Conformational Changes in Rhodopsin

MOVEMENT OF HELIX F DETECTED BY SITE-SPECIFIC CHEMICAL LABELING AND FLUORESCENCE SPECTROSCOPY

Thomas D. Dunham and David L. Farrens

From the Departments of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, Oregon 97201-3088

A recent proposal for the formation of functionally active rhodopsin has placed critical importance on a movement of one of its transmembrane helices (Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Science 274, 768–770). We investigated this hypothesis using a series of eight rhodopsin mutants containing single reactive cysteine residues in the region (helix F) where movement was previously detected. The cysteine mutants were studied in two ways, by measuring their reactivity to a cysteine-specific reagent (PyMPO-maleimide), and by labeling the cysteines with a fluorescent label (monobromobimane) followed by fluorescence spectroscopic analysis. The chemical reactivity data showed sequence-specific variations in reactivity for the mutants in the dark state, resulting in a pattern suggestive of an α helix. Interestingly, only upon photoactivation to the MII form did residues found on the inner "face" of this helix react with the PyMPO-maleimide. The ability of the dark state mutants to react with the fluorescent label monobromobimane followed a similar pattern. Furthermore, fluorescence measurements indicate that a bimane label on the inner face of the helix (at V250C) detects changes in the polarity of its environment and accessibility to a fluorescence quenching agent upon MII formation. Viewed together, the data provide further direct evidence that rhodopsin activation involves a conformational change at helix F.

G-protein-coupled receptors (GPCRs) are membrane proteins that act as the initial input stages in many sensory systems. Arguably, the best characterized GPCR is the bovine visual photoreceptor, rhodopsin (1). Approximately half the amino acids in rhodopsin are found in a cluster of seven membrane-spanning helices (see Fig. 1). The rhodopsin "antagonist" (11-cis-retinal) is covalently bound in the middle of these helices, inactivating the protein in the dark state. Rhodopsin becomes activated when it absorbs light (absorption λmax = 500 nm) and the retinal is converted to an all-trans form (2). This activated rhodopsin species, called MII (λmax = 380 nm), is the only form of rhodopsin that can readily bind and activate the G-protein transducin and initiate the subsequent biochemical events involved in vision.

A central question remains as to why only the MII form of rhodopsin can bind and activate transducin. It has been noted that transducin can be activated by peptide analogues of the cytoplasmic loops of rhodopsin (3), suggesting that dark state rhodopsin is inactive because the loops are physically inaccessible. Thus, a reasonable hypothesis is that MII formation involves some kind of conformational change that exposes the cytoplasmic loops and allows transducin to bind and become activated.

Recently, the nature of these conformational changes has been studied by Khorana, Hubbell, and co-workers using an approach called site-directed spin labeling (SDSL). Briefly, their SDSL studies involved constructing mutant rhodopsin proteins with unique reactive cysteine residues in the cytoplasmic domain in loops C-D and E-F (4, 5), labeling these cysteines with a methanethiosulfonate spin label, and then measuring the electron paramagnetic resonance (EPR) spectrum of the labeled mutant in the dark state and MII state. By assessing the mobility and environment of each spin label, a probable structure of the cytoplasmic domain could be proposed. Interestingly, their experiments suggested that many conformational changes in MII occur near helix F (6, 7). A model was proposed, suggesting MII formation involves an outward movement of this helix away from helix C (7, 8). The potential importance of this movement was implied from observations that linking helix F to helix C (either by disulfide bonds or metal chelating agents) abolished transducin activation (8, 9). Furthermore, a wealth of data is accumulating that suggests a movement of this helix plays a crucial role in forming the active, signaling state in other GPCRs.

In this paper, we directly tested the hypothesis that helix F movement occurs in rhodopsin upon MII formation. We measured differences in chemical reactivity of cysteines engineered into this helix, and studied fluorescence labels attached to these cysteines. Our results clearly detect a conformational change in helix F, and the results are consistent with a previously proposed movement of this helix upon rhodopsin activation. Finally, the methods we describe here can be broadly applied to studies of other GPCRs that may be available in limited quantities.

#References#

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2. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Dr., Portland, OR 97201-3098. Tel.: 503-494-0583; Fax: 503-494-8393.

The abbreviations used are: GPCR, G-protein-coupled receptor; MES, 2-(4-morpholino)ethanesulfonic acid; DM, dodecyl maltoside; PyMPO, N-(maleimidylethyl)-5-((4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate; MII, metarhodopsin II; mBBr, monobromobimane; SDSL, site-directed spin labeling.

Note that results from SDSL studies of helices A and B have not yet been reported, although such studies are currently under way (W. L. Hubbell and H. G. Khorana, personal communication).
FIG. 1. Suggested secondary structure model of rhodopsin. The cysteine residues introduced into this protein (5) are shown in black. The model also shows in gray the native cysteine residues at positions 140, 316, 322, and 323 that were changed to serines to produce a protein with no “background” reactive cysteines. The cytoplasmic side is on the top in this drawing. Labels A–G refer to the helices below.

**EXPERIMENTAL PROCEDURES**

**Materials**

All buffers were purchased from either Fisher or Sigma, except the spectroscopy grade HEPES that was purchased from United States Biochemical Corp. PyMPO-maleimide and mBBr were purchased from Molecular Probes (Eugene, OR). DM was purchased from Anatrace (Maumee, OH), and GBX red light filters from Eastman Kodak Corp. Polyacrylamide columns (2-ml bed volume) were purchased from Pierce. The 9-mer peptide corresponding to the rhodopsin C terminus was acquired from the Emory University Microchemical Facility (Atlanta, GA). The 1D4 antibody was purchased from the National Cell Culture Institute. L-Cysteine was purchased from Sigma, and 4-

**Construction and Expression of Rhodopsin Cysteine Mutants—**The single cysteine rhodopsin mutants used in the present study have been described previously (5). Briefly, the mutants were constructed in the plasmid PMT4, containing a synthetic gene of rhodopsin in which the potentially reactive “background” cysteine residues 140, 316, 322, and 323 were replaced with serines (10, 11) (see Fig. 1). The mutant rhodopsin proteins were transiently expressed in COS cells using the DEAE-dextran method (12, 13, 14). Two days after transfection, the cells were harvested and stored at −80 °C.

**Purification of Rhodopsin Mutants—**All steps in this procedure were done in dark room conditions under filtered red light. Purification of mutant rhodopsins followed previously described procedures (5, 11) except that samples were purified using small disposable polystyrene columns. Before purification, the columns were prepared by adding 400 μl of the 1D4-coupled antibody bead slurry (0.5% bead v/v slurry). The columns were stored in Buffer B at 4 °C and primed with 5 ml of Buffer A before use.

For a typical purification, the harvested cells from five plates of transfected COS-1 cells were thawed in an ice bath for 30 min, then solubilized in 5 ml of Buffer A + 0.5 mM phenylmethylsulfonyl fluoride and placed on a nutator at 4 °C for 1 h. The samples were then centrifuged at 4,000 × g for 10 min, and the supernatant removed. This was followed by a second centrifugation for 15 min at 20,000 × g at 4 °C. The supernatant from this second centrifugation was applied to the 1D4 columns and allowed to flow through by gravity. The pass-through was collected and reapplied to the column a total of five times. The columns were next washed with 5 ml of Buffer C, then with 40 ml of Buffer D, followed by another 40 ml of Buffer E. During the last 5 ml of washing with Buffer E, a 27-gauge 0.5-inch needle was attached to the column to slow the flow rate further. The samples were eluted in 200-μl fractions of Buffer F containing 250 μM 9-mer peptide. A spectrum of each elution sample was measured using a Shimadzu UV-1601 spectrophotometer, and the purified samples were then stored at either 4 °C in the dark or at −80 °C for longer term storage.

**Reactivity Measurements of Rhodopsin Cysteine Mutants with PyMPO—**The reactivity of the rhodopsin cysteine mutants in the dark state was probed by reacting the samples (at pH 6.8 in 0.05% DM, 10 mM NaHCO₃, 1 mM EDTA) with a 50-fold excess of PyMPO (Fig. 2A). The reaction was carried out for 16 h at room temperature while the mutants were bound to the 1D4 antibody columns. This approach allowed extensive washing of the beads to remove any unreacted PyMPO label. After elution from the column, PyMPO labeling was determined for each mutant by comparing its absorption spectrum with that of the labeled sample, allowing the amount of attached PyMPO to be determined from the remaining absorbance at 380 nm (using an extinction coefficient of 23,000 for PyMPO).

**Reactivity Measurement of Purified Rhodopsin Cysteine Mutant V250C with PyMPO in the MII State—**Three μg of mutant rhodopsin in 70 μl was reacted with 20-fold excess PyMPO for 1 min at pH 7.2 (100 mM HEPES and 0.05% DM) and 4 °C in either the dark (−) or MII (+) state (bleached for 30 s). The reaction was then quenched by the addition of L-cysteine to a 1 mM final concentration. Samples were resolved by SDS-polyacrylamide gel electrophoresis. The amount of label incorporation was determined using a Bio-Rad Gel Documentation Instrument by comparing the signal intensity with that of a series of PyMPO-labeled rhodopsin standards of known concentration. The background mutant, # (containing no reactive cysteine residues) (10, 11), was used to show specificity in PyMPO labeling. After visualization of the gels, the samples were subjected to Coomassie staining to check for equal distribution of protein in each of the sample wells.

**Labeling of Rhodopsin Mutants with Monobromobimane (mBBr)—**Labeling of the cysteine mutants with mBBr (Fig. 2B) was carried out with the samples bound to the 1D4 beads, similar to previous methods (10, 14). In the brief, the 1D4 beads in Buffer C were incubated with each solubilized mutant in 15-ml Falcon tubes at 4 °C on a nutator for 5 h. The suspension was then centrifuged at 4,000 × g for 2.5 min and the supernatant removed. A series of washes were performed by adding the respective buffer to the rhodopsin-bound 1D4 beads, followed by removal of the supernatant as described above. The washes were as follows: 10 ml of Buffer D, 10 ml of Buffer D (10-min incubation), 10 ml...
of Buffer F (10-min incubation). The 1D4-bound mutants were then incubated with a 20-fold excess of mBBr in Buffer F at pH 6.7 for 18 h at 4 °C. Before elution from the column, the reaction of the mBBr with samples was quenched by the addition of a 20-fold excess of t-cysteine. The beads were then washed with 12 ml of Buffer F containing varying amounts of detergent (0.025%, 0.2%, 0.2%, and 0.025% DDM) before continuing with the normal purification protocol (see above).

Characterization of mBBr-labeled Rhodopsin Mutants—The mBBr incorporation in each mutant was determined by comparison to a standard curve of mBBr reacted with t-cysteine. Covalent attachment of the labels was verified by subjecting the samples to SDS-polyacrylamide gel electrophoresis analysis visualized by UV irradiation. The effect of labeling on each mutant was assessed by measuring its absorption spectra and rates of retinal release from MII. Spectra were measured using a Shimadzu 1601 UV-visible spectrophotometer both in the dark state and in the MII state after photoconverting the samples for 30 s with >500 nm light using a 150-watt fiber optic illuminator (Techni-Quip Corp). The MII stability was assessed by measuring the time course of retinal release after MII formation (14) using a Photon Technologies QM-1 steady-state fluorescence spectrophotometer. Each measurement was carried out at 20 °C using 125 μl of a 0.25 μM mutant sample in 5 mM MES, pH 6.0 and 0.05% DM. After photoconverting the samples to the MII state (see above), the retinal release measurements were carried out by exciting the sample for 3 s (excitation wavelength = 295 nm, 1/4-nm bandpass slit setting) then blocking the excitation beam for 42 s, to avoid photobleaching the samples. This cycle was repeated for 90 min during each measurement. The sensitivity of the measurements was enhanced by removing the emission monochromator and monitoring the tryptophan fluorescence emission through a combination of one >310-nm long pass filter and a 298–435-nm band pass filter. Results were analyzed, using Sigma Plot (Jandel Scientific) to obtain the τQ and values for retinal release. All fits had χ2 values ranging from 0.99 to 1.1.

Fluorescence Excitation Scans of mBBr-labeled Mutants K248C–R252C—Excitation scans were carried out using the PTI QM-1 fluorescence spectrophotometer described above. The excitation bandpass was 1/3 nm, and the fluorescence emission was detected through two >470-nm long pass filters (Oriel). Each measurement used 200 μl of a 2-μg sample (250 nm). The samples were first scanned in the dark state and then again after photoconverting to the MII state. Two such scans from 300 to 450 nm (step size = 1 nm, integration time of 0.25 s) were averaged for each mutant, and two separate sample preparations were measured. Under these conditions, less than 2% bleaching of the samples was observed as judged by UV-visible spectroscopy. Excitation maxima were determined by importing the spectra into the program LabCalc (Galactic Industries), and then taking the first derivative of the spectra, using a 21-point window. The peak maximum was taken to be the crossover point, i.e. the wavelength where the first derivative crossed zero, going from positive to negative.

Fluorescence Lifetime Measurement of mBBr-labeled Rhodopsin Mutant V250C—Fluorescence lifetime measurements of the bimane-labeled rhodopsin mutants were carried out using a PTI Laserstrobe fluorescence lifetime instrument. Measurements were taken at 10 °C, using 381-nm excitation pulses (full width at half maximum (FWHM) ~1.5 ns) while monitoring the emission through two long pass filters (>450 nm and >470 nm). Measurements used 225 μl of a 1 μM sample placed in a 4-mm black jacketed cuvette, and represented three averages of 5 shots per point, collected in 100 channels. Under these conditions, each measurement took less than 5 min and resulted in less than 2% of the rhodopsin spectra being converted to MII, as judged by UV-visible spectroscopy. Fluorescence lifetimes of the samples in the MII state were measured as described above, except the samples were irradiated with >490 nm light for 1 min before measurement. The fluorescence decays were fit to a single exponential using the commercial PTI program. Note that less than 8% of the sample had decayed to opsin and free retinal during the 5-min duration of the measurement, as calculated from the retinal release rates at 10 °C.

Fluorescence Quenching Measurements—Steady-state fluorescence quenching measurements were carried out using the PTI steady-state fluorescence spectrophotometer and KI as the fluorescence quenching agent. Measurements used 125 μl of a 0.25 μM sample in a 2-mm cuvette. Excitation was set at 380 nm (1/3-nm bandpass setting). The emission was monitored through two >470-nm long pass filters. Five separate samples with different KI concentrations (ranging from 0 to 25 mM) were measured. The salt concentration in the sample was kept constant at 25 mM by the addition of a corresponding amount of KCl, and Na2S2O3 was added to 10 mM to inhibit formation of I−. Measurements of the MII state were carried out after bleaching the samples with >490 nm light for 30 s, as described previously.

The data were plotted as fluorescence intensity versus concentration of quenching agent, to calculate the Stern-Volmer quenching constant, \( K_{SV} \).

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F_0 \cdot F = 1 + K_{SV} \cdot [Q] \quad (Eq. 1)
\]

\[
F_0 \cdot F = 1 + k_q \cdot \tau_Q \cdot [Q] \quad (Eq. 2)
\]

\( F_0 \) and \( F \) are the intensities of the fluorescence before and after addition of the KI quenching agent (Q), respectively. The plots were analyzed using Sigma Plot (Jandel Scientific). The \( K_{SV} \) values thus obtained were then used with the measured fluorescence lifetimes (\( \tau_Q \)) to determine the bimolecular quenching constant, \( k_q \), from the following form of the Stern-Volmer relationship (15).

RESULTS

Expression, Purification, and Spectral Characterization of Single Cysteine Rhodopsin Mutants K248C–I256C—In the present paper, we have used rhodopsin mutants containing unique, single cysteine residues in the cytoplasmic end of helix F to investigate whether movement occurs in this region of the protein upon MII formation. The mutants (K248C–I256C, shown in Fig. 1) were previously constructed (5) in the “backbone” mutant (θ) containing no other reactive cysteine residues (10). The mutants were transiently expressed in COS-1 cells and purified using an immunoaffinity procedure (12, 13). Similar to the previous study, we found these mutants are all wild-type-like, as judged by their ability to bind 11-cis-retinal and form a 500-nm-absorbing species. Further, each mutant could be obtained in the correctly folded form, as judged by the \( A_{280\,nm}/A_{500\,nm} \) ratio (which ranged from 1.6 to 1.8).

Accessibility of the Rhodopsin Cysteine Mutants to the Cysteine-specific Reagent PyMPO-maleimide—The accessibility of the cysteine mutants K248C–I256C in dark state rhodopsin mutants was investigated using the cysteine-specific reagent PyMPO-maleimide (Fig. 2A). As anticipated, this large, rigid molecule demonstrated differences in its ability to penetrate and react with cysteine residues in sterically constrained regions of the protein (Fig. 3). The spectra of each PyMPO-labeled mutant (purified after reaction with for 16 h with a 50-fold excess of PyMPO) were shown in Fig. 3A, and a plot of the labeling efficiency is shown in Fig. 3B. While several mutants (K248C, E249C, T251C, R252C, and I255C) showed a near one-to-one labeling efficiency with the PyMPO, several others (V250C, M253C and V254C, I256C) showed little to no reactivity. The reactivity of V250C with PyMPO in the MII state was explored further (described below).

Measurement of Light-dependent Changes in Reactivity of Mutant V250C with PyMPO—Mutant V250C, which showed...
almost no reactivity with PyMPO in the dark state, was next studied to see whether it might show increased reactivity in the MII state (Fig. 4). Indeed, a dramatic increase in labeling of this residue was observed after the sample was converted to the MII state. In contrast, the control “background” mutant showed no reactivity toward the PyMPO either in the dark state or after photobleaching to the MII form. The extent of PyMPO labeling was determined to be one PyMPO per mutant.

**Fig. 3.** Reactivity of PyMPO-maleimide with dark state rhodopsin cysteine mutants. A, spectrum of each PyMPO-labeled mutant. Each spectrum shows the labeled mutant, a wild-type ideal rhodopsin spectrum, and the difference between the two, from which the amount of PyMPO was calculated. B, plot of the number of PyMPO labels/mutant determined from A. Details are given under “Experimental Procedures.”
V250C by comparison to a series of rhodopsin standards previously labeled with PyMPO (Fig. 4, B and C). The specificity of the PyMPO for residue V250C was established further by monitoring the time course of the labeling reaction (Fig. 4D). The results from these experiments show that one PyMPO specifically reacts with the cysteine residue at V250C, and does so only upon MII formation. Note that, during the 5-min time span used in this labeling experiment, less than 3% of the sample has decayed from MII to opsin (calculated from a $t_{1/2}$ for MII = 115 min at 4°C; Ref. 14).

**Labeling the Rhodopsin Mutants with Monobromobimane and Characterizing the Samples**—The single cysteine mutants were next labeled with the cysteine-specific label monobromobimane for subsequent fluorescent spectroscopic studies. Monobromobimane was chosen because it is a relatively small label (approximately the size of a spin label or tryptophan residue) with a long fluorescence lifetime and high quantum yield. Additionally, monobromobimane is sensitive to changes in the polarity of the surrounding solvent (16).

The labeling efficiency for each mutant was determined by comparison to a “standard” graph of bimane (Table I). Mutants K248C through M252C could each be reacted with approxi- mately one bimane label per protein. However, the labeling efficiencies of mutants I255C and I256C were much less than one label per protein, and consistent with the PyMPO labeling results, mutants M253C and V254C showed essentially no reactivity with the bimane label. Thus, the latter four mutants were not used in further analysis. Note that only $\sim 0.2$ mBBr/protein were observed to react with the “background” cysteine-less mutant $\theta$, consistent with previous reports showing little reactivity of $\theta$ with different cysteine-specific reagents (10, 11).

The effect of the bimane label on each mutant was characterized by measuring the labeled mutant’s absorption spectra and rate of retinal release from the MII state. None of the absorption spectra for any of the mutants showed obvious differences with respect to wild-type rhodopsin in either the dark state or MII state (Table I). Furthermore, all of the bimane-labeled mutants displayed retinal release rates similar to wild-type rhodopsin (Table I). These results suggest that no dramatic perturbation of the protein structure was induced by the bimane label.

**Fluorescence Excitation Scans of Monobromobimane-labeled Mutants**—Fluorescence excitation spectra were measured for the mutants to assess the environment surrounding each bimane label (Fig. 5). The excitation and emission spectra of bimane are sensitive to the polarity of the surrounding solvent (16). In our own experiments, we have found that the excitation maxima of bimane can range from 388 nm in water (dielectric constant = 78.5) to 371 nm in dioxane (dielectric constant $\sim 2.2$). We used this property of bimane to monitor conformational changes by detecting shifts in the excitation maxima of the attached bimane labels. This approach has a key advantage for rhodopsin studies. During fluorescence excitation measurements, the samples are subjected to a minimal amount of measuring beam light, due to the small bandpass excitation slit settings used to obtain high wavelength resolution data. UV-visible spectra taken before and after the measurements showed less than 2% bleaching of the rhodopsin samples had occurred (data not shown).

As can be seen in Table I, four of the mutants (K248C, E249C, T251C, and R252C) showed small changes in their conformational behavior with respect to wild-type rhodopsin in either the dark state or MII state (Table I). Furthermore, all of the bimane-labeled mutants displayed retinal release rates similar to wild-type rhodopsin (Table I). These results suggest that no dramatic perturbation of the protein structure was induced by the bimane label.

**Fluorescence Quenching Studies of V250C-mBBr in the Dark State and MII Form**—The conformational change detected at V250C-mBBr was further investigated by fluorescence quenching studies. As can be seen from the Stern-Volmer plot of these studies (Fig. 6A), steady-state fluorescence quenching by KI is much greater for the MII species. However, these results must be corrected for the differences in fluorescence lifetimes of the dark state and MII state V250C-mBBr, as shown in Fig. 6B. The most appropriate way to do this is to compare bimolecular quenching constants ($k_q$) rather than the Stern-Volmer quenching constants, $K_{SV}$, obtained from the steady-state fluorescence measurements. This can be accomplished by using

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the relationship \( k_q = K_{SV}/\tau_0 \). Note that the different lifetimes are most likely due to the decreased fluorescence overlap (and thus energy transfer) from bimane to retinal in the MII state.

The Stern-Volmer quenching constants \( K_{SV} \) obtained from the steady-state fluorescence quenching measurements, the fluorescence lifetimes \( \tau_0 \), and the bimolecular quenching constant \( k_q \) calculated for V250C-mBBr in the dark and MII states are given in Table II. Note that the higher \( k_q \) obtained for V250C-mBBr in the MII state clearly shows that the bimane label experiences more collisions with the aqueous quenching agent I\(^-\) than it does in the dark state. These results again clearly show that some conformational change has occurred in the protein at this location. Details of these measurements are given under “Experimental Procedures.”

### DISCUSSION

In this paper, we set out to test the proposal that a movement of helix F in rhodopsin occurs upon MII formation (7–9). Our experiments employed a series of eight mutants, each of which contained a single reactive cysteine in a different part of helix F (Fig. 1). We used two different methods to analyze these mutants. The reactivity of the engineered cysteine residues was investigated (4, 17, 18) using a large, rigid cysteine reactive reagent, PyMPO (Fig. 2A), and the fluorescence properties of a solvent-sensitive fluorescent label (mBBr, Fig. 2B) attached to the cysteine residues was studied (15).
The first striking observation from these studies was that the cysteine residues varied in their reactivity toward PyMPO. Furthermore, the reactivity varied in a sequence-specific fashion (Fig. 3) that is consistent with models showing this region of rhodopsin to be an α helix (7, 8, 19, 20). Fig. 7A indicates on a rhodopsin model (7) the location of the mutants’ reactivity with PyMPO.

The results also provide direct evidence for a conformational change around helix F during MII formation. For example, residues on the inner face of this helix could only react with the PyMPO in the MII form (V250C (Fig. 4) and also M253C (data not shown)). Similarly, a fluorescent bimane label at V250C also detected changes both in the polarity of its environment (Fig. 5) and in its accessibility to fluorescence quenching agents (Fig. 6) upon formation of the MII state. While our results are consistent with the previously proposed outward tilt and/or rotation of helix F (8) (shown in schematic form in Fig. 7B), they do not resolve the exact nature of the movement or rule out a possible concomitant movement of helix C (6, 21). One thing clear from these studies is that the packing of the inner face of helix F is quite tight. Neither the PyMPO nor the mBBr could react with some residues on the inner face of this helix, even after extended incubation times (16 h). In contrast, the reaction of PyMPO with V250C in the MII state was complete in less than 30 s (Fig. 4D).

The hydrophobic shift detected by the bimane label at V250C is noteworthy and has not been reported before. This shift, going from an apparent dielectric constant of ~44 to ~17, is most likely is due to a restructuring of the protein that introduces the bimane label into new contacts with neighboring hydrophobic residues. Examination of recent rhodopsin models (19–22) shows several “hydrophobic patches” in the helices neighboring V250C. The role that a new, more hydrophobic patch may play in transducin activation is unknown. However, it is tempting to speculate that it may be involved in increasing Arg-135 exposure during MII formation. Arg-135 is a highly conserved pair of residues required for normal transducin binding and activation (23–26). The new region of hydrophobicity might change the hydrogen bonding propensity and/or location of Arg-135 in the MII state.

Several other indirect clues from biochemical and mutagenesis studies have suggested a movement of helix F is required to form an active MII rhodopsin species. For example, linking helix F with helix C, through either disulfide linkages (8) or metal chelating agents (9) abolishes the ability of the mutants to activate transducin. Substitutions of M257L on the inner face of this helix have also been found to result in constitutively active mutants, presumably through a sterical mechanism (27).

A growing body of evidence from mutagenesis and modeling studies suggests that activation of other GPCRs involves the same helix (28–32). Interestingly, studies of the m2 muscarinic receptors suggest the region studied in the present paper (which includes the so-called VTIL region; Ref. 32), might become more exposed upon receptor activation (33), and thus allow direct coupling with the C-terminal sequence of α₁b (34). The movement we describe here may be involved in a similar way, effectively exposing this region in rhodopsin and enabling direct coupling with the G-protein transducin. Alternatively, a helix F/helix VI movement may be required to reorient the C-terminal tail (35). Recently, a movement of the same region in helix VI in the β-adrenergic receptor was suggested from fluorescence spectroscopy studies (36). This latter result raises the intriguing possibility that a movement of helix F/helix VI is a conserved and primary step in the activation of GPCRs.

In future work we intend to define further the nature of the conformational change we detect here in helix F, and to attempt to resolve whether the helix movement involves exclusively a rotation or a tilt, or some combination of the two movements. It would also be of interest to test the functional role of the movement by studying defective rhodopsin mutants.
Finally, we point out that the approaches described in this paper allow experiments requiring only minimal amounts of sample (2–3 μg), and thus should work with other GPCR systems of limited availability. The combination of PyMPO as a probe with SDS-polyacrylamide gel electrophoresis analysis is especially advantageous, as it provides a simple, sensitive, and direct method to measure conformational changes in GPCRs.

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