Stability of Dark State Rhodopsin Is Mediated by a Conserved Ion Pair in Intradiscal Loop E-2*

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The rhodopsin crystal structure reveals that intradiscal loop E-2 covers the 11-cis-retinal, creating a “retinal plug.” Recently, we noticed the ends of loop E-2 are linked by an ion pair between residues Arg-177 and Asp-190, near the highly conserved disulfide bond. This ion pair appears biologically significant: it is conserved in almost all vertebrate opsins and may occur in other G-protein-coupled receptors. We report here that the Arg-177/Asp-190 ion pair is critical for the folding and stability of dark state rhodopsin. We find ion pair mutants that regenerate with retinal are functionally and spectrally wild-type-like yet thermally unstable in their dark state because of rapid hydrolysis of the retinal Schiff base linkage. Surprisingly, Arrhenius analysis indicates that the activation energies for the hydrolysis pathways are similar between the ion pair mutants and wild-type rhodopsin. Furthermore, the ion pair mutants do not show increased reactivity toward hydroxylamine, suggesting that their instability is not caused by an increased exposure to bulk solvent. Our results indicate that the loop E-2 ion pair is important for rhodopsin stability and thus suggest that retinitis pigmentosa observed in patients with Asp-190 mutations may in part be the result of thermally unstable rhodopsin proteins.

Rhodopsin, the dim light photoreceptor of rod cells, is the best characterized member of the superfamily of G-protein-coupled receptors (GPCRs), 1 (1–8). Rhodopsin consists of a chain of 348 amino acids, approximately half of which form a cluster of seven membrane-spanning helices located within the membrane (Fig. 1). The rhodopsin chromophore, 11-cis-retinal, resides in the middle of these helices attached to lysine 296 through a protonated Schiff base link (9, 10). Interactions of amino acid side chains, as well as water molecules within the chromophore-binding pocket with the retinal, result in the 500 nm absorbance maxima for dark state rhodopsin (11, 12). Dim light vision begins when the 11-cis-retinal chromophore in rhodopsin absorbs a photon and is converted to all-trans-retinal. This change in retinal configuration initiates a series of photo-intermediates and conformational changes in the protein, culminating in a 380 nm absorbing species called metarhodopsin II (MII), the “active conformation” which is able to bind and activate the G-protein transducin (3, 6, 13).

Recently, high resolution crystal structures of rhodopsin have been obtained (2, 14, 15). These structures confirm some of the previous hypotheses about the rhodopsin structure, such as the general arrangement of the transmembrane helices, the locations of the disulfide bond, and glycosylation sites (16–21). However, they also revealed several surprises. One of the most intriguing aspects was the high degree of order in the intradiscal loops (the equivalent to the extracellular loops in other GPCRs and hereby denoted as such). Especially intriguing is loop E-2, which connects helices IV and V (residues 173–198) and forms a twisted β-hairpin that lies alongside the retinal chromophore, potentially forming a “lid” or “plug” across the retinal-binding pocket (2, 14, 15) (Fig. 1). This unexpected finding has led to a number of new questions. What role does the structure of loop E-2 play in the stability and function of rhodopsin? Does it help provide a place for retinal to bind, or does retinal binding induce structure in loop E-2? If the loop E-2 structure is present in the apoprotein (opsin), how does retinal get in and out of the binding pocket?

We have recently begun to address some of these questions, and in the process noticed an ion pair, Arg-177/Asp-190, is present on the ends of loop E-2 (Fig. 1B). This ion pair, Arg-177/Asp-190, is conserved in almost all vertebrate rhodopsins and may also be present in other GPCRs (Fig. 2). Furthermore, the potentially important functional role of this ion pair is suggested by the fact that mutations at residue Asp-190 in rhodopsin are found in patients with autosomal dominant retinitis pigmentosa (ADRP) (22–26).

In this work we report our investigations into the structural and functional role of the Arg-177/Asp-190 ion pair. Our primary finding is that the ion pair helps stabilize the dark state rhodopsin structure. We find that mutations to the ion pair either result in opsin proteins that do not regenerate with 11-cis-retinal or, if they do regenerate, undergo rapid retinal Schiff base hydrolysis in the dark state. Surprisingly, the active MII signaling state and MII decay processes are not af-
Materials

 Except where noted below, all buffers and chemicals were purchased from either Fisher or Sigma. Protease inhibitor tablets and GTP-S were purchased from Roche Molecular Biochemicals. DM was purchased from Anatrace (Maumee, OH), and GBX red light filters were from Eastman Kodak Co. Polystyrene columns (2-ml bed volume) were purchased from Pierce. Frozen bovine retinas were from J. A. Lawson Co. (Lincoln, NE). Transducin was purified from rod outer segments as described previously (27). DNA oligonucleotides were purchased from Qugen/Operon (Alameda, CA). Restriction endonucleases were from New England Biolabs (Beverly, MA). 11-cis-Retinal was a generous gift from Dr. R. Crouch (Medical University of South Carolina and NEI, National Institutes of Health). The rho1D4 antibody was purchased from the National Cell Culture Center (Minneapolis, MN). The nonapeptide corresponding to the C terminus of rhodopsin was acquired with the Emory University Microchemical Facility (Atlanta, GA). Cuvettes were purchased from Uvonics (Plainview, NY). Bandpass filters and long-pass filters were purchased from Oriel (Stratford, CT). The 30% acrylamide/bisacrylamide solution (37.5:1) was purchased from Bio-Rad. Goat anti-mouse (H+L) conjugated with peroxidase and SuperSignal West Pico Luminol/Enhancer Solution were obtained from Pierce.

Buffers

The definitions of the buffers used are as follows: PBSSC (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM Na2HPO4, pH 7.2); buffer A (1% DM and PBSSC (pH 7.2)); buffer B (2 mM ATP, 0.1% DM, 1 mM NaCl, and 2 mM MgCl2, pH 7.2); buffer C (0.05% DM and PBSSC (pH 7.0)); buffer D (0.05% DM and 5 mM MES (pH 6.0)); buffer E (5 mM Tris-HCl, 2 mM EDTA (pH 7.2)); and buffer F (20 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM EDTA (pH 7.2)).

Construction and Expression of Rhodopsin Mutants

Site-directed mutagenesis was performed using a cassette-based strategy as described previously in the pMT4 plasmid (28, 29), as well as overlap extension PCR (30) to generate EcoRI and NotI fragments containing either the R177C, R177K, R177Q or D190C, D190E, D190N mutations in the synthetic bovine rhodopsin gene (28). The primers for the primers are as follows: R177C, 5'-CTGTGGCATGTTGTCAC-ATCCGGAGG3'; R177K, 5'-GCTGTGGCATGTTGTCAC-ATCCGGAGG3'; R177Q, 5'-GCTGTGGCATGTTGTCAC-ATCCGGAGG3'; and D190N, 5'-CGGATCGAGTACTACACGCCGC3'. The sequences of the primers are as follows: R177C, 5'-CTGTGGCATGTTGTCAC-ATCCGGAGG3'; R177K, 5'-GCTGTGGCATGTTGTCAC-ATCCGGAGG3'; R177Q, 5'-GCTGTGGCATGTTGTCAC-ATCCGGAGG3'; and D190N, 5'-CGGATCGAGTACTACACGCCGC3'. The primers were purchased from Genentech and were annealed by heating to 95°C and then allowing to cool slowly. The primer sets were then subcloned into the pMT4 plasmid. The correct products were verified by dideoxynucleotide sequencing. The mutant rhodopsin proteins were transiently expressed in COS-1 cells using the DEAE-dextran method, and cells were harvested 56–72 h after transfection as described previously (33, 34).

Purification of Rhodopsin Mutants

Mutant rhodopsin proteins were expressed and harvested essentially as described previously (34). Briefly, five 15-cm plates of transfected COS-1 cells were washed twice with 7 ml of cold PBSSC buffer per plate, pelleted, and subsequently resuspended in 10 ml of cold PBSSC (pH 6.5) containing 0.5 mM PMFS. The opsin mutants were then reconstituted with 11-cis-retinal at 4 °C for 1 h, and an additional 5 μl of 11-cis-retinal was then added and regeneration allowed to proceed for an additional 1 h (35). The purification of the rhodopsin mutants proceeded essentially as the original procedure (33), except small polystyrene columns were used for washes and elution (36). Cells were solubilized in 5 ml of buffer A containing 0.5 mM PMFS at 4 °C for 1 h and then centrifuged to pellet the unsolubilized fraction. The supernatant was mixed with 400 μl of rho1D4 antibody-Sepharose beads (binding capacity ~1 μg of rhodopsin/μg of resin) in buffer B containing 0.5 mM PMFS and incubated at 4 °C for 4–5 h. The slurry was subsequently transferred to polystyrene columns and washed once with 50 ml of buffer C followed by a 40-ml wash with buffer D by gravity filtration. Samples were eluted in 550–μl
Intradiscal Ion Pair Mediates Thermal Stability of Rhodopsin

fractions of buffer D containing 200 μM nonapeptide corresponding to the rhOD4 antibody epitope (the last nine amino acids of the C terminus of rhodopsin). A spectrum of each elution fraction was recorded (described below), and the purified samples were either used immediately or snap-frozen in liquid N2 and stored at –80 °C.

Inmunoblot Analysis of Rhodopsin Mutant Cell Membranes

COS cells expressing rhodopsin mutants were pelleted and resuspended in 1 ml/plate of buffer E and homogenized on ice. The homogenates were then centrifuged at 40,000 × g for 45 min at 4 °C, and the pellets were washed with 5 ml of buffer F and subsequently resuspended in buffer F. Protein concentrations of the resuspended membrane pellets were determined by a modified DC protein Assay from Bio-Rad. The manufacturer’s instructions were followed except for the addition 1.45% SDS to each well. Aliquots of the membrane preparation were snap-frozen and stored at –80 °C until use. SDS-PAGE was performed according to Laemmli (37), using a 5% stacking gel and a 10% resolving gel. The protein bands were electrotransferred onto Immobilon-P transfer membranes (Millipore) and detected using the rhOD4 monoclonal antibody as described previously (19). Protein expression levels were determined using a Bio-Rad Phosphorimager, and pixel densities were determined using a GS-525 molecular imaging system using supplied software.

UV-visible Absorption Spectroscopy

All UV-visible absorption spectra were recorded with a Shimadzu UV-1601 spectrophotometer at 20 °C using a bandwidth of 2 nm, a response time of 1 s, and a scan speed of 500 mm/min unless otherwise noted. For concentration calculations, a molar extinction coefficient value (ε650) for WT rhodopsin was taken to be 40 600 M⁻¹ cm⁻¹ (38). The samples were photobleached in buffer D by illumination for 30 s (at a 6-Hz flash rate) with a Machine Vision Strobe light source (EG & G) equipped with a wavelength λ >490-nm long-pass filter. This light treatment was found to be adequate for full conversion of all samples. Extinction coefficients were determined for each dark state mutant species as described previously in buffer D at 15 °C (34, 39). The presence of a protonated Schiff base (PSB) in the MII state for each mutant was verified by adding H₂SO₄ to a pH of 1.9 immediately after photobleaching and then measuring the absorbance spectrum to assay the presence of a spectral species at 440 nm (which indicates a PSB) (40).

Thermal Bleaching of Rhodopsin Samples

Absorbance Measurements—Thermal decay rates were followed by UV-visible spectroscopy in buffer D. Specific temperatures were maintained using water-jacketed cuvette holders connected to a circulating water bath. Temperature was monitored through emersion of a digital thermometer into the sample chamber, with an accuracy of approximately ±0.2 °C. Thermal stability of the mutants was determined by first measuring the samples from 650 to 250 nm at 1-min intervals at a given temperature. Thermal decay rates were then measured by monitoring the decrease of the 500 nm absorbing dark state species from these measurements over time (41–43). Base-line drift was corrected for by normalizing all spectra to an absorbance of 0 at 650 nm.

Fluorescence Measurements—Thermal decay rates were also measured by monitoring the increase in tryptophan fluorescence at 330 nm, caused by the release of retinal from the chromophore-binding pocket (44). The experimental set up was similar to that of the retinal release assay (described below) except that the samples were not photobleached. All thermal decay data was analyzed using mono-exponential decay (absorbance experiments) or mono-exponential rise to maxima (fluorescence experiments) fitting algorithms in Sigma Plot (Jandel Scientific software).

Thermodynamic Calculations of Thermal Decay Rates

Activation energies (E_a) were determined by applying rate data to the Arrhenius equation: \( k = A e^{-E_a/RT} \). Thermodynamic parameters \( \Delta H^\circ, \Delta S^\circ \), and \( \Delta G^\circ \) were calculated from the rate data as described previously (41, 45). Briefly, the following thermodynamic Equations 1–3 were used,

\[
\Delta G^\circ = -(2.3) RT \log(hk T) \quad \text{(Eq. 1)}
\]
\[
E_a = \Delta H^\circ - T \Delta S^\circ \quad \text{(Eq. 2)}
\]
\[
\Delta G^\circ = \Delta H^\circ - T \Delta S^\circ \quad \text{(Eq. 3)}
\]

where \( R \) is the universal gas constant; \( T \) is the temperature; \( h \) is the thermal decay rate, \( k \) is Planck’s constant, and \( h_k \) is the Boltzmann constant.

Measurement of the Rate of Retinal Release and/or MII Decay by Fluorescence Spectroscopy

The MII stability was assessed by measuring the time course of retinal release occurring after MII formation using a Photon Technologies QM-1 steady state fluorescence spectrophotometer (44). Each measurement was carried out using 100 μl of a 0.25 μM mutant sample in buffer D, and sample temperature was maintained as described above. After the samples were photobleached to the MII state (see above), the retinal release measurements were carried out at the appropriate temperature by exciting the sample for 3 s (excitation wavelength λ = 295 nm, 12-nm bandwidth slit setting) and then blocking the excitation beam for 42 s to avoid further photobleaching the samples. Tryptophan fluorescence emission was monitored at 330 nm (12-nm bandwidth slit setting), and this cycle was repeated for up to 100 min during each measurement. To determine the t½ values for retinal release, experimental data were analyzed using a mono-exponential rise to maxima fit in Sigma Plot (Jandel Scientific software). In this manner a series of MII decay rates was obtained at 5, 10, 15, 20, 25, 30, and 35 °C, and their rates were applied to the Arrhenius equation, \( k = A e^{-E_a/kT} \), to determine the activation energy (E_a) of the retinal release process for each mutant rhodopsin.

Determination of Transducin (G_{T}) Activation Rates

Activation of G_{T} by rhodopsin was monitored using fluorescence spectroscopy at 1°C as described previously (34, 46–48). The excitation wavelength λ = 295 nm, and fluorescence emission was monitored at 340 nm (12-nm bandwidth). Photobleached mutant rhodopsin (see above) was added to a concentration of 5 nM to the reaction mixture consisting of 250 mM GTP_{S}, 10 mM Tris (pH 7.2), 2 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, and 0.01% DM, and the mixture allowed to stir for 300 s. The reaction was then initiated with the addition of GTP_{S} to a final concentration of 5 μM, and the increase in fluorescence was followed for an additional 2000 s. To calculate the initial activation rates, the slopes of the initial fluorescence increase following GTP_{S} addition were determined through the data points covering the first 60 s.

Effect of R177Q Thermal Decay on Ability to Activate Transducin

Activation of G_{T} by rhodopsin was monitored as described above. Activation assays were first performed on freshly thawed WT and R177Q stocks. The stocks were next incubated in the dark at 37 °C to facilitate thermal decay of the 500 nm absorbing species, and aliquots were withdrawn at the indicated time points and assayed for G_{T} activation.

Hydroxylamine Reactivity

Hydroxylamine reactivity of the dark state was determined for purified rhodopsins by monitoring the rate of 500 nm absorbance decrease after the addition of hydroxylamine (pH 6.0) to the samples in buffer D to a final concentration of 50 mM at the indicated temperatures (49). Base-line drift was corrected as described above (see "Thermal Bleaching of Rhodopsin Samples").

RESULTS

Rationale for Choice of Loop E-2 Ion Pair Mutations—Amino acid mutations were constructed based on their ability to disrupt or potentially restore the ion pair charge interaction, while introducing minimal steric perturbation. Thus, residue Arg-177 was mutated to R177K (conserved charge) and R177Q (neutral substitution). Residue Asp-190 was mutated to D190E, D190A, D190G, and D190Y, because previous reports analyze other ADRP-associated point mutations at site Asp-190. Arg-177 was mutated to R177K (conserved charge) and R177Q (neutral substitution). Residue Asp-190 was mutated to D190E, D190A, D190G, and D190Y, because previous reports suggest these mutations are defective in folding, trafficking, and/or chromophore binding (23, 24, 26).

Characterization of Rhodopsin Mutants—Expressed ion pair mutant rhodopsins were analyzed for expression levels, proper post-translational modifications, ability to bind 11-cis-retinal, and photobleaching properties. Immunoblot analysis of mu-
tants expressed from transfected COS cells indicates all mutants expressed to similar levels comparable with that of wild-type rhodopsin (Fig. 3A). Mutants R177C, D190C, and D190E did not regenerate in our hands and were therefore not further characterized. These mutants also had abnormal glycosylation patterns in comparison to WT rhodopsin in that they did not exhibit the characteristic glycosylation smear pattern when expressed in COS cells (Fig. 3A). Furthermore, mutants defective in chromophore binding tended to form large molecular weight aggregates relative to both WT and other mutants (Fig. 3A).

Immunoblot analysis of recombinant rhodopsins purified using the rho1D4 monoclonal antibody reveal a band pattern similar to that of wild-type rhodopsin, with an apparent molecular mass of ~40 kDa and the characteristic heterogeneous glycosylation smear due to overexpression in a COS cell system (Fig. 3B), (50). Mutants capable of regenerating with 11-cis-retinal formed normal photobleaching behavior with respect to formation of a blue-shifted λmax ≈ 380 nm species (characteristic of the MII intermediate) (9). Acidification of these photobleached samples generated a λmax = 440 nm species, indicating the presence of a protonated retinal Schiff base (PSB) (40). These results are compiled in Table I, and a representative example of the photobleaching behavior is shown for mutants R177Q and D190N (Fig. 4A). Similar to the single mutants R177Q and D190N, the R177Q/D190N double mutant shows wild-type behavior in terms of expression levels, post-translational modifications, and chromophore binding. However, it did exhibit perturbed photobleaching properties. Although capable of forming both a spectral MII species and a PSB, following illumination a residual species with a λmax of ~480 nm persisted up to 10 h after illumination (data not shown). The cause of this is not known, although similar effects have been reported for other rhodopsin point mutations such as G90S and L226C (29, 34, 51).

Retinal Release Rates and Activation Energies for Metarhodopsin II Decay Measured by Fluorescence Spectroscopy—To determine potential effects the ion pair mutations may have on the stability of the MII active signaling species of rhodopsin, the activation energies for retinal release were determined. The rate of retinal release occurring during the decay of the MII species was measured using a fluorescence-based assay at 20 °C (44). Under the conditions used for this assay, the t1/2 of retinal release for WT rhodopsin at 20 °C in buffer D was 13 ± 0.5 min (n = 3), comparable with the 13–15-min values reported previously (34, 43, 48, 52) for both ROS-purified and COS-expressed rhodopsin. Somewhat unexpectedly, the corresponding t1/2 values for the ion pair mutant rhodopsins were similar to that of WT rhodopsin. The values for each of the mutants are compiled in Table I. The activation energy for the metarhodopsin II decay process was obtained by monitoring the rate of fluorescence increase in buffer D at seven different temperatures (5, 10, 15, 20, 25, 30, and 35 °C). The rate of fluorescence increase in all cases was temperature-dependent, and Arrhenius plots of these measurements indicated a temperature-dependent linear relationship for all mutants (Fig. 4B). From these plots an activation energy (Ea) of 20.2 kcal/mol was obtained for purified WT rhodopsin in DM, in good agreement with values reported previously (34, 48, 53). Arrhenius plots of the retinal release rates for the ion pair mutants show nearly equal Ea values (Fig. 4B and Table I).

Transducin Activation by Ion Pair Mutants—To assess the potential functional effects, the ion pair mutants were tested for their ability to activate transducin using a fluorescence-based assay that measures the increase in tryptophan fluorescence of the Gαs-GTPyS species (46, 48, 54). All ion pair mutations that regenerated with retinal are functionally active, and representative examples are presented in Fig. 4C. The results for transducin activation are compiled in Table I as initial rates of fluorescence increase relative to WT rhodopsin.

Thermal Stability in the Dark State—The most dramatic perturbation induced by the ion pair mutations was on the stability of the dark state structure. Thermal stabilities of dark state WT and ion pair mutant rhodopsins were determined by measuring the loss of the 500 nm absorbing species over time as described under “Experimental Procedures.” An example of this assay is depicted for mutant R177Q at 37 °C in Fig. 5A. The loss of the 500 nm species directly correlates with a loss of ability to activate transducin (Fig. 5, B and C). Additionally, the decrease in absorbance at 500 nm reflects a loss of the chromophore Schiff base linkage as judged by decay of the acid-denatured 440 nm species over the duration of the thermal decay assay (Fig. 5, D and E). Furthermore, we conclude the retinal is leaving the chromophore binding pocket after the hydrolysis because the rate of the loss of the 500 nm absorbing

FIG. 3. Immunoblot analysis of mutant rhodopsins. A, immunoblot of COS cell membrane fractions expressing mutant rhodopsin proteins, probed using the rho1D4 antibody. The full-length ROS, COS-expressed WT, and ion pair mutants run at molecular mass values of ~38 kDa. The lower molecular weight bands most likely reflect under- or unglycosylated opsins (19). B, immunoblot of purified ion pair rhodopsin mutants. Purified recombinant rhodopsin mutants were prepared as described under “Experimental Procedures” and probed using the rho1D4 antibody. ROS purified rhodopsin was included as a control. Notice that purification removes the lower weight species.
species correlates with the rate of tryptophan fluorescence increase, and irradiation of the sample with light following a plateau in signal does not cause a further fluorescence increase (Fig. 5F) (44).

All of the ion pair mutants showed significantly expedited rates of thermal decay in comparison to WT rhodopsin as judged by their loss in absorbance at 500 nm and increase in fluorescence at 330 nm (Table II). A comparison of the thermal decay rates at 55 °C monitored by absorbance is shown in Fig. 6. Note that the thermal decay rate of ROS-purified rhodopsin was similar to that of WT recombinant rhodopsin purified from COS cells (38.5 ± 3.0 and 37 min at 55 °C, 44).

**TABLE I**

| Species | Light Absorbance | Dark Absorbance | Fluorescence Increase | Activation Energy | Relative Rate | Relative Rate |
|---------|------------------|-----------------|-----------------------|-------------------|--------------|--------------|
| WT      | 500              | 500             | 1.0                   | 15.0              | 0.5          | 1.0          |
| R177C   | ND               | ND              | ND                    | ND                | ND           | ND           |
| R177K   | 500              | 500             | 1.6                   | 40,600            | 20.2         | 1.0          |
| R177Q   | 501              | 385             | 1.8                   | 40,000            | 20.3         | 0.93         |
| D190C   | ND               | ND              | ND                    | ND                | ND           | ND           |
| D190N   | 501              | 385             | 1.8                   | 35,000            | 20.8         | 0.83         |
| D190E   | ND               | ND              | ND                    | ND                | ND           | ND           |
| R177Q/D190N | 497         | 380             | 1.8                   | 38,000            | 20.8         | 0.64         |

| *λ*<sub>max</sub><sup>a</sup> | Light | Dark | λ<sub>280</sub>/A<sub>500</sub> | t<sub>1/2</sub> MII decay | MII Decay | Relative Rate |
|-----------------------------|-------|------|-----------------------------|------------------------|-----------|--------------|
| WT                          | 500   | 381  | 1.6                         | 40,600                 | 13.0 ± 0.5| 20.2         |
| R177C                       | ND    | ND   | ND                          | ND                     | ND        | ND           |
| R177K                       | 500   | 385  | 1.6                         | 40,000                 | 12.5 ± 0.8| 20.3         |
| R177Q                       | 501   | 385  | 1.8                         | 41,000                 | 12.8 ± 0.3| 20.8         |
| D190C                       | ND    | ND   | ND                          | ND                     | ND        | ND           |
| D190N                       | 497   | 385  | 1.8                         | 35,100                 | 11.6 ± 0.4| 20.8         |
| D190E                       | ND    | ND   | ND                          | ND                     | ND        | ND           |
| R177Q/D190N                 | 497   | 380  | 1.8                         | 38,000                 | 10.5 ± 0.3| 20.8         |

A, characterization of ion pair mutants by UV-visible spectroscopy: DS, dark state; hν, photobleached metarhodopsin II state; PSB, protonated Schiff base (at pH 1.9). Spectral property values are reported in Table I. B, Arrhenius plots of retinal release rates from the MII state of ion pair mutant rhodopsins. The rate constants were obtained from the retinal release assays (see “Experimental Procedures”) performed in buffer D at pH 6.0, with temperatures ranging from 5 to 35 °C. For comparison, values obtained for wild-type rhodopsin are overlaid in black open circles fit with black lines. The activation energy (E<sub>a</sub>) values determined from these assays are reported in Table I. C, example of transducin activation by ion pair mutants. Transducin activation was measured by monitoring the increase in G<sub>T</sub> tryptophan fluorescence that occurs upon MII stimulation of the G<sub>T</sub>-GTP-γ-S complex formation. The arrow indicates the time of GTP-γ-S addition. The relative rates of transducin activation of the mutants compared with WT rhodopsin are reported in Table I.

**FIG. 4.** Disrupting the Arg-177/Asp-190 ion pair does not impair MII stability or function in mutants that regenerate with 11-cis-retinal. Left panels show representative results for mutant R177Q, and right panels show representative results for mutant D190N.

- **B.** A, characterization of ion pair mutants by UV-visible spectroscopy: DS, dark state; hν, photobleached metarhodopsin II state; PSB, protonated Schiff base (at pH 1.9). Spectral property values are reported in Table I. B, Arrhenius plots of retinal release rates from the MII state of ion pair mutant rhodopsins. The rate constants were obtained from the retinal release assays (see “Experimental Procedures”) performed in buffer D at pH 6.0, with temperatures ranging from 5 to 35 °C. For comparison, values obtained for wild-type rhodopsin are overlaid in black open circles fit with black lines. The activation energy (E<sub>a</sub>) values determined from these assays are reported in Table I. C, example of transducin activation by ion pair mutants. Transducin activation was measured by monitoring the increase in G<sub>T</sub> tryptophan fluorescence that occurs upon MII stimulation of the G<sub>T</sub>-GTP-γ-S complex formation. The arrow indicates the time of GTP-γ-S addition. The relative rates of transducin activation of the mutants compared with WT rhodopsin are reported in Table I.

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<sup>a</sup> All *λ*<sub>max</sub> values are determined from the 1st derivative of the raw spectral data and estimated to within ± 1 nm.

<sup>b</sup> Extinction coefficients determined in buffer D at 15 °C; for further details see “Experimental Procedures.”

<sup>c</sup> MII decay assays performed in buffer D as described under “Experimental Procedures.” Values from three independent experiments are presented as the mean ± S.E.

<sup>d</sup> The relative initial rate of G<sub>T</sub> activation is represented by the rate of fluorescence increase obtained from the slope of the fluorescence measurements in the first 60 s after addition of GTP-γ-S relative to that of WT rhodopsin.

<sup>e</sup> The extinction coefficient for WT was assumed to be 40,600 M<sup>−1</sup> cm<sup>−1</sup> (38).

<sup>f</sup> Mutants R177C, D190C, and D190E did not bind 11-cis-retinal to form a stable pigment.

<sup>g</sup> ND, not determined because mutant did not regenerate with 11-cis-retinal.
Fig. 5. Rapid thermal decay of R177Q mutant correlates with loss of functional ability, loss of Schiff base linkage, and release of chromophore. Top panels, rapid loss of 500 nm absorbance for R177Q mutant correlates with loss in ability to activate transducin. A, representative spectra of mutant R177Q depicting loss of 500 nm absorbing species and gain in 380 nm species over time at 37 °C in buffer D. Spectra are plotted in 30-min intervals, with t = 0 as a darker line. B, transducin activation assays performed on mutant R177Q incubated in the dark at 37 °C and assayed at the indicated time points. The arrow indicates the time of GTPγS addition. C, loss of 500 nm absorbing species (black circles) correlates with loss of functional activity. The relative rates of transducin activation of mutant R177Q following thermal decay at 37 °C for 0, 5, or 14 h is plotted (open triangles) as the percent of initial R177Q activity. Bottom panels, loss of 500 nm absorbance in R177Q mutant is due to retinal Schiff base hydrolysis and release of retinal from the chromophore-binding pocket. D, the initial (t = 0 h) dark state absorbance spectra (solid line) and acid-denaturing spectra (dashed line) of mutant R177Q, at 37 °C, shows the presence of protonated Schiff base. E, after 14 h and loss of 500 nm absorbance species (solid line), no protonated Schiff base is obtained upon acidification (dashed line), indicating retinal linkage has been hydrolyzed. F, thermal decay of the 500 nm absorbing species (t0 = 198 min see C) correlates with the rate of retinal release from the chromophore-binding pocket, as monitored by increase in fluorescence at 330 nm from dark state species (t0 = 187 min). At the end of the experiment the sample was photobleached with white light, with no noticeable increase in tryptophan fluorescence, suggesting the retinal has completely left the binding pocket.

### Table II

Thermodynamic parameters for the dark state thermal decay of wild-type and ion pair mutant rhodopsins.

All experiments performed in buffer D as described under “Experimental Procedures.”

| Temperature | 37 °C | 55 °C |
|-------------|------|------|
|             | 500 nm decay t0<sup>a</sup> | E<sub>a</sub> thermal decay<sup>a</sup> | ΔG<sup>‡</sup> | ΔΔG<sup>‡</sup> | ΔH<sup>‡</sup> | Fluorescence increase t0<sup>a</sup> |
|             | min | kcal/mol | kcal/mol | kcal/mol | kcal/mol |
| WT          | 3100 ± 45 | 16.1 | 25.7 | -1.7 | 15.5 |
| R177Q       | 165 ± 10  | 30.6 | 24.0 | -1.7 | 20.0 |
| R177K       | 230 ± 15  | 26.2 | 24.2 | -1.5 | 25.6 |
| D190N       | 224 ± 15  | 17.2 | 24.2 | -1.5 | 16.6 |
| R177Q/D190N | 219 ± 17  | ND<sup>c</sup> | ND<sup>c</sup> | ND<sup>c</sup> | ND<sup>c</sup> |

|             | 500 nm decay t0<sup>a</sup> | E<sub>a</sub> thermal decay<sup>a</sup> | ΔG<sup>‡</sup> | ΔΔG<sup>‡</sup> | ΔH<sup>‡</sup> |
|-------------|------|------|------|------|------|
|             | min | kcal/mol | kcal/mol | kcal/mol | kcal/mol |
| WT          | 38.5 ± 3.0 | 38 | 103 | 24.5 | 102.3 |
| R177Q       | 0.9 ± 0.2  | 2.1 | 115.5 | 22.1 | -2.4 | 114.8 |
| R177K       | 2.5 ± 0.2  | 3.3 | 94.1 | 22.7 | -1.8 | 93.4 |
| D190N       | 2.4 ± 0.4  | 3.3 | 106.9 | 22.8 | -1.7 | 106.2 |
| R177Q/D190N | 1.4 ± 0.4  | 2.7 | ND<sup>c</sup> | ND<sup>c</sup> | ND<sup>c</sup> | ND<sup>c</sup> |

<sup>a</sup> Dark state thermal decay rates obtained from absorbance measurements at respective temperatures. Values from three independent experiments are presented as the mean ± S.E.

<sup>b</sup> Activation energies (E<sub>a</sub>) and thermodynamic parameters (ΔG<sup>‡</sup>, ΔΔG<sup>‡</sup>, ΔH<sup>‡</sup>) were obtained from linear regression of Arrhenius plots (Fig. 7), for further details see “Discussion.”

<sup>c</sup> ND, not determined.

<sup>d</sup> Increase in tryptophan fluorescence was monitored once at 330 nm to monitor dark state thermal decay rates.

respectively. The activation energies for the thermal absorbance decay processes were determined by monitoring the loss of the 500 nm absorbing species over time at 7 different temperatures (37, 41, 45, 47.5, 50, 52.5, and 55 °C). In all cases, the rate of loss in 500 nm absorbance was temperature-dependent, and Arrhenius plots indicate a similar temperature-dependent relationship for all mutants (Fig. 7). The Arrhenius plots are clearly concave, suggesting at least two different rate-limiting processes may occur during the temperature-dependent absorbance decay. With this in mind,
two linear regressions were used to approximate the activation energies for the two apparent processes (55–47.5 and 47.5–37 °C, respectively). From this analysis, the $E_a$ for WT rhodopsin was determined to be 16 kcal/mol at 37 °C and 103 kcal/mol at 55 °C. The thermodynamic parameters $E_a$, $\Delta G^\ddagger$, $\Delta H^\ddagger$, and $\Delta S^\ddagger$ were estimated from the rate data for WT and mutant rhodopsins using equations described previously (see Table II) (41, 45).

Hydroxylamine Reactivity—Hydroxylamine reactivity experiments showed that the ion pair mutants were not more susceptible to hydroxylamine in the dark state. These assays were carried out for purified ROS rhodopsin and each mutant sample, and the decay of the dark state 500 nm absorbing species was monitored in buffer D at either 20, 37, or 55 °C over time following the addition of hydroxylamine (pH 6.0) to a final concentration of 50 mM. WT rhodopsin purified from retinal sources and from expressed COS cells was found to be inert to hydroxylamine in the dark state, as described previously (49).

FIG. 6. Ion pair mutants show much faster rates of thermal decay than wild-type rhodopsin. Plot shows the loss of 500 nm absorbance at 55 °C as a function of time for WT rhodopsin and ion pair mutants (WT, filled circles; R177K, filled diamonds; R177Q, open squares; D190N, open triangles; R177Q/D190N, filled hexagons). Data were fitted to a mono-exponential decay function (see “Experimental Procedures”), and the $t_{1/2}$ value for the process was determined for each mutant over a range of different temperatures. The values are given in Table II.

FIG. 7. Arrhenius plots of dark state thermal decay rates show ion pair mutants have similar activation energies for retinal hydrolysis yet faster rates than wild-type rhodopsin. The rate constants were obtained from the dark state thermal absorbance decay experiments (see Fig. 6), performed in buffer D, with the temperatures ranging from 37 to 55 °C (WT, filled circles; R177K, filled diamonds; R177Q, open squares; D190N, open triangles). The concave plot suggests two different processes may lead to thermal decay. The $E_a$ and thermodynamic values approximated for these processes are presented in Table II.

Intriguingly, none of the ion pair mutants exhibited any increased reactivity toward hydroxylamine treatment in the dark state at 20, 37, and 50 °C (Fig. 8).
Early studies by Khorana and others (50, 55–59) led to the hypothesis that the rhodopsin intradiscal domain plays a crucial role in maintaining proper protein folding, correct post-translational modifications, trafficking, and 11-cis-retinal binding. Consistent with this theory, a number of point mutations that naturally occur in this region result in ADRP, an inherited human disease causing retina degeneration (23, 24, 26, 60–62). As noted in the Introduction, the rhodopsin crystal structures reveal a high degree of order and structure in the intradiscal region. This region of the protein is proposed to be structurally critical for maintaining the electrostatic and hydrogen-bonded network surrounding the retinal chromophore (15). Most notably, loop E-2 forms a twisted β-sheet which lies across the retinal chromophore (2, 14, 15). Through analysis of the loop E-2 region we noticed an ion pair Arg-177/Asp-190 is present on either end of this loop structure. Additionally, we noticed that residue Arg-177 is hydrogen-bonded to the backbone carbonyl of residue P7, a residue found at the turn of loop E-1 in the N terminus of the protein. The fact that residues Arg-177 and Asp-190 interact with many residues within the intradiscal region of rhodopsin suggests that the ion pair may play a significant role in maintaining the structural integrity of the “retinal plug” domain. The published rhodopsin crystal structures show very little difference in the region around the Arg-177/Asp-190 ion pair (analysis of all three structures using the program Swiss-PDB Viewer shows that the 87 amino acid side chains within 16 Å of Arg-177 show a root mean square deviation of 1.0 Å or less (2, 14, 15)). However, we do notice a difference in the placement of residue Asn-200, which exhibits a different rotameric flip between structures 1HZX, 1L9H, and 1F88, and thus exhibits alternate hydrogen bonding to residue Asp-190 in the different structures (2, 14, 15). The present report details our studies on the structural and functional effects caused by disrupting the Arg-177/Asp-190 intradiscal ion pair located on either end of loop E-2 (Fig. 1B).

**General Characteristics of Mutants**—The majority of the single ion pair mutants we created, expressed to similar levels comparable with WT rhodopsin, underwent proper glycosylation (as judged by whole cell lysate immunoblotting, Fig. 3) and regenerated with 11-cis-retinal. Additionally, with the exceptions of mutants R177C, D190C, and D190E, the mutants were properly folded, as judged by their ability to bind the 11-cis-retinal chromophore, and produced a wild type like A490/A600 ratio (Table I). The fact that most of the Asp-190 mutations were unable to bind retinal is in agreement with previous reports of other Asp-190 mutations, which also were found to be defective in retinal binding (23, 26, 58). One possible reason for the sensitivity of this site to mutations may be that residue Asp-190 is partially buried and makes contacts with several residues (Ile-189 and Tyr-Y191), which form part of the retinal binding pocket (Fig. 1B) (2, 15, 52). Abrogation of these contacts by mutations to residue Asp-190 may thus distort the retinal-binding pocket thereby making it inaccessible or sterically unfavorable for proper binding of 11-cis-retinal. Additionally, it is also possible that the R177C and D190C mutants are not able to regenerate with 11-cis-retinal because improper disulfide bonds are formed in the final folded structure, as these residues are in close proximity to the conserved Cys-110/Cys-187 disulfide pair, as well as residue Cys-185 (Fig. 1B). This explanation is supported by recent findings, which show that improper disulfide bonds form as a result of mutations to this region (63–65). Furthermore, the fact that the glycosylation patterns of whole cell lysate immunoblots of mutants R177C, D190C, and D190E are different suggests the misfold-
Arrhenius Analysis Indicates the Thermal Decay in Rhodopsin May Occur through More Than One Pathway—Interestingly, the Arrhenius analysis of the thermal decay rates shows a concave plot, with all of the mutants decaying much faster than WT rhodopsin. To our knowledge, this behavior has not been reported previously for rhodopsin. Concave Arrhenius plots can be attributed to several factors, although the most common interpretation is that at least two different rate-limiting steps are involved (73). With this interpretation in mind, we fit our WT rhodopsin data assuming two different activation energies may be present, and we find $E_a$ values of $-16$ kcal/mol for the lower temperature range ($37-47.5^\circ$C) and $-103$ kcal/mol for the higher temperature range ($47.5-55^\circ$C). As shown in Table II, the ion pair mutants all showed similar $E_a$ values ranging from 17 to 31 kcal/mol for the lower temperature range and 94–107 kcal/mol for the higher temperature range. Finally, thermodynamic analysis of the Arrhenius data show that the ion pair and WT rhodopsins have similar $\Delta H^\ddagger$ values (see Table II), suggesting the perturbation caused by the mutations are generally entropic in nature (34, 41, 74).

It is informative to compare the thermal decay activation energies from this study with previous findings. For example, our $E_a$ of 103 kcal/mol for WT rhodopsin (at the higher temperatures) is in excellent agreement with the $102.1 \pm 5.8$ kcal/mol value reported previously by Khorana and co-workers (41) for WT using a similar experimental set up. Furthermore, the $E_a$ value of 16 kcal/mol we observe at the lower temperatures is similar to the $20.2$ kcal/mol value obtained for the retinal release process that occurs during MII decay (34, 44), and to the $E_a$ of hydrolysis for model Schiff base retinal compounds (75), as well as the $E_a$ for retinal binding in rhodopsin (76).

What might be the cause of the different $E_a$ values obtained for the Arrhenius analysis at different temperatures? The higher activation energy barriers at higher temperatures may reflect the less favorable conditions for hydrolysis of the Schiff base linkage present in the interior of unfolded rhodopsin. In other words, thermal denaturation of the protein occurs which may reposition key amino acids involved in Schiff base formation and hydrolysis, thereby increasing the $E_a$ observed at the higher temperatures. Alternatively, the lower $E_a$ observed at the lower temperatures suggests that at these temperatures another process also contributes to Schiff base hydrolysis, one that occurs more efficiently at lower temperatures (such as proton tunneling) than the process that dominates at higher temperatures. Another possibility is that the pre-exponential factor in the Arrhenius analysis has changed; the pre-exponential factor is related to steric factors and/or the efficiency with which the collisions lead to a productive reaction (73).

Thermodynamic Significance of the Arg-177/Asp-190 Ion Pair—The $\Delta G^\ddagger$ values for the ion pair mutants are approximately $-1.5$ to $-2.4$ kcal/mol, similar to the $-2.9 \pm 1.2$ kcal/mol reported for the C110A/C187A mutation of the critical rhodopsin disulfide bond (41). Thus, in terms of dark state stability, abrogation of the ion pair results in free energy changes comparable with those observed for the loss of the Cys-110/Cys-187 disulfide bond. This is perhaps not surprising, because the Arg-177/Asp-190 ion pair is in close proximity to the intradiscal Cys-110/Cys-187 disulfide bond (Fig. 1B). However, it is important to note that the ion pair mutations we report here apparently have little effect on the stability of the MII structure, in contrast to the disulfide bond that appears crucial for MII stability (41). This latter point is of interest; to the best of our knowledge, the Arg-177/Asp-190 ion pair mutants represent a previously unidentified class of rhodopsin mutants which alter the stability and structure of the dark state yet have little to no effect on the stability of the MII state. One explanation for this phenomena may be that the loop E-2 region changes structure during MII formation and the ion pair is no longer present in the MII state, thus the stability of MII would not be affected by mutations to the Arg-177/Asp-190 ion pair. We note that although the Arg-177 and Asp-190 residues point away from the helical bundle, it is also possible that mutations to this ion pair may affect the positioning or orientation of helices 5 and 6. However, there is some precedence for conformational changes in loop E-2, as Ridge et al. (77) have previously shown residue Cys-185 becomes accessible to chemical labeling only in the MII state.

Disrupting the Arg-177/Asp-190 Ion Pair Does Not Appear to Increase the Exposure of the Retinal Schiff Base to Bulk Solvent—We initially interpreted our results to mean that disrupting the Arg-177/Asp-190 ion pair causes a loosening of the loop E-2 structure, and thus we hypothesized the increased rate of Schiff base hydrolysis was due its increased exposure to external solvent. However, to our surprise, we found that the presence of 50 mM hydroxylamine had no effect on any of the mutants tested, even when tested at three different temperatures (Fig. 8). The lack of increased hydroxylamine sensitivity argues against the hypothesis that the thermal instability induced in the ion pair mutants is due to a structural perturbation that renders their Schiff base more susceptible to attack by the bulk solvent. Rather, the hydroxylamine data suggest that the Arg-177/Asp-190 ion pair in the intradiscal domain of rhodopsin mediates thermal stability of the dark state structure through some other mechanism.

Speculation on the Role of Arg-177/Asp-190 in Stabilizing Rhodopsin—Previous chemical models of the retinal-binding and release pathways have speculated that the protonated retinal Schiff base linkage can spontaneously hydrolyze and thus is in dynamic equilibrium with retinal covalently bound to rhodopsin (75, 78). One interpretation of our data may be that the ion pair stabilizes the retinal plug structure. If the retinal plug functions to block the release of free 11-cis-retinal produced through spontaneous hydrolysis (through a steric mechanism which confines the transiently hydrolyzed retinal to the chromophore binding pocket), disruption of this structure might lead to an apparent increase in Schiff base hydrolysis rates. In this scenario the function of the retinal plug is to effectively force the transiently formed free 11-cis-retinal to remain in the binding pocket and reform a Schiff base linkage with rhodopsin. Alternatively, the ion pair may enhance rhodopsin stability by constraining the conformation of a network of water molecules and residues attached to loop E-2 (Glu-181 to Ser-186) that directly link loop E-2 with the retinal Schiff base through residue Glu-113 (15, 52). Restraining the flexibility of this region may thus make rhodopsin more stable by inhibiting transient formation of the tetrahedral carbinolamine intermediate thought to be involved in the transition state of Schiff base hydrolysis (75, 79). We are presently carrying out further experiments to test these hypotheses.

Conclusions—The Arg-177/Asp-190 ion pair located on either end of intradiscal loop E-2 appears to be important for maintaining dark state rhodopsin stability, although it does not appear to play a critical role in formation or stability of the active MII species. These results illustrate the importance of the rhodopsin structure revealed by x-ray crystallography (2, 14, 15). With the structure of rhodopsin in hand, it is now possible to assess the previously unappreciated functional role

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3 Normally, if solvent-accessible, a retinal Schiff base is rapidly cleaved by hydroxylamine, and this property has frequently been used as a measure of accessibility of the retinal Schiff base linkage in rhodopsin (41, 42, 52, 68, 72).
of interactions that occur within the protein to provide receptor stability and allow receptor activation and attenuation.

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Stability of Dark State Rhodopsin Is Mediated by a Conserved Ion Pair in Intradiscal Loop E-2
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