Retinitis Pigmentosa Mutants Provide Insight into the Role of the N-terminal Cap in Rhodopsin Folding, Structure, and Function*

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Autosomal dominant retinitis pigmentosa (ADRP) mutants (T4K, N15S, T17M, V20G, P23A/H/L, and Q28H) in the N-terminal cap of rhodopsin misfold when expressed in mammalian cells. To gain insight into the causes of misfolding and to define the contributions of specific residues to receptor stability and function, we evaluated the responses of these mutants to 11-cis-retinal pharmacological chaperone rescue or disulfide bond-mediated repair. Pharmacological rescue restored folding in all mutants, but the purified mutant pigments in all cases were thermo-unstable and exhibited abnormal photobleaching, metarhodopsin II decay, and G protein activation. As a complementary approach, we superimposed this panel of ADRP mutants onto a rhodopsin background containing a juxtaposed cysteine pair (N2C/D282C) that forms a disulfide bond. This approach restored folding in T4K, N15S, V20G, P23A, and Q28H but not T17M, P23H, or P23L. ADRP mutant pigments obtained by disulfide bond repair exhibited enhanced stability, and some also displayed markedly improved photobleaching and signal transduction properties. Our major conclusion is that the N-terminal cap stabilizes opsin during biosynthesis and contributes to the dark-state stability of rhodopsin. Comparison of these two restorative approaches revealed that the correct position of the cap relative to the extracellular loops is also required for optimal photochemistry and efficient G protein activation.

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§The abbreviations used are: ADRP, autosomal dominant retinitis pigmentosa; GPCR, G protein-coupled receptor; 7TM, seven transmembrane; ER, endoplasmic reticulum; ROS, rod outer segment(s); GT, transducin; DDM, n-dodecyl β-maltoside; Bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; GTPγS, guanosine 5′-O-(γ-thio)triphosphate; MII, metarhodopsin II; EL, extracellular loop.
As part of the visual cycle in rod cells, 11-cis-retinal, synthesized by retinal pigment epithelial cells, is delivered to ROS and combines with rod opsin to form rhodopsin. However, it is not known when or where de novo synthesized rod opsin first encounters and combines with 11-cis-retinal, an event that can influence folding, especially for ADRP mutants susceptible to 11-cis-retinal-mediated pharmacological chaperone rescue (17, 18).

ADRP rhodopsin mutants were previously classified according to their expression profiles in transfected HEK-293S or COS-1 cells (19, 20). Type I rod opsin mutants fold normally and reach the cell surface, whereas Type II mutants have reduced expression levels, traffic abnormally, and form pigment inefficiently (19). The absence of 11-cis-retinal during rod opsin folding in these and many similar studies has led us and others to question the relevance of these folding outcomes because, in rod cells, 11-cis-retinal is naturally abundant and may circulate to the ER located in rod cell inner segments. Pharmacological rescue was previously shown to restore pigment formation in ADRP mutant T17M (21), as well as T4R and P23A/H/L (22). Here, we have extended these analyses to all known human ADRP rhodopsin mutations in the N terminus (T4K, T17M, N15S, V20G, P23A/H/L, and Q28H), and for the first time, we have assessed the active-state properties of these rescued mutant pigments.

As the mutant pigments resulting from pharmacological rescue were found to be defective, we used an alternative strategy to repair the defects in the N-terminal cap. Our hypothesis was that mutations within the N terminus of rhodopsin destabilize the entire protein by decoupling key interactions of the cap with...
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the extracellular loops. Stabilization of the transmembrane helices and connecting loops with bound ligand can overcome these defects, as we have shown here. However, to test our hypothesis directly, we introduced a disulfide bond in an attempt to tether the damaged cap to the 7TM domain. This was achieved by superimposing this panel of ADRP mutants onto a rhodopsin background containing a juxtaposed cysteine pair (N2C/D282C) known to form a disulfide bond (Cys-2–Cys-282) that increases the thermostability of rod opsin (23).

For a subset of these ADRP mutants, the N2C/D282C background restored folding of rod opsin, and the resulting pigments had much improved thermostability, photobleaching behavior, and activation of the G protein transducin (G₄). These findings highlight how the extracellular domain of rhodopsin has evolved, under selection pressure constraints requiring high sensitivity vision in dim light, to contribute to receptor stability and signaling efficiency.

EXPERIMENTAL PROCEDURES

Materials and Buffers—Bovine retinas were purchased from J. A. Lawson Co. (Lincoln, NE). 11-cis-Retinal was provided by Rosalie K. Crouch (Storm Eye Institute, Medical University of South Carolina). n-Dodecyl β-D-maltoside (DDM) was purchased from Anatrace (Maumee, OH). Bis-tris propane, BES, GTPγS, 9-cis-retinal, and hydroxylamine hydrochloride were purchased from Sigma. PMSF was purchased from Fluka. Disposable polystyrene columns (2-ml bed volume) were purchased from Thermo Scientific. Restriction endonucleases were purchased either from New England Biolabs or Thermo Scientific. The nonapeptide corresponding to the nine amino acid residues (TETSQVAPA) at the C terminus of rhodopsin was purchased from Peptide Protein Research. Sepharose 4B beads were purchased from Amersham Biosciences. The HEK-293S cell line was provided by J. Nathans (The John Hopkins School of Medicine), as was pRSV-TAg (SV40 large T-antigen expression plasmid). DMEM, DMEM/Ham’s F-12 medium, FBS, trypsin, l-glutamine, penicillin, and streptomycin were purchased from PAA Laboratories Ltd.

The compositions of the buffers used were as follows: Buffer A, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄ (pH 7.2); Buffer B, Buffer A + 0.1 mM PMSF; Buffer C, Buffer A + 0.1% DDM; Buffer D, 10 mM Bis-tris propanoic acid, 140 mM NaCl, and 0.1% DDM (pH 7.2); Buffer E, 10 mM Bis-tris propanoic acid and 0.1% DDM (pH 7.2); Buffer F, Buffer E at pH 6; Buffer G, Buffer D + 100 μM epitope nonapeptide; Buffer H, Buffer E + 100 μM epitope nonapeptide; Buffer I (2×), 50 mM BES, 250 mM NaCl, and 1.5 mM Na₂HPO₄ (pH 7.02); Buffer J (20×), 2.5 mM CaCl₂; Buffer K, 10 mM Tris (pH 7.2), 2 mM MgCl₂, 100 mM NaCl, 1 mM DTT, and 0.012% DDM; and Buffer L, 50% glycerol, 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, and 5 mM MgOAc.

Preparation of ROS Membranes—Rhodopsin in ROS membranes was prepared from frozen bovine retinas by sucrose density gradient centrifugation (24).

Construction of Opsin Mutants—Site-directed mutagenesis using complementary primer pairs was performed with a synthetic opsin gene in the expression plasmid pMT4 (25) as the target template. All mutants were also superimposed onto the N2C/D282C rhodopsin background (23) by KpnI-PstI (restriction endonuclease sites) fragment exchange. Mutations were verified by DNA sequencing of both strands of the entire opsin gene.

Cell Culture and Transfection—HEK-293S cells were maintained in DMEM/Ham’s F-12 medium supplemented with heat-treated (55 °C, 30 min) 10% (v/v) FBS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK-293S cells were grown in a humidified incubator under a 5% CO₂ atmosphere at 37 °C. Calcium phosphate precipitation-mediated transfection was performed as follows using a modification to a procedure developed by Chen and Okayama (26) and O'Mahoney and Adams (27). On the day before transfection, confluent cell monolayers of HEK-293S cells were trypsinized and seeded at a density of 2–2.5 × 10⁶ cells in 22.5 ml of DMEM (supplemented as described above) in a 15-cm tissue culture dish. 24 h after cells were seeded, a transfection mixture (2.5 ml) containing plasmid DNA and Buffers I and J was added to the pre-confluent cell monolayer. To prepare the transfection mixture, plasmid pMT4 containing opsin genes (54 μg) and pRSV-TAg (6 μg) was diluted in sterile water (1.125 ml). Buffer J (125 μl) was then added slowly with continuous mixing. Finally, Buffer I was added over 1 min with Vortex mixing, and the mixture was immediately added dispersed over the cell monolayers. The cells were then kept for 19 h in a humidified incubator set at 1% CO₂ and 37 °C. After this incubation period, the spent medium was removed, and the cells were carefully washed once with unsupplemented DMEM (20 ml). The cells were subsequently fed with DMEM (25 ml) containing supplements and kept for a further 48 h in a humidified incubator set at 5% CO₂ and 37 °C prior to harvest.

Treatment of Transfected Cells with Pharmacological Chaperones—HPLC-purified retinal isomers were added to transfected cells using a slight modification to the method described previously (17). Briefly, 11-cis-retinal or 9-cis-retinal, prepared as 100 mM solutions in cell culture-grade dimethyl sulfoxide, was dispensed over transfected HEK-293S cells using safelight illumination conditions (Kodak No. 2 filter). These retinoids were added to cells in two 10 μM installments, 24 and 48 h after removal of the transfection mixture, to give a final retinoid concentration of 20 μM. The culture dishes were wrapped with a single layer of aluminum foil to maintain dark conditions during incubation. Cells were harvested under safelight illumination 24 h after the final addition of retinoids.

Generation of Rhodopsin Pigment and Detergent Solubilization of Cells—Transfected HEK-293S cells obtained from a single 15-cm tissue culture dish were washed twice with 10 ml of ice-cold Buffer A prior to resuspension in 0.9 ml of chilled Buffer B. Pigment formation and subsequent procedures were carried out in a dark room illuminated by Kodak No. 2 safelight filters. Cell suspensions were treated with 11-cis-retinal or 9-cis-retinal (5 μM) added in two installments over 3 h at 4 °C with end-over-end nutator mixing. Whole cells were solubilized with 1% (w/v) DDM for 1 h at 4 °C with end-over-end mixing. The solubilized cell extracts containing pigment were centrifuged at 14,000 rpm for 30 min at 4 °C to remove insoluble material prior to purification of pigments. A fraction of the supernatant was kept for...
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Measurement of the Rate of Metarhodopsin II Decay in Rhodopsin—Metarhodopsin II (MII) decay was monitored at 20 °C as described previously (31) using instead a PerkinElmer Life Sciences LS 50 fluorometer. Excitation of rhodopsin tryptophans (0.25 μM in Buffer F) was at 280 nm (2.5-nm slit width), and emission was monitored at 330 nm (15-nm slit width). Rhodopsin was converted to MII by photobleaching the sample for 30 s with light (>495 nm). Fluorescence was recorded at 30-s intervals with a 2-s integration time for 120 min. Data were normalized and best fit to either a single- or double-component rise-to-plateau exponential function (SigmaPlot 12.0). Derived rate constants were used to calculate the half-life of MII decay.

GT Activation Assay—Freshly prepared (1–2 days old), similarly aged rhodopsin mutants with no measurable loss of pigment during storage were used for this assay. GT was purified from ROS membranes according to the method of Kühn (32), except that the final supernatant containing GT was concentrated using a 60-ml Jumbosep™ centrifugal concentration device fitted with a 10-kDa cutoff filter (Pall Corp.) before dialysis against the storage buffer (Buffer F). The activation of GT by rhodopsin MII was measured by monitoring the increase in intrinsic fluorescence of GT that accompanies the exchange of GDP for GTP (33). GT activation was measured at 20 °C in Buffer K using the PerkinElmer Life Sciences LS 50 fluorometer as described above. The fluorometer was equipped with an electronic stirrer (Model 300, Rank Brothers Ltd.) set to mix the reaction cuvette contents at 240 rpm. Excitation of GT was at 280 nm, and emission was recorded at 340 nm. The interval for data collection was 3 s, and the reaction was followed for 1 h. The reaction mixture contained 250 nM GT in 1 ml of Buffer K. Rhodopsin (20 nM) was added and allowed to equilibrate for 1 min before bleaching the sample for 30 s with light (>495 nm). GTPγS (5 μM) was then added to initiate the reaction. The relative initial rate of GT activation was determined from the slope of the increase in relative fluorescence over the first 60 s following the addition of GTPγS. The GT activation rates for ADRP mutants were expressed as a percentage of WT rhodopsin initial activation rates. To reduce the Cys-2–Cys-282 disulfide bond, rhodopsin pigments were incubated with DTT (50 mM final concentration) on ice for 1 h prior to commencement of the GT activation assay.

Packing Analysis—The packing analysis of the N-terminal domain of rhodopsin was carried out using the method of occluded surfaces (34). The occluded surface method calculates packing values at the level of individual atoms, amino acids, or entire proteins. Packing values range from 0.0 to 1.0, corresponding to totally exposed and totally occluded environments. Hexagonally packed spheres have a maximum packing value of 0.8 due to the void space that exists where the spheres are not in direct contact (35). In the occluded surface calculation, van der Waals surfaces are drawn around each atom in the protein, and normals are constructed that extend outward until they reach another surface or a length of 2.8 Å, the diameter of a water molecule. The cutoff of 2.8 Å between amino acid surfaces accounts for the possibility that water can occupy that space, and therefore, the corresponding surface is defined as being non-occluded. The definition of the occluded surface packing value takes into account the normalized occluded (or buried) surface area weighted by the distance to the occluding neighbors.

RESULTS

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examination by UV-visible difference spectroscopy and SDS-PAGE for an assessment of rhodopsin yield.

Rho-1D4 Immunoaffinity Purification of Rhodopsin—Rhodopsin was purified from solubilized bovine ROS membranes or from detergent-solubilized transfected HEK-293S cell extracts using Rho-1D4-Sepharose 4B beads with modifications to the methods described previously (25, 28, 29). All purification procedures and manipulations were carried out at 4 °C. 50 μl of Rho-1D4-Sepharose beads with a binding capacity of ~1 μg of rhodopsin/μl of settled beads was added to the cell lysate and mixed end-over-end at 4 °C for 2 h to allow binding. The suspension was packed into a 2-ml disposable column and washed with 40 ml of Buffer D (high salt), followed by 20 ml of salt-free Buffer E. Correctly folded rhodopsin was purified by elution with salt-free Buffer H (0.5 ml) for up to three elutions. Once elution of rhodopsin pigment was complete, rod opsin was recovered from the column using Buffer G (high salt). Pigm-

tents were stored in the dark at 4 °C.

UV-visible Absorption Spectroscopy of Rhodopsin and Photobleaching—UV-visible spectra of pigments purified in Buffer H were recorded at 20 °C using a PerkinElmer Life Sciences A35 UV-visible spectrophotometer equipped with water-jacketed cell holders. Spectra were acquired between 250 and 650 nm using a 2-nm bandwidth, a response time of 1 s, and a scan speed of 480 nm/min. The molar extinction coefficient value used to calculate rhodopsin yields was 40,600 M–1 cm–1 at 500 nm (30). Purified rhodopsin samples were photobleached at 20 °C by direct illumination in a cuvette using a Schott KL 1500 compact fiber optic light guide fitted with a >495-nm long-pass filter. Rhodopsin samples were manually bleached for 30 s, followed immediately by UV-visible spectroscopy. This process was repeated four times. Finally, the protonated Schiff base was trapped by acidification of the sample (500 μl) by the addition of 2 N H2SO4 (2 μl).

Thermal Stability of Detergent-solubilized Rhodopsin Mutants—Puriﬁed rhodopsin pigments in Buffer H were monitored by UV-visible spectroscopy. Samples were kept at either 37 or 55 °C for 2 min in quartz cuvettes to allow for temperature equilibration. UV-visible spectra were recorded at 2-min intervals for 30 min and afterward at 20-min intervals for at least another 24 h, except as stated otherwise. Normalized data were best fit using the single- or double-component exponential decay function of SigmaPlot 12.0, and these values were used to calculate the half-life (t1/2) of the mutant rhodopsin pigments. Pigments that decayed rapidly at 55 °C were monitored at 5-s intervals using the time drive function on the spectrometer.

Rhodopsin Sensitivity to Hydroxylamine—Schiff base hydrolysis of rhodopsin by hydroxylamine (22) was measured in Buffer F; the final concentration of hydroxylamine hydrochloride (titrated to pH 6.0 using NaOH) was 50 mM. UV-visible absorbance spectra were collected at the same time intervals described above.

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RESULTS

Pharmacological Chaperone Rescue of Misfolded ADRP Rhodopsin Mutants—HEK-293S cells transfected with ADRP rhodopsin mutants were incubated in the presence or absence of
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11-cis-retinal during expression. Harvested cells were treated with 11-cis-retinal, and rhodopsin pigments were purified from solubilized cell extracts by Rho-1D4-Sepharose immunoaffinity chromatography (see "Experimental Procedures"). Preferential elution of correctly folded rhodopsin was achieved using salt-free Buffer H. Eluted rhodopsin fractions were examined by UV-visible absorbance spectroscopy, and the results are presented in Fig. 2A and Table 1. These N-terminal ADRP mutants made very small or undetectable amounts of pigment. These very low yields are attributed to receptor misfolding and inefficient formation of correctly folded opsin capable of combining with 11-cis-retinal to form pigment. However, when 11-cis-retinal was supplied to transfected cells during opsin biosynthesis, all of these ADRP mutants made WT-like rhodopsin pigment, but the expression levels varied (Fig. 2A, Table 1). The yields of ADRP rhodopsin mutants T4K and P23A reached WT levels, whereas all other mutants were recovered at levels ~50% that of WT rhodopsin. WT rhodopsin expression levels were unaffected by the presence of 11-cis-retinal during opsin expression.

For comparison, we also performed pharmacological rescue experiments using the 9-cis-retinal isomer; similar results were obtained, except expression levels were lower, and the purified pigments had blue-shifted absorbance maxima (data not shown). Our results for T17M and P23A/H/L are in full agreement with previous 11-cis-retinal pharmacological rescue experiments (17, 18, 21, 22, 36). We now included T4K, N15S, V20G, and Q28H to demonstrate that all known ADRP mutants in the N-terminal tail are susceptible to 11-cis-retinal pharmacological rescue.

Restoration of ADRP Rhodopsin Mutants in the N2C/D282C Background—The N2C/D282C mutation was previously shown to confer thermal stability to purified rod opsin apoprotein.

**TABLE 1**

| Mutant | Yield* | \( \lambda_{\text{max}} \) | \( A_{280}/A_{\lambda_{\text{max}}} \) |
|--------|--------|-----------------|-----------------|
| WT (ROS)* | NA† | 500 | 1.6 ± 0.1 |
| WT (HEK-293S)* | 42 ± 2.3 | 499 | 1.6 ± 0.1 |
| WT (HEK-293S)† | 42 ± 3.9 | 499 | 1.7 ± 0.1 |
| N2C/D282C* | 41 ± 2.4 | 500 | 1.6 ± 0.1 |
| N2C* | 40 ± 2.1 | 497 | 1.8 ± 0.1 |
| D282C* | 39 ± 1.7 | 499 | 1.8 ± 0.1 |
| T4K (WT)* | 6 ± 3.4 | 500 | 2.1 ± 0.3 |
| T4K (WT)† | 30 ± 2.6 | 500 | 1.8 ± 0.1 |
| T4K (N2C/D282C)* | 35 ± 2.9 | 500 | 1.7 ± 0.1 |
| N15S (WT)* | 4 ± 2.1 | 498 | 2.7 ± 0.3 |
| N15S (WT)† | 18 ± 1.3 | 499 | 2.1 ± 0.1 |
| N15S (N2C/D282C)* | 31 ± 2.5 | 499 | 1.7 ± 0.1 |
| T17M (WT)* | 4 ± 1.4 | 499 | 2.4 ± 0.1 |
| T17M (WT)† | 15 ± 3.4 | 499 | 1.9 ± 0.1 |
| T17M (N2C/D282C)* | 4 ± 1.3 | 499 | 3.5 ± 0.3 |
| T17M (N2C/D282C)† | 7 ± 1.2 | 498 | 1.8 ± 0.0 |
| V20G (WT)* | 3 ± 0.9 | 497 | 5.8 ± 0.1 |
| V20G (WT)† | 14 ± 2.2 | 499 | 1.9 ± 0.1 |
| V20G (N2C/D282C)* | 25 ± 3.1 | 498 | 1.8 ± 0.1 |
| P23A (WT)* | 4 ± 2.4 | 499 | 2.8 ± 0.1 |
| P23A (WT)† | 37 ± 1.3 | 499 | 1.8 ± 0.1 |
| P23A (N2C/D282C)* | 41 ± 2.4 | 499 | 1.7 ± 0.1 |
| P23H (WT)* | 1 ± 2.3 | 491 | 2.9 ± 0.1 |
| P23H (WT)† | 14 ± 3.3 | 497 | 1.8 ± 0.1 |
| P23H (N2C/D282C)* | 2 ± 1.1 | 498 | 4.1 ± 0.3 |
| P23H (N2C/D282C)† | 6 ± 2.1 | 498 | 1.9 ± 0.3 |
| P23L (WT)* | 1 ± 1.9 | 494 | 7.5 ± 0.0 |
| P23L (WT)† | 14 ± 3.1 | 498 | 1.9 ± 0.1 |
| P23L (N2C/D282C)* | 2 ± 1.5 | 497 | 3.9 ± 0.3 |
| P23L (N2C/D282C)† | 7 ± 2.7 | 497 | 1.8 ± 0.2 |
| Q28H (WT)* | 2 ± 2.4 | 495 | 4.3 ± 0.1 |
| Q28H (WT)† | 10 ± 3.4 | 497 | 1.9 ± 0.1 |
| Q28H (N2C/D282C)* | 24 ± 2.8 | 499 | 1.7 ± 0.1 |

* Average rhodopsin yield from a 15-cm tissue culture dish containing transiently transfected HEK-293S cells grown in the absence (∗) or presence (†) of 11-cis-retinal.

† Absorbance maxima (\( \lambda_{\text{max}} \)) in the visible region.

\( A_{280}/A_{\lambda_{\text{max}}} \) ratio of pigments obtained from low salt elution (\( A_{280}/A_{\lambda_{\text{max}}} = 1.6 \) for pure rhodopsin).

†† NA, not applicable.

**FIGURE 2. Restoration of folding in ADRP mutants either by 11-cis-retinal pharmacological chaperone rescue or by disulfide bond-mediated repair in the N2C/D282C background.** UV-visible absorption spectra of purified rhodopsin pigments from transfected HEK-293S cells were determined as described under “Experimental Procedures.” Representative spectra from three independent experiments are shown. A, ADRP mutants in the WT opsin gene background. The individual panels show purified pigments obtained from transfected cells grown in the absence (black traces) or presence (red traces) of 11-cis-retinal during opsin biosynthesis. B, the panels show spectra of purified pigments obtained from cells transfected with ADRP mutants in the WT (black traces) or N2C/D282C (blue traces) opsin gene background. C, bar chart showing the relative amounts of pigment obtained as described for A and 8.
(23) and the pigment of rhodopsin mutant N15D (37). Our hypothesis was that correct folding in this panel of ADRP rodopsin mutants would be repaired if we could re-establish the stability of the 7TM domain by improving association of the defective cap with extracellular loops (ELs) 1–3. To test this idea, these ADRP mutants were subcloned into the N2C/D282C background. Transient transfection experiments were performed as described under “Experimental Procedures,” but 11-cis-retinal was not added until after the transfected cells were harvested. Pigments were purified and analyzed by UV-visible absorbance spectroscopy as described above, and the results are presented in Fig. 2B and Table 1. The rhodopsin double mutant N2C/D282C and the single mutants N2C and D282C all formed pigments at levels comparable to WT rhodopsin, findings that are in agreement with Xie et al. (23). WT levels of pigment were also obtained from ADRP mutant P23A in the N2C/D282C background. Several other ADRP mutants also formed rhodopsin pigment in this N2C/D282C background, but at lower levels compared with WT. These mutants were T4K (90% of WT), N15S (80% of WT), and both Q28H and V20G (both ~50% of WT). Pigment formation was negligible or absent for ADRP mutants T17M, P23H, and P23L in the N2C/D282C background. The relative expression levels of these mutants restored by the two separate approaches are shown in Fig. 2C. The T17M, P23H, and P23L mutants in the N2C/D282C background formed some pigment when 11-cis-retinal was present during expression; however, these levels of expression were lower than those observed in the WT background under 11-cis-retinal rescue conditions (Table 1).

Thermal Stability of Restored ADRP Rhodopsin Mutant Pigments—Thermal stability measurements were performed using the purified pigments obtained either by 11-cis-retinal pharmacological rescue or by N2C/D282C repair. The decrease in absorbance at 500 nm at 37 or 55 °C as a function of time was monitored, and the data collected were used to determine half-life values (see “Experimental Procedures”). When kept at 37 °C, WT rhodopsin purified from HEK-293S cells has a half-life of ~58 h. This value is comparable to the thermal decay profile at 37 °C of WT rhodopsin prepared from COS-1 cells described in a previous study (38). All ADRP mutant pigments obtained by 11-cis-retinal pharmacological rescue were much less stable at 37 °C compared with WT rhodopsin (Fig. 3A and Table 2). In addition, the thermal decay profiles of these mutant pigments had two kinetic components as revealed by curve fitting of the data. The most stable ADRP mutant at 37 °C was P23A (τ1/2 = 14 h for component I), whereas the most unstable was Q28H (τ1/2 = 19 min for component I). The observed order of stability for these mutant pigments in the WT rhodopsin background at 37 °C was as follows: P23A > N15S > T4K > V20G > T17M > P23H > P23L > Q28H.

To investigate the consequences of tethering the defective cap mutants to the extracellular surface, we measured the thermal stability of the purified ADRP mutant pigments in the N2C/D282C rhodopsin background. At 37 °C, rhodopsin N2C/D282C had a τ1/2 of >4 days (115.5 h), whereas WT rhodopsin had a τ1/2 of ~3 days (58 h). The increased thermal stability of rhodopsin N2C/D282C is attributable to the additional disulfide bond (Cys-2–Cys-282) in the extracellular domain that forms between the two engineered cysteine residues (Fig. 1A). The ADRP mutant pigments obtained using the N2C/D282C background all displayed higher thermal stabilities than those lacking the additional N2C/D282C mutations obtained by rescue with 11-cis-retinal. For example, the P23A mutant had a half-life of ~14 h at 37 °C, whereas the corresponding P23A (N2C/D282C) mutant had a half-life of 58 h. P23A in the N2C/D282C background was also treated with DTT before monitoring pigment stability. In this case, the thermal stabilization effect of the N2C/D282C background was reversed, and the pigment subsequently regained a thermal decay profile similar to that observed in the WT background (Fig. 3 and Table 2). We also conducted thermal stability measurements at 55 °C, and the relative stabilities of the mutants at that temperature were similar to those at 37 °C (Fig. 3B and Table 2).

Susceptibility of ADRP Rhodopsin Mutants to Hydroxyamine—To examine the physical accessibility of the retinal Schiff base–Lys-296 linkage to the bulk solvent, hydroxyamine was used as a probe. The loss of absorbance at 500 nm in the presence of 50 mM hydroxyamine at 37 °C was examined by UV-visible absorbance spectroscopy (see “Experimental Procedures”). WT rhodopsin in the dark showed some resistance to attack by hydroxyamine (τ1/2 = 11.5 h) compared with all of the purified ADRP mutant pigments, which were more susceptible (Fig. 3C and Table 3). The increase in susceptibility to hydroxyamine attack for ADRP rhodopsin mutants in the WT rhodopsin background is in agreement with results from previous studies (22). We now report that those ADRP mutants repaired in the N2C/D282C background were more resistant to hydroxyamine than those in the WT background. For example, P23A in the WT rhodopsin background (τ1/2 = 55.9 min) was 6-fold more sensitive than P23A in the N2C/D282C background (τ1/2 = 330.1 min). Similarly, the V20G mutant in the WT background (τ1/2 = 10.6 min) acquired an increased resistance to hydroxyamine (τ1/2 = 80.6 min) in the N2C/D282C background. In addition, mutant pigments obtained by pharmacological rescue in the WT background exhibited double-component decay kinetics in the presence of hydroxyamine compared with single-component decay kinetics exhibited by mutants repaired by the N2C/D282C background.

Photobleaching Properties of Purified Rhodopsin Mutant Pigments—UV-visible absorbance spectroscopy was used to examine the photobleaching properties of immunoaffinity-purified mutant pigments (see “Experimental Procedures”). Spectra were recorded before and after illumination (>495-nm light) as described under “Experimental Procedures.” Purified WT rhodopsin (visible λmax = 500 nm) was readily photoconverted to the MII 380-nm form (39, 40). Further illumination of the sample for up to 2 min resulted in no further spectral change. Subsequent acidification of the sample moved the absorption maximum from 380 to 440 nm, which corresponds to the formation of a protonated Schiff base (41). The rhodopsin N2C/D282C mutant displayed photobleaching profiles similar to WT rhodopsin (Fig. 4), as did the single N2C and D282C pigments (data not shown). When 11-cis-retinal-reated ADRP mutant pigments were illuminated, conversion to the 380-nm species was incomplete, and a small but detectable shoulder (380–480 nm) with a peak at ~450 nm was evident.
Further 30-s pulses of illumination were required for complete conversion to the 380-nm form. The degree of resistance to photobleaching varied among the ADRP mutants. Some mutants (T17M, P23A, P23L, P23H, and Q28H) possessed a higher content of a photobleaching-resistant form, whereas others (T4K, V20G, and N15S) photobleached more readily. Upon acidification, several of the photobleached ADRP mutant pigments did not convert fully to the 440-nm species. This property is best explained by the fast decay of MII and release of all-trans-retinal that occur in these mutants prior to sample acidification. A summary of the photobleaching properties of the ADRP rhodopsin mutants is reported in Table 4. These data are consistent with those described in a recent study (22), as well as in earlier reports (20, 42).

We next examined the photobleaching properties of purified ADRP mutant pigments in the N2C/D282C background. Mutants T4K, N15S, V20G, and P23A in the N2C/D282C background now displayed WT-like photobleaching profiles (Fig. 4). By contrast, when these ADRP mutants were superimposed onto the single N2C or D282C (control) background, their photobleaching profiles remained abnormal (data not shown).

**MII Decay Rates of Rhodopsin Mutants** — The rate of rhodopsin MII decay was measured by following the kinetics of all-trans-retinal release. The assay exploits the fluorescence of native tryptophan residues in proximity to retinal that is quenched when retinal is present (see “Experimental Procedures”) (31). The results for selected mutants are shown in Fig. 5A, and all data are compiled in Table 4. The $t_{1/2}$ of MII decay for WT rhodopsin purified from HEK-293S cells was $\sim$15.8 min, a value similar to that reported for rhodopsin purified from ROS or transfected COS-1 cells (13, 43). In our experiments, the $t_{1/2}$ of MII decay for ROS was 16.5 min. The $t_{1/2}$ of MII decay for the N2C/D282C mutant was 14.7 min, a value comparable to that for WT rhodopsin purified from HEK-293S cells. The $t_{1/2}$ values
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Table 2
Thermal stability of ADRP mutant pigments

Values are the means ± S.D. from three independent experiments. Data were collected at time intervals as described under "Experimental Procedures," except at 37 °C (1), which were recorded every 6 min. Pigments were obtained by the addition of 11-cis-retinal after transient transfection (10) or during opsin biosynthesis (10).

| Mutant          | Component I | Component II | Component I | Component II |
|-----------------|-------------|--------------|-------------|--------------|
|                 | t1/2 (h)    | %            | t1/2 (h)    | %            |
| WT (ROS)        | ND          | ND           | ND          | ND           |
| WT (HEK-293S)   | 57.8 ± 3.3  | 100          | 74.7 ± 5.2  | 100          |
| N2C/D282C       | 58.3 ± 4.2  | 100          | 72.5 ± 3.9  | 100          |
| N2C/D282C       | 115.5 ± 13  | 100          | 630 ± 150   | 100          |
| N2C/D282C       | 57.5 ± 5.1  | 100          | 69.9 ± 5.1  | 100          |
| N2C/D282C       | 62.5 ± 7.5  | 100          | 173 ± 11    | 100          |
| N2C/D282C       | 58.7 ± 6.4  | 100          | 84.4 ± 6.3  | 100          |
| T4K (WT)        | 3.2 ± 0.3   | 60.5         | 0.45 ± 0.3  | 39.5         |
| T4K (N2C/D282C) | 43.7 ± 5.2  | 100          | 67.9 ± 15   | 100          |
| N155 (WT)       | 5.8 ± 0.5   | 61.6         | 0.56 ± 0.5  | 38.4         |
| N155 (N2C/D282C) | 3.85 ± 3.9 | 100          | 87.7 ± 9.1  | 100          |
| T17M (WT)       | 1.4 ± 0.3   | 51.7         | 0.65 ± 0.3  | 48.3         |
| T17M (N2C/D282C) | 23.1 ± 2.3 | 64.2         | 4.13 ± 2.3  | 35.8         |
| V20G (WT)       | 1.6 ± 0.4   | 51.5         | 1.51 ± 0.4  | 48.5         |
| V20G (N2C/D282C) | 4.8 ± 1.5  | 100          | 128.4 ± 21  | 100          |
| P23A (WT)       | 1.44 ± 0.8  | 76.8         | 0.82 ± 0.8  | 23.2         |
| P23A (N2C/D282C)| ND          | ND           | ND          | ND           |
| P23A (N2C/D282C)| ND          | ND           | ND          | ND           |
| P23A (N2C/D282C)| 58.6 ± 11  | 100          | 165 ± 19    | 100          |
| P23A (N2C/D282C)| 15.5 ± 0.7  | 66.7         | 0.58 ± 0.7  | 33.3         |
| P23H (WT)       | 1.7 ± 0.3   | 76.7         | 0.22 ± 0.3  | 23.3         |
| P23H (N2C/D282C) | 1.5 ± 0.3  | 50.1         | 0.13 ± 0.3  | 49.9         |
| V20G (WT)       | 0.8 ± 0.3   | 78.1         | 0.18 ± 0.3  | 21.9         |
| V20G (N2C/D282C) | 2.5 ± 0.5  | 82.9         | 0.21 ± 0.5  | 17.1         |
| P23H (WT)       | 0.3 ± 0.1   | 50.4         | 0.21 ± 0.1  | 49.6         |
| Q28H (N2C/D282C) | 1.5 ± 1.3  | 100          | 13.5 ± 5.3  | 100          |

a Half-life of pigments were derived by curve fitting data to either the single- or double-component exponential decay function of SigmaPlot 12.0.

The percentage contribution of each component of decay is shown.

b ND, not determined.

c Pigment was incubated with DTT before initiating thermal decay.

d Mutant is repaired by N2C/D282C.

Table 3
Stability of mutant pigments in the presence of hydroxylamine at 37 °C

Half-life values of pigments and their contribution as a percentage were derived by curve fitting data to the single- or double-component exponential decay function of SigmaPlot 12.0. Pigments were obtained by the addition of 11-cis-retinal after transient transfection (10) or during opsin biosynthesis (10). Values are the means ± S.D. from three independent experiments.

| Mutant          | Component I | Component II |
|-----------------|-------------|--------------|
|                 | t1/2 (h)    | %            |
| WT (HEK-293S)   | 11.6 ± 13   | 100          |
| N2C/D282C       | 10.5 ± 15   | 100          |
| N2C/D282C       | 3.7 ± 1.4   | 100          |
| D282C           | 3.0 ± 1.1   | 100          |
| T4K (WT)        | 0.8 ± 0.2   | 49.8         |
| T4K (N2C/D282C) | 3.3 ± 0.7   | 100          |
| N155 (WT)       | 1.3 ± 0.3   | 47.8         |
| N155 (N2C/D282C)| 4.6 ± 0.4  | 100          |
| T17M (WT)       | 0.2 ± 0.1   | 59.6         |
| T17M (N2C/D282C)| 3.2 ± 0.3  | 80.2         |
| V20G (WT)       | 0.2 ± 0.1   | 80.3         |
| V20G (N2C/D282C) | 3.9 ± 0.6 | 100          |
| P23A (WT)       | 1 ± 0.1     | 60           |
| P23A (N2C/D282C)| 5.5 ± 0.3  | 100          |
| P23H (WT)       | 1 ± 0.1     | 17.8         |
| P23H (N2C/D282C)| 0.24 ± 0.1 | 50           |
| P23L (WT)       | 0.35 ± 0.1  | 17.2         |
| P23L (N2C/D282C)| 0.14 ± 0.1 | 50           |
| Q28H (WT)       | 0.21 ± 0.1  | 38.2         |
| Q28H (N2C/D282C)| 1.71 ± 0.7 | 100          |

of MII decay for the single N2C and D282C mutants were 15.5 and 18.1 min, respectively.

The MII decay kinetics of the 11-cis-retinal-rescued human ADRP mutants in the N terminus have not been examined previously. They were found to be complex: curve fitting of the data revealed the presence of two MII decay kinetic components (slow and fast). For example, the P23A MII decay profile has a slow component (t1/2 = 18.3 min, 64.8% contribution) and a fast component (t1/2 = 2.3 min, 35.2% contribution). Similarly, the P23H MII decay profile had a slow component (t1/2 = 13.1 min, 63.5% contribution) and a fast component (t1/2 = 1.1 min, 36.5% contribution). A summary of the MII decay profiles is compiled in Table 4. All of the MII decay rates for ADRP mutants modified by N2C/D282C repair were faster compared with WT rhodopsin. However, unlike the double-component decay kinetics observed for ADRP mutants in the WT background, they now exhibited single-exponential decay kinetics. The MII decay profiles of V20G (12.4 min), T4K (12.1 min), and N155 (9.8 min) in the N2C/D282C background were fit to a single-component exponential decay function.

To further prove that a disulfide bond between Cys-2 and Cys-282 is responsible for the single-component MII decay kinetics acquired by the ADRP mutants in the N2C/D282C rhodopsin background, we performed the same experiments using pigments pretreated with DTT. This reducing agent had no effect on the MII decay kinetics of WT opsin purified from ROS (t1/2 = 16.5 min) or from transfected HEK-293S cells (t1/2 = 15.7 min). Similarly, DTT had only a small effect on the rate of MII decay of rhodopsin N2C/D282C (t1/2 = 14.5 min) or the single mutant N2C or D282C (t1/2 = 15.5 and 18.0 min, respectively). By contrast, DTT had a pronounced effect on the rate of MII decay of ADRP mutants rescued in the N2C/D282C rhodopsin background. The MII decay profiles of ADRP mutants in the N2C/D282C background treated with DTT displayed
double-component decay kinetics. Interestingly, the major component decayed faster than that observed in the WT rhodopsin background. For example, N15S (N2C/D282C) MII decay had a single component (t_1/2/H11005/9.8 min), but after DTT treatment, its MII decay profile included two components of 6.2 min (79.8%) and 0.47 min (20.2%). Similarly, the MII decay of V20G (N2C/D282C), which had a half-life of 12.4 min, was converted to two components of 4.9 min (60.8%) and 1 min (39.2%) by DTT. To conclude this analysis, we conducted MII decay rate analysis for several ADRP mutants in the single-cysteine (N2C or D282C) rhodopsin background as shown in Table 4. These mutants decayed in a manner very similar to the ADRP mutant in the WT rhodopsin background. For example, the decay of V20G MII comprised two kinetic components: 18.9 min (66.7% contribution) and 4.49 min (33.3% contribution). These MII half-life values are comparable to those in the V20G/D282C mutant, which were 12.6 min (57.4%) and 4.4 min (42.6%), respectively. Similarly, the N2C/V20G mutant MII decay had two kinetic components of 10.1 min (69.3%) and 1.45 min (30.7%).

Activation of G_T by ADRP Rhodopsin Mutants — To examine the signal transduction properties of the N-terminal ADRP mutants, we examined their ability to activate G_T using a fluorescence-based assay (33) as described under “Experimental Procedures.” This assay measures the increase in relative fluorescence of Trp-207 in the G_T α-subunit that occurs during MII-catalyzed exchange of GDP for GTP upon activation (33, 44, 45). The results for selected mutants are shown in Fig. 5 (B and C), and all data are compiled in Table 4. The values of G_T activation are expressed relative to that of WT MII (100%). Upon light activation, the MII species of all detergent-purified ADRP mutants showed a markedly reduced level of G_T activation compared with WT MII. For example, the relative G_T activation rates for T4K, N15S, and P23A mutant MII species were 25, 8.9, and 15.3% of WT MII, respectively (Table 4). Only the V20G mutant MII species retained a significant amount of G_T-activating ability (50.8% of WT MII). The rate of G_T activation by N2C/D282C rhodopsin MII was 79.8% of the WT MII level. This finding is in agreement with the value reported by Oprian and co-workers (23), who used a filter binding-based G_T activation assay. We found that the MII forms of purified ADRP mutants restored in the N2C/D282C rhodopsin background had improved levels of G_T activation. ADRP mutants T4K and N15S in the N2C/D282C rhodopsin background exhibited G_T activation rates that were 78.2 and 73.4% of WT MII, respectively, cf. 25 and 9% in the absence of the disulfide bond formed by N2C and D282C. These values are comparable to the value of N2C/D282C MII (79.8% of WT). The activation of G_T by ADRP rhodopsin mutants in the single N2C or D282C rhodopsin background was essentially the same as that observed in the WT rhodopsin background. We next measured G_T activation by the ADRP N2C/D282C mutant in the presence of DTT. As shown in Fig. 5B and Table 4, DTT had no effect on G_T activation by WT MII. The N2C/D282C rhodopsin G_T activation level was reduced by 10% in the presence of DTT, whereas the activation rates with the single N2C or D282C mutant treated with DTT were ~5% lower. In those ADRP mutants in which G_T activation was improved in the N2C/D282C background,
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TABLE 4
MII decay and G
i activation rates by ADRP rhodopsin mutants

| Mutant          | MII (λmax) | Component I | Component II | G
i activation |
|-----------------|------------|-------------|--------------|--------------|
|                 |            | %          | %            | Initial rates | % of WT |
|                 |            | min        | min         | %           |
| WT (ROS)        | 380        | 16.5 ± 0.3 | 100         | ND           | ND      |
| WT (ROS)        | NA         | 16.5 ± 0.3 | 100         | ND           | ND      |
| WT (HEK-293S)  | 381        | 15.8 ± 0.5 | 100         | 0.124 ± 0.01 | 100     |
| WT (HEK-293S)  | NA         | 15.7 ± 0.5 | 100         | 0.199 ± 0.02 | 96.0    |
| N2C/D282C      | 380        | 14.8 ± 0.6 | 100         | 0.099 ± 0.03 | 79.8    |
| N2C/D282C      | NA         | 14.5 ± 0.6 | 100         | 0.089 ± 0.02 | 71.8    |
| N2C/D282C      | 387        | 15.6 ± 0.9 | 100         | 0.096 ± 0.02 | 77.4    |
| N2C/D282C      | NA         | 15.5 ± 0.9 | 100         | 0.089 ± 0.02 | 71.8    |
| D282C           | 382        | 18.1 ± 0.3 | 100         | 0.089 ± 0.02 | 71.8    |
| D282C           | NA         | 18.0 ± 0.3 | 100         | 0.097 ± 0.02 | 63.7    |
| T4K (WT)        | 380        | 17.3 ± 0.7 | 52.1         | 0.031 ± 0.05 | 25.0    |
| T4K (N2C/D282C) | 380        | 12.1 ± 0.7 | 100         | 0.097 ± 0.01 | 78.2    |
| T4K (N2C/D282C) | NA         | 6.8 ± 0.9  | 94.5         | 0.038 ± 0.02 | 30.7    |
| N155 (WT)       | 379        | 17.5 ± 0.5 | 53.7         | 0.011 ± 0.003 | 8.9    |
| N155 (N2C/D282C) | NA         | 9.8 ± 1.2  | 100         | 0.091 ± 0.01 | 73.4    |
| T17M (WT)       | 379        | 16.5 ± 0.3 | 70.3         | 0.012 ± 0.007 | 9.7    |
| T17M (N2C/D282C) | NA         | 6.2 ± 0.8  | 79.8         | 0.035 ± 0.004 | 28.2    |
| V2OG (WT)       | 379        | 18.9 ± 0.7 | 66.7         | 0.063 ± 0.008 | 50.8    |
| V2OG (N2C/D282C) | 380       | 12.4 ± 0.9 | 100         | 0.088 ± 0.002 | 71.0    |
| V2OG (N2C/D282C) | NA         | 6.9 ± 1.3  | 60.8         | 0.054 ± 0.01 | 43.6    |
| V2OG (N2C/D282C) | NA         | 10.1 ± 0.9 | 69.3         | 0.032 ± 0.005 | 25.8    |
| V2OG (N2C/D282C) | NA         | 12.6 ± 0.5 | 57.4         | 0.031 ± 0.003 | 25.0    |
| P23A (WT)       | 377        | 18.3 ± 0.3 | 68.4         | 0.019 ± 0.002 | 15.3    |
| P23A (N2C/D282C) | 380        | 7.4 ± 1.3  | 100         | 0.085 ± 0.03 | 68.6    |
| P23A (N2C/D282C) | NA         | 6.8 ± 0.7  | 78.1         | 0.051 ± 0.01 | 41.1    |
| P23A (N2C/D282C) | NA         | 11.8 ± 0.6 | 64.1         | 0.017 ± 0.002 | 13.7    |
| P23A (N2C/D282C) | NA         | 13.4 ± 0.5 | 54.1         | 0.031 ± 0.001 | 14.5    |
| P23A (N2C/D282C) | 378        | 13.1 ± 0.9 | 63.5         | 0.037 ± 0.002 | 29.8    |
| P23A (N2C/D282C) | 378        | 11.6 ± 0.6 | 59.2         | 0.017 ± 0.008 | 13.7    |
| P23A (N2C/D282C) | 377        | 10.5 ± 0.3 | 75.6         | 0.028 ± 0.002 | 22.6    |
| P23A (N2C/D282C) | 382        | 7.5 ± 0.3  | 100         | 0.035 ± 0.005 | 28.2    |
| P23A (N2C/D282C) | NA         | 8.7 ± 0.5  | 79.5         | 0.023 ± 0.004 | 26.6    |

a Absorbance maximum of pigment after 30 s of illumination (>495 nm).

b Half-life of MII decay of purified pigments obtained as described in the legend to Fig. 5. Half-life values were derived by curve fitting data to the single- or double-component rise-to-plateau exponential function using SigmaPlot 12.0. In all cases, R2 > 0.99.

c When two (MII) decay components were identified, the t1/2 of the additional component (c) and the proportion of each component as a percentage (c and c) are indicated.

d Initial rates of transducin activation were derived using fluorescence increase measured over the first 60 s after GTPγS addition. a.u., arbitrary units.

e Initial rate of G
i activation relative to WT rhodopsin (100%).

f ND, not determined; NA, not applicable. Pigments pretreated with DTT.

DTT typically reversed this effect. In the case of Q28H, signal transduction properties were not noticeably improved by the N2C/D282C background, and DTT had no effect.

DISCUSSION

GPCRs are a large superfamily of cell surface receptors that respond to diverse ligands (46). The extracellular domain of GPCRs usually mediates the recognition and entry of the specific ligands that regulate signaling activity. However, visual pigments, such as rhodopsin, are unique GPCRs that have evolved into binary molecular on-off switches that contain a light-sensitive covalently bound retinylidene ligand. Unlike most other GPCRs, the extracellular surface of the opsin is not the site of ligand entry; instead, retinal enters (11-cis) and exits (all-trans) via the 7TM domain (47). As a consequence, we propose that the extracellular domain has been free to evolve into a compact structure that contributes to the extraordinary thermostability and very low dark activity of rhodopsin. The N terminus is an integral component of this domain (Fig. 1), and to investigate its specific role, we performed a detailed analysis of ADRP mutations in this segment.

The fact that 11-cis-retinal ligand can function as a pharmacological chaperone for all known ADRP mutations in the N terminus of rhodopsin suggests that the N-terminal cap is not absolutely required for coordinating assembly of the 7TM bundle and formation of the ligand-binding pocket (5, 6, 48). Instead, we propose that the cap packs onto the extracellular domain after assembly of the helical bundle and then contributes to the general stability of the ligand-free opsin state, as well as ligand-bound rhodopsin pigment. The differences we observed in the absolute expression levels of the different mutants under pharmacological rescue conditions may be attributable to the severity of the mutational defect, the efficiency of pharmacological rescue, the efficiency of trafficking to the cell surface, and/or the stability of the resultant rescued pigment.

All of the N-terminal ADRP mutants obtained by pharmacological rescue with 11-cis-retinal formed pigments with WT rhodopsin-like dark-state spectral properties, showing that the 7TM helices adopt the correct topology and that EL-2 attains the correct fold. However, the rapid thermal decay of these mutant pigments indicates that a functional cap is required for structural stability. The degree of thermal instability was dependent upon the position of the mutation and the amino acid change and can be understood, in part, by how the folded N-terminal cap packs onto the extracellular surface of the
The crystal structure of the rhodopsin extracellular domain reveals the position of the N terminus above EL-2, sandwiched between EL-1 and EL-3. The ADRP sites are situated at the core of this structure (Fig. 1), with Pro-23 and Gln-28 mediating key interactions with EL-2. The three most unstable pigments, Q28H, P23L, and P23H, have two of the highest packing values in the N terminus, consistent with their position within the core of the N-terminal cap (Table 5) and their tight interactions with both EL-1 and EL-2. Previously, mutations designed to impair the Arg-177–Asp-190 salt bridge in the EL-2 lid also gave rise to unstable rhodopsin pigments (49); however, expression levels were normal, as was susceptibility to attack by hydroxylamine and GT activation.

### FIGURE 5

**A**. The MIII decay profiles of selected ADRP mutant pigments in the WT opsin background obtained by 11-cis-retinal rescue were determined in the absence (red lines) or presence (brown lines) of DTT. The MIII decay profiles of mutants prepared using the N2C/D282C background were also determined in the presence (green lines) or absence (blue lines) as indicated. The increase in tryptophan fluorescence that occurs as the photo-bleached pigment (MIII) decays was monitored as described under “Experimental Procedures.” Data were fit to either the single- or double-exponential rise-to-plateau function (SigmaPlot 12.0) for determination of half-life values. Data from these experiments are compiled in Table 4. Each graph is a representative result collected using three independently purified samples.

**B**. GT activation profiles of selected ADRP mutants. The mutant pigments used were prepared as described for **A**, and GT assays were performed using a fluorescence assay as described under “Experimental Procedures.” Red lines, WT background; brown lines, WT plus DTT; blue lines, N2C/D282C background; green lines, N2C/D282C plus DTT. The traces shown are representative of three independent experiments.

**C**. Comparison of relative initial rates of GT activation by light-activated ADRP mutants prepared using 11-cis-retinal rescue in the WT background (red bars) or the N2C/D282C rhodopsin background without (blue bars) or with (green bars) DTT treatment. The initial rate of GT activation by WT rhodopsin was taken as 100%. The results shown are the means ± S.D. from three independent experiments and are reported in Table 4.

### TABLE 5

**Packing values within the N-terminal cap and inter-residue contacts with EL-1–3**

| Position | Residue | Packing value | Rays contacting ELs<sup>a</sup> |
|----------|---------|---------------|-------------------------------|
|          |         |               | EL-1 | EL-2 | EL-3 |
| 1        | Met     | 0.083         | 0          | 103  |      |
| 2        | Asn     | 0.274         | 103  |      |      |
| 3        | Gly     | 0.452         | 1      |      |      |
| 4        | Thr<sup>b</sup> | 0.430 | 1      |      |      |
| 5        | Glu     | 0.222         | 32    |      |      |
| 6        | Gly     | 0.454         | 2      |      |      |
| 7        | Pro     | 0.140         | 2      |      |      |
| 8        | Asn<sup>b</sup> | 0.343 | 12    | 62   |      |
| 9        | Phe     | 0.501         | 61    |      |      |
| 10       | Tyr     | 0.372         | 163   | 40   |      |
| 11       | Val     | 0.606         | 37    |      |      |
| 12       | Pro     | 0.475         | 53    | 106  |      |
| 13       | Phe     | 0.368         | 17    |      |      |
| 14       | Ser     | 0.289         | 5     |      |      |
| 15       | Asn<sup>b</sup> | 0.330 | 1      |      |      |
| 16       | Lys     | 0.093         | 1     |      |      |
| 17       | Thr<sup>b</sup> | 0.172 | 2      |      |      |
| 18       | Gly     | 0.306         | 183   | 3    |      |
| 19       | Val<sup>b</sup> | 0.358 | 1      |      |      |
| 20       | Val<sup>b</sup> | 0.555 | 1      |      |      |
| 21       | Arg     | 0.289         | 24    |      |      |
| 22       | Ser     | 0.573         | 1     |      |      |
| 23       | Pro<sup>b</sup> | 0.579 | 2      |      |      |
| 24       | Phe     | 0.508         | 238   | 43   |      |
| 25       | Glu     | 0.291         | 33    |      |      |
| 26       | Ala     | 0.456         | 56    |      |      |
| 27       | Pro     | 0.367         | 89    |      |      |
| 28       | Gln<sup>b</sup> | 0.594 | 53    | 48   |      |
| 29       | Tyr     | 0.306         | 1     |      |      |
| 30       | Tyr     | 0.463         | 1     |      |      |
| 31       | Leu     | 0.518         | 1     |      |      |
| 32       | Ala     | 0.364         | 1     |      |      |
| 33       | Glu     | 0.309         | 1     |      |      |

<sup>a</sup> In the occluded surface calculation, rays are drawn from the van der Waals surfaces and terminate when they contact the van der Waals surface of a neighboring residue. Here, we list the number of rays from the selected amino acid on the N-terminal cap and terminating on EL-1, EL-2, or EL-3.

<sup>b</sup> Sites of ADRP mutation. Asn-15 is a site for N-glycosylation and forms hydrogen bonds to Thr-4, Val-20, and Gly-18. These interactions appear to contribute to maintenance of the extracellular horn of rhodopsin, as do those involving Thr-17, the side chain of which hydrogen bonds with the backbone amine of Val-19. Pro-23 and Gln-28 mediate interactions of the N terminus with both EL-1 and EL-2.
All mutant pigments obtained by pharmacological rescue displayed impaired photobleaching (Fig. 4), as reported previously (22). Furthermore, the light-activated mutant pigments had abnormal MII decay profiles with two kinetic components. Similar MII decay profiles have been described previously for other rhodopsin mutants, including those that cause ADRP (50, 51). Although the molecular basis of the double-component decay profile is not known, it could be due to incomplete phototransformation of the mutant pigments to MII (Fig. 4). These observations point to several additional roles for the N-termi-
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The purified T4K, N15S, V20G, P23A and Q28H mutant pigments obtained using the N2C/D282C rhodopsin background had much improved thermostability, WT-like photobleaching properties, and single-component MI1 decay kinetics. Some (T4K, N15S, V20G, and P23A) activated G_{T} to an extent comparable to the control pigment (WT N2C/D282C), but G_{T} activation by repaired Q28H did not improve. Taken together, these observations indicate that the Cys-2–Cys-282 bond tethers the defective caps in place, and although this is sufficient for improved stability and photoactivation, the fit is not always optimal. In certain experiments, the improved function was reversed by treating the pigments with DTT to reduce the Cys-2–Cys-282 disulfide bond (Figs. 3 and 5 and Tables 2 and 4), providing further evidence that the Cys-2–Cys-282 disulfide bond is responsible for the repaired phenotype. Although disulfide bond-mediated repair has no obvious use as a therapeutic tool, our findings indicate that regions other than the orthosteric ligand–binding site (57), such as the N terminus, should be considered as future drug targets for stabilization of ADRP rhodopsin mutants.

A direct comparison of the two approaches, pharmacological rescue and N2C/D282C repair, highlights the role of the N-terminal cap in rhodopsin structure and function and sheds light on likely defects arising from ADRP mutations in this region. In both cases, we propose that repair/rescue is likely to occur relatively late in the protein-folding process. This is because rescue requires a native-like binding pocket, whereas repair requires that folding has reached a stage such that the two cysteine residues, separated by 280 amino residues in the rod opsin polypeptide chain, acquire close proximity. However, we cannot rule out the possibility that disulfide bond formation between Cys-2 and Cys-282 happens early in folding due to flexibility of the N-terminal segment prior to adopting its compact folded-state structure. We thus propose that these N-terminal ADRP mutations do not prevent assembly of the 7TM bundle, but rather lower the stability of the final folded rod opsin structure.

Our observations allowed us to construct a model to help explain rhodopsin misfolding and restoration of function (Fig. 6) and to draw the following conclusions. 1) A functional cap is required to stabilize the 7TM bundle of rod opsin (Fig. 6A). 2) Defects in the cap result in instability of the assembled 7TM bundle and retention of unstable rod opsin by the ER prior to ER-associated protein degradation (Fig. 6B). 3) Instability in these ADRP mutants can be compensated by stabilizing the 7TM bundle by occupancy of the retinal ligand pocket during opsin biosynthesis (Fig. 6C) or, in certain cases, repaired by securing the defective lid by a Cys-2–Cys-282 disulfide bridge (Fig. 6D). 4) The cap also stabilizes the positions of EL-1–3, which are integral to coordinated movement of the 7TM domain, receptor activation, and signaling. In conclusion, the dual strategy we have used here in attempts to restore folding in ADRP rhodopsin mutants is significant because it has shed light on the multifunctional role of the N terminus in rhodopsin and provides much needed insight into mechanisms by which mutations in this domain might initiate ADRP.

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