint from FTIR spectroscopy ($\theta_{\text{FTIR}}^{\text{alk}}$) (9) (Figs. 3C, 4B). Although the Meta II_bH⁺ state is predominant at acid pH values, a significant population of Meta II_a and Meta II_b states exists at alkaline pH (9).
- 17 Interpret the results in structural terms. Note that Asp⁸³ is a marker for the H1/H2/H7 inter-helical network, whereas Glu¹²² is a marker for the H3/H5 inter-helical network. Data for the retinal SB and Asp⁸³ marker bands are explained in terms of deprotonation of the PSB, which triggers activating structural changes in the hydrogen-bonded H1/H2/H7 helical network. Use the amide I and Glu¹²² marker bands for investigating changes in the H3/H5 helical network. (E.g. chromophore movement towards helix H5 occurs with an outward tilt of H6 as seen by spin-label EPR spectroscopy (32).) Note that protonation of Glu¹³⁴ of the ERY motif stabilizes the activated receptor due to a second protonation switch, and explains the pH dependence of the activation (9).
- 18 Conduct analogous FTIR studies of rhodopsin in various recombinant membranes, e.g. sandwich samples of rhodopsin recombined with POPC or the synthetic lipid DOPC (see Note 15). Typical data (Fig. 4C) show that for rhodopsin/POPC membranes (1:100 protein/lipid molar ratio) the alkaline end point values are generally lower compared to the retinal disk membranes. There is a striking downshift of the pK_a to lower values, as noted above (Fig. 3D). Additional sample data for rhodopsin/DOPC recombinant membranes (Fig. 4D) show the pH dependence of the retinal SB band follows closely the structural changes monitored by the Asp⁸³ FTIR difference band. For the Glu¹²² or the amide difference bands (Fig. 4D) the alkaline endpoints are substantially lower than for the retinal SB or Asp⁸³ marker bands.

¹⁵1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) is a synthetic lipid with a phosphocholine head group at the *sn*-3 position and two unsaturated oleoyl (18:1 ω 9) chains at the glycerol *sn*-2 and *sn*-1 positions. It differs from the natural lipids in that it has a single type of head group, with two identical unsaturated acyl groups lacking polyunsaturation as in the case of DHA chains.

- 19 Explain the combined FTIR and UV-visible results by structural changes in hydrogen-bonded networks of rhodopsin (see above). According to the scheme in Fig. 1A, membrane lipids significantly perturb the relative populations of the various Meta II conformational substates (see Note 16).

4. Data Analysis and Interpretation

The results of FTIR and UV-visible spectroscopy are combined to investigate the complex pH-dependent population curves for conformational equilibria among the Meta I, Meta II_a, Meta II_b, and Meta II_bH⁺ photoproduct states (9, 10). Influences of membrane lipids are analogous to mutating the protein. Further interpretation of the UV-visible and FTIR results in terms of lipid-protein interactions involves an activated ensemble mechanism (Fig. 1B).

4.1. Reduction and analysis of experimental UV-visible and FTIR spectral data

1. Use the individual marker bands in UV-visible or FTIR spectroscopy to quantify the fractional Meta II values (θ) as calculated by the formula:

$$\theta = (\Delta A - \Delta A_{\text{Meta I}}) / (\Delta A_{\text{Meta II}_b\text{H}^+} - \Delta A_{\text{Meta I}}) \quad (1)$$

where $\theta \equiv \theta_{\text{UV-vis}}$ or θ_{FTIR} (10). Note that ΔA is the pH-dependent absorption intensity for a marker band in either UV-visible or FTIR difference spectra of the photoproduct minus dark state. E.g. in UV-visible studies the spectral difference

ΔA can be monitored at $\lambda = 380$ nm (characteristic of Meta II) or $\lambda = 480$ nm (indicative of Meta I). Equivalently θ can be calculated using the molar extinction coefficients of the photoproducts (8). In FTIR studies, the marker bands are at 1768 cm^{-1} (Asp⁸³), 1745 cm^{-1} and 1727 cm^{-1} (Glu¹²²) and 1644 cm^{-1} (amide I of Meta II) (Fig. 4A). In the above formula, $\Delta A_{\text{Meta I}}$ and $\Delta A_{\text{Meta II}_b\text{H}^+}$ correspond to the Meta I minus dark state and Meta II_bH⁺ minus dark state reference (basis) spectra, e.g. measured at 10 °C and pH 9.5 and 30 °C and pH 6.0, respectively (Figs. 2C, 2D) (see Note 17).

2. In the case of UV-visible spectrophotometry, use Eq. (1) to calculate the fractional Meta II value $\theta_{\text{UV-vis}}$ due to the protonated Schiff base, using data over the absorption range from 300 to 650 nm (Fig. 2) (9). Typically ΔA at 380 nm is characteristic of Meta II and 480 nm is indicative of Meta I. Alternatively, the experimental difference spectra are fit as a linear combination of Meta I and Meta II_bH⁺ reference (basis) spectra (Fig. 2). Assuming two component-spectra the coefficients are $\theta_{\text{UV-vis}}$ for the Meta II fraction and $(1 - \theta_{\text{UV-vis}})$ for the Meta I fraction (9). Use nonlinear regression fitting software (e.g. Matlab; Mathworks,

¹⁶Selective stabilization of photoproducts by membrane lipids affords a way of structurally characterizing the activation mechanism of rhodopsin. For rhodopsin in DOPC membranes the Meta II_a substate is stabilized, in which PSB deprotonation has occurred prior to helix H6 tilt. It provides a missing link between opening the PSB salt bridge in the transition from Meta I, and the subsequent tilt of H6 in forming Meta II_b as detected by spin-label EPR spectroscopy (32). A very similar selective stabilization of the Meta II_a substate is also found for recombinants of rhodopsin with DOPE/DOPC lipid mixtures (10).

¹⁷When possible carry out all experiments using rhodopsin from a single batch of retinas to avoid sample variability that may affect the quantitative spectral data reduction and analysis. Alternatively multiple batches can be combined if necessary.

Natick MA, USA or Origin; OriginLab, Northampton, MA, USA) to obtain the coefficients (9).

3. In FTIR spectroscopy, the fractional Meta II value θ_{FTIR} is calculated from the difference bands in the conformation-sensitive region between 1800 and 1600 cm^{-1} (Fig. 3A). Again there are two possibilities. The Meta II fraction θ_{FTIR} can be calculated as a linear combination of reference (basis) spectra for the Meta I and Meta II_bH⁺ states (Fig. 3A). (It is assumed that the Meta II spectral signature is nearly the same for the Meta II_a, Meta II_b, and Meta II_bH⁺ substates as in Fig. 3B.) However, use of only two basis spectra may be an oversimplification, since the changes detected by the various marker bands may differ (10). Alternatively, use Eq. 1 to calculate the fractional Meta II value θ_{FTIR} from the intensities of the individual Asp⁸⁴, Glu¹²², or the amide I marker bands as a function of pH (Fig. 4).
4. Fit the data obtained for the Meta II fraction as a function of pH from UV-visible or FTIR spectroscopy to various binding isotherms for hydronium ions (use non-linear regression software). Typically, at relatively low temperature (0–10 °C) the spectral results are described by the Henderson-Hasselbalch equation:

$$\theta = 10^{\text{p}K_a - \text{pH}} / (1 + 10^{\text{p}K_a - \text{pH}}) \quad (2)$$

The above formula describes a two-state, acid-base equilibrium between Meta II_bH⁺ (conjugate base) and Meta I (conjugate acid) states, with the acid

endpoints at $\theta_{\text{UV-vis}}^{\text{acid}}$ or $\theta_{\text{FTIR}}^{\text{acid}} = 1$ and the alkaline endpoints at $\theta_{\text{UV-vis}}^{\text{alk}}$ or $\theta_{\text{FTIR}}^{\text{alk}} = 0$ (Figs. 3C,D) (see Note 18).

5. At higher temperatures (range of 20–37 °C), however, the alkaline endpoint values of the pH titration curves typically do not fall to zero (Figs. 3C,D). Fit the fractional Meta II values θ obtained from the UV-visible and/or FTIR marker bands at higher temperatures to a modified Henderson-Hasselbalch function (9) as given by:

$$\theta = (\theta^{\text{alk}} + 10^{\text{p}K_a - \text{pH}}) / (1 + 10^{\text{p}K_a - \text{pH}}) \quad (3)$$

Here θ ($\equiv \theta_{\text{UV-vis}}$ or θ_{FTIR}) is the Meta II fraction and θ^{alk} ($\equiv \theta_{\text{UV-vis}}^{\text{alk}}$ or $\theta_{\text{FTIR}}^{\text{alk}}$) is the alkaline endpoint of the pH titration curve (see Note 18). (Note that Eq. 3 is more general than Eq. 2 which corresponds to the limit that $\theta^{\text{alk}} \rightarrow 0$.)

¹⁸Additional data analysis uses the following thermodynamic relations. The standard free energy change ($G^{\circ'}$) is related to both the standard enthalpy change ($H^{\circ'}$) and the standard entropy change ($S^{\circ'}$) through the relation $G^{\circ'} = H^{\circ'} - T S^{\circ'}$. The standard states (symbol $\circ'$) correspond to a one molar solution of the components (Meta I or Meta II) at pH 7. All symbols have their usual definitions (R = gas constant, T = temperature, P = pressure). The standard free energy change is given by $G^{\circ'} = -RT \ln K_{eq}'$ where K_{eq}' is given by $\theta/(1-\theta)$ and $\theta \equiv \theta_{\text{UV-vis}}$ or θ_{FTIR} as obtained from the spectral data reduction. The standard enthalpy change is given by $H^{\circ'} = -R(\ln K_{eq}' / 1/T)P$ and the standard entropy change is given by $S^{\circ'} = -(G^{\circ'} / T)P$. Last, the change in standard heat capacity at constant pressure is given by $\Delta C_P^{\circ'} = -(\partial \Delta G^{\circ'} / \partial T)_P$. For further specifics see Refs. (5, 39).

4.2. Example of how to interpret results in terms of lipid-membrane protein interactions

1. The experimental data from UV-visible and FTIR spectroscopy are used to investigate influences of the membrane lipid head group and acyl chain composition on rhodopsin activation (2–5, 7, 33). Lipid substitutions involve varying the head group size and charge as well as the degree of (poly)unsaturation and bulkiness of the acyl chains. Membrane recombinants are selected to distinguish between chemically specific effects versus non-specific lipid-protein interactions due to material properties of the bilayer (1, 5, 9, 10).
2. Generally the membrane lateral pressure profile (Fig. 5A) originates from a balance of opposing attractive and repulsive forces acting at the level of the polar head groups and the acyl chains of the phospholipids. Head group repulsions and interfacial attraction give an optimal separation for the polar region. Within the hydrocarbon core, van der Waals attraction plus steric repulsion yield a preferred distance between the chains. Relate the lipid influences on the metarhodopsin equilibrium (e.g. Fig. 3) to the pressures from head groups of various sizes, and to the bulkiness or degree of polyunsaturation of the acyl groups.
3. Note the lateral pressure profile corresponds to the spontaneous (intrinsic) curvature of a monolayer leaflet (designated as H_0) (Fig. 5B) (1, 5). The value of H_0 can be positive (curvature towards hydrocarbon, as in the case of micelles or normal hexagonal phases of amphiphiles), zero (no curvature, as in the textbook bilayer), or negative (curvature towards water, as in the case of microemulsions or reverse hexagonal phases of amphiphiles) (Fig. 5B). Correlate the shifting of the Meta I–Meta II equilibrium by membrane lipids (see above) with their propensity to form nonlamellar phases, such as the reverse hexagonal (H_{II}) or cubic phases (34). Test whether the influences of bulky unsaturated acyl chains, such as DHA, can be mimicked by relatively small head groups such as PE, and vice versa.
4. Interpret the experimental UV-visible and FTIR results for rhodopsin in terms of models for lipid-membrane protein interactions, such as the flexible surface model (FSM) (1). The idea behind the FSM is that mismatch of the spontaneous (intrinsic) curvature of a monolayer leaflet to the curvature at the proteolipid interface governs the free energies of proteins such as rhodopsin (1, 21) or ion channels (35–37) (Fig. 5C). The FSM describes a competition between the elastic energy due to deformation of the membrane lipids (within the stress field of the bilayer) and the solvation energy of the proteolipid boundary (formulated in terms of the proteolipid surface tension), e.g. due to hydrophobic mismatch (8). Matching the membrane curvature H to the spontaneous monolayer curvature H_0 provides a driving force for protein conformational transitions that explains many of the lipid influences in membranes (1) (see Note 20).

²⁰By connecting the membrane protein shape transitions to changes in membrane lipid curvature forces, a continuum picture explains the influences of membrane lipids on protein functions by the theory of elasticity. The spontaneous (intrinsic) curvature H_0 may be identical with the physical monolayer curvature (denoted by H), or deformation (strain) of the membrane may occur yielding curvature elastic stress or frustration (5). The competition or balance of the curvature free energy (stress/strain) with the acyl chain stretching

5. Relate the curvature free energy of the membrane lipid bilayer to the equilibrium constant for the transition between the Meta I and Meta II photoproducts of rhodopsin (21, 33) and further interpret the experimental findings using the FSM (see Note 21).

Acknowledgments

The author is indebted to A. V. Botelho, J. J. Kinnun, J. W. Lewis, K. Martínez-Mayorga, B. Mertz, A. V. Struts, and R. Vogel for discussions. Research in the laboratory of the author is supported by the U.S. National Institutes of Health and is gratefully acknowledged.

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energy (due to solvation of the proteolipid interface) explains how membrane lipids affect protein conformational energetics in the case of rhodopsin, and is applicable to (mechanosensitive) ion channels and other membrane proteins.

²¹Values of K_{eq}' and/or pK_a determined for the extended mechanism (see above) (9, 40) can be further interpreted in terms of standard free energy changes. Notably the lateral pressure profile (41) does not correspond to any measurable quantity—it is calculated theoretically (42). By contrast, the spontaneous curvature can be found experimentally (43, 44). The membrane lipid curvature free energy can be calculated using experimental estimates of the bending modulus (k_C) and spontaneous curvature (H_0) (33, 45, 46). Use the Helfrich formula $g_C = k_C(H - H_0)^2 + \bar{k}_C K$ as described (1). Here g_C is the curvature free energy per unit area and c_1 and c_2 are the two principal curvatures of the surface. In addition $H \equiv (c_1 + c_2)/2$ is the mean curvature, H_0 is the spontaneous (intrinsic) curvature in the absence of strain, k_C is the bending rigidity (modulus), and $\bar{k}_C$ is the saddle curvature modulus. Note that the curvature free energy is simply given by $g_C \approx k_C(H - H_0)^2$ assuming only a single curvature (c_1) contributes. The curvature c_1 can be calculated from the inverse radius of the hexagonal phase cylinders in the presence of hydrocarbon (43).

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Fig. 1.

Structural and thermodynamic model for rhodopsin shows activation of a prototypical G protein-coupled receptor (GPCR) in a membrane environment. (A) Rhodopsin in the dark-state (Protein Data Bank code 1GZM) (25) has extracellular (*e*) and cytoplasmic (*c*) membrane sides. Large-scale conformational changes of rhodopsin involve the E2 extracellular loop and H5 and H6 helices and initiate the visual process. Volumetric expansion of the receptor within the membrane is described by an activated ensemble mechanism (26). (B) Extended reaction scheme for the dynamically activated receptor involves an ensemble of photoproduct substates (PSB $\equiv$ protonated Schiff base; SB $\equiv$ Schiff base; 134 $\equiv$ Glu¹³⁴). Light absorption yields 11-*cis* to *trans* isomerization of the retinal ligand, followed by equilibration of Meta I with different isochromic Meta II substates. The Meta I state has a PSB and a deprotonated Glu¹³⁴ residue. Deprotonation of the PSB results in the Meta II_a state. In Meta II_b a tilt of helix H6 occurs. Last, in Meta II_bH⁺ proton uptake by Glu¹³⁴ occurs giving a pH-dependent driving force for receptor activation. Note that Meta II_b and Meta II_bH⁺ are conformational substates that activate the G protein transducin (G_t). (C) The extended reaction scheme (panel B) predicts a complex titration behavior, with Meta I, Meta II_a, and Meta II_b present at alkaline pH. A pH-dependent equilibrium with Meta II_bH⁺ occurs at lower pH due to proton uptake by Glu¹³⁴ from the aqueous solvent.

Fig. 2.

UV-visible spectroscopy characterizes deprotonation of the retinylidene Schiff base in the Meta I–Meta II equilibrium of rhodopsin in membranes. Results are for hydrated sandwich samples of membranes between CaF_2 windows (used for FTIR; see below) acquired with a diode array spectrophotometer. (A) Light absorption by the dark state ($\lambda_{\text{max}} = 500 \text{ nm}$) forms the Meta I photoproduct ($\lambda_{\text{max}} = 485 \text{ nm}$) at low temperature (10°C) and alkaline pH (9.5) with a protonated Schiff base. (B) Illumination of the dark state ($\lambda_{\text{max}} = 500 \text{ nm}$) favors the Meta II_bH⁺ photoproduct ($\lambda_{\text{max}} = 380 \text{ nm}$) at higher temperature (20°C) and acidic pH (5.0) with a deprotonated Schiff. (C) UV-visible difference spectra (photoproduct minus dark state) at low temperature (10°C) reveal pH dependence of the Meta I–Meta II equilibrium. The pH values are indicated next to the spectra. The equilibrium lies completely to the side of Meta I at alkaline pH (10.0) (evident from lack of 380-nm absorption), and completely to the side of Meta II_bH⁺ at acidic pH (5.0) (shown by the 380-nm absorption and depletion at 500 nm). (D) UV-visible difference spectra showing pH dependence of the Meta I–Meta II equilibrium at higher temperature (30°C). The spectra are at the same pH values as in panel C. At higher temperature the equilibrium is not fully shifted to Meta I at alkaline pH. The residual absorption at 380 nm (arrow) is due to the Meta II_a and Meta II_b photoproducts and is $\approx$ independent of pH 9.0 (non-zero alkaline endpoint). (E) Fraction of rhodopsin with a deprotonated Schiff base ($\theta_{\text{UV-vis}}$) plotted as a function of pH. The pH titration curves represent the amount of the Meta II_a, Meta II_b, and Meta II_bH⁺ substates in equilibrium with Meta I (see Fig. 1B). Significant deviations from classical Henderson-Hasselbalch pH behavior are evident, with a striking temperature-dependent increase of the alkaline endpoint. (Experimental data from Ref. (9)).

Fig. 3.

Fourier transform infrared (FTIR) spectroscopy describes structural changes in rhodopsin occurring upon photoactivation in membranes. Representative data are shown for hydrated sandwich samples between CaF_2 windows. (A) Reference (basis) FTIR difference spectra (photoproduct minus dark state) obtained for rhodopsin in native disk membranes, corresponding to the Meta II_bH⁺ substate ($T=20$ °C, pH 5.0) or the Meta I state ($T=10$ °C, pH 9.5). The conformation-sensitive region is enclosed in the box. (B) Comparison of alkaline endpoint spectra at different temperatures with synthetic FTIR spectra calculated as a linear combination of the Meta I and Meta II_bH⁺ basis spectra (panel A). Note that the Meta II_b and Meta II_bH⁺ photoproducts share similar FTIR signatures. (C) pH titration curves for rhodopsin in native disk membranes showing plots of Meta II fraction from FTIR spectroscopy θ_{FTIR} (filled circles with solid lines) compared to Meta II fraction $\theta_{\text{UV-vis}}$ from UV-visible spectrophotometry (open symbols with dashed lines, from Fig. 2E). (No distinction is made among Meta II substates.) The alkaline endpoint fractions $\theta_{\text{FTIR}}^{\text{alk}}$ of the FTIR-based pH titration curves at 30 and 37 °C are less than the values $\theta_{\text{UV-vis}}^{\text{alk}}$ obtained from the UV-visible titration curves. (D) Analogous FTIR and UV-visible pH titration curves for rhodopsin reconstituted into POPC membranes show Meta II fractions θ_{FTIR} and $\theta_{\text{UV-vis}}$, respectively. Note the significant influences of the lipid composition versus the native disk membranes in panel C, indicating their role in the visual transduction process. (Experimental data from Ref. (9).)

Fig. 4.

Combination of FTIR and UV-visible spectroscopy reveals an ensemble of conformational substates in rhodopsin activation. Data are for fully hydrated sandwich samples (10). (A) The FTIR marker bands monitor the conformation of the receptor in pH-dependent experiments. The amide I band (due to C=O stretch of peptide groups) is sensitive to the secondary structure; Asp⁸³ detects the interhelical H1/H2/H7 network; Glu¹²² manifests the H3/H5 interhelical network; and Glu¹³⁴ of the ERY motif characterizes the H3/H6 network. (B) Plots of Meta II fraction θ ($\equiv \theta_{\text{FTIR}}$ or $\theta_{\text{UV-vis}}$) versus pH for rhodopsin in native retinal disk membranes at 30 °C obtained from the various marker bands. (No distinction is made among Meta II substates.) Only small differences are seen in the alkaline endpoint values for the various marker bands. (C) Meta II fraction θ for rhodopsin in membranes with the natural phospholipid POPC at 30 °C as determined by UV-visible and FTIR spectroscopy. For rhodopsin/POPC membranes minor differences are evident in the alkaline endpoint values for the marker bands. Compared to the disk membranes, the pK_a is strongly shifted to lower values for rhodopsin in the POPC recombinant membranes, and the alkaline endpoint values are lower. (D) Meta II fraction θ ($\equiv \theta_{\text{FTIR}}$ or $\theta_{\text{UV-vis}}$) versus pH for rhodopsin recombined with the synthetic phospholipid DOPC at 30 °C obtained from the various UV-visible and FTIR marker bands. For rhodopsin/DOPC membranes the alkaline endpoint values for the retinal SB and Arg⁸³ bands exceed those for the amide I and Glu¹²² marker bands, revealing multiple conformational states. Compared to POPC the pK_a is strongly shifted to higher values and approaches the disk membranes. (Experimental data from Ref. (10).)

Fig. 5.

Example of interpretation of experimental UV-visible and FTIR spectra in terms of lipid-protein interactions. The flexible surface model (FSM) describes how membrane lipids act as agonists or antagonists of rhodopsin function due to non-specific material properties of the bilayer (1). (A) Biophysical properties of membrane lipids involve a balance of attractive and repulsive forces pertinent to average lipid shapes and membrane curvature. The optimum lipid shape is due to the lipid polar head groups and non-polar acyl chains—it is visualized as the frustum of a right circular cone or cylinder. The upper base represents the head groups and the lower base is due to the chains. (B) Average lipid shape is closely tied to the concept of spontaneous curvature of an individual lipid monolayer (leaflet) (1). The spontaneous curvature (H_0) is due to bending of a neutral (pivotal) plane where the area is constant. It can be towards hydrocarbon ($H_0 > 0$), planar ($H_0 = 0$), or towards water ($H_0 < 0$) as indicated. The local curvature stress is proportional to $k_C |H - H_0|^2$ where k_C is the curvature elastic modulus (analogous to Hooke's Law). (C) The FSM explains functional influences of membrane lipid-protein interactions by elastic deformation of a membrane lipid film (d_p = thickness adjacent to proteolipid interface; d_L = thickness of unperturbed bilayer). Bending of the neutral plane together with expansion/compression competes with the solvation energy of the proteolipid interface. The concept of curvature matching explains membrane lipid influences by nonspecific material properties of the lipid bilayer (frustration). Note the general principles of membrane curvature deformation (1) as revealed by rhodopsin are applicable to other membrane proteins, including GPCRs, transporters, and ion channels.