Retinitis Pigmentosa Rhodopsin Mutations L125R and A164V
Perturb Critical Interhelical Interactions

NEW INSIGHTS THROUGH COMPENSATORY MUTATIONS AND CRYSTAL STRUCTURE ANALYSIS

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L125R, a severe retinitis pigmentosa rhodopsin missense mutation, results in rhodopsin protein misfolding, retinal degeneration, and ultimately blindness. The initiating structural events leading to this protein misfolding are unknown. Through the use of compensatory mutations, in conjunction with crystal structure-based molecular analysis, we established that the larger and positively charged Arg replacing Leu125 sterically hinders both the adjacent Trp126 and a critical interhelical interaction between transmembrane III (TM III) and transmembrane V (TM V; Glu122 and His311 salt bridge). Further, analysis of another retinitis pigmentosa mutation, A164V (TM IV), indicates that the larger Val interferes with residues Leu119 and Ile123 on TM III, leading to the disruption of the same critical Glu122-His311 salt bridge (TM III-TM V interaction). Combined, these localized defects in interhelical interactions cause structural changes that interfere with the ability of opsins to bind 11-cis-retinal. These distortions ultimately lead to the formation of an abnormal disulfide bond, severe protein instability, aggregation, and endoplasmic reticulum retention. In the absence of a crystal or NMR structure of each retinitis pigmentosa mutation, compensatory mutagenesis and crystal structure-based analysis are powerful tools in determining the localized molecular disturbances. A detailed understanding of the initiating local perturbations created by missense mutations such as these, not only identifies critical factors required for correct folding and stability, but additionally opens avenues for rational drug design, mimicking the compensatory mutations and stabilizing the protein.

Rhodopsin, the dim light-activated photoreceptor in the retina, is considered to be a prototypical G protein-coupled receptor (1). A substantial understanding of the structure of rhodopsin has been obtained from electron cryomicroscopy (2), electron paramagnetic resonance (3, 4), and biochemical studies (5). More recently, the 2.8-Å resolution crystal structure of rhodopsin has been obtained from electron cryomicroscopy (2), electron paramagnetic resonance (3, 4), and biochemical studies (5). Additional analyses have examined the direct effect of vitamin A, as well as other retinoids, on the protein folding and stability properties of specific retinitis pigmentosa mutants. A treatment protocol of Vitamin A plus 11-cis-retinal improved stability of the T17M mutant protein and delayed photoreceptor degeneration in a transgenic mouse model expressing that retinitis pigmentosa mutant (10). Additional studies have shown that improved folding of P23H, the most common retinitis pigmentosa mutant in North America, can be achieved with both 11-cis-7-ring-retinal (11) and 9-cis-retinal (12) addition.

As a representative G protein-coupled receptor, rhodopsin is an integral membrane protein composed of three distinct regions: the intracellular (cytoplasmic), transmembrane, and extracellular (intradiscal) regions (Fig. 1). The intradiscal loop connecting helices IV and V contains Cys187 and forms a plug upon which 11-cis-retinal lies (7). Cys187 also forms a highly conserved disulfide bond with Cys140, which is essential for the correct folding, stability, and function of rhodopsin (13–15). The intradiscal domain is structurally coupled to the transmembrane (TM) domain. Thus, mutations in the intradiscal domain can severely affect the ability of rhodopsin to bind 11-cis-retinal in the TM domain. Similarly, mutations within the TM domain can lead to the formation of an abnormal Cys185-Cys187 disulfide bond, resulting in irreversible protein misfolding. Within the transmembrane domain, 11-cis-retinal covalently binds to opsin via a protonated Schiff base at the ε-amino group of Lys296 located in TM VII (16, 17). This interaction is stabilized by a negatively charged counterion at Glu113 within TM III (18, 19). Furthermore, the transmembrane domain is also essential in conveying a conformational change induced by cis-trans isomerization of 11-cis-retinal to the cytoplasmic domain.

Most RP mutations lead to varying degrees of rhodopsin misfolding, particularly those found in the intradiscal and...
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The degree of misfolding can be gauged by the inability of opsin to bind 11-cis-retinal. L125R (TM III) is one of the most severe RP mutations, as measured by the total inability of the expressed mutant opsin to bind 11-cis-retinal (complete misfolding), retention in the endoplasmic reticulum, poor expression, and lack of glycosylation. This complete loss of retinal binding and correct opsin folding is depicted in the total inability of the expressed mutant opsin to bind 11-cis-retinal (complete misfolding). The molecular basis for the initiation of such protein misfolding among the RP rhodopsin mutations is unknown. Furthermore, a thorough understanding of these RP mutations can also provide a wealth of information on the structure and function of rhodopsin, particularly when combined with crystal structure analysis.

In this study, we examine the initiating structural events that lead to misfolding for the two severe RP mutations L125R and A164V, in the third and fourth transmembrane helices (TM III and TM IV), respectively (20, 22, 23). We postulate that there may be common regional mechanisms affecting critical interhelical interactions that lead to misfolding. Using compensatory mutations (based upon crystal structure analysis; Table I), we have determined the specific conformations adopted by the mutant that causes severe misfolding, as characterized by decreased absorption following illumination of rhodopsin protein. Protein samples were introduced into the synthetic rhodopsin gene in the pMT4 expression vector (25) through PCR mutagenesis. Complementary sets of oligonucleotide primers (−30 bases each) were constructed using the change in codon 125 from (CTG (Leu) → CGG (Arg)) and in codon 164 from (GCT (Ala) → GTT (Val)). The reaction mixture contained 5 μl of 10X Pfu reaction buffer, 500 ng of wild-type construct, 500 ng of each primer, 0.2 mM dNTPs, 1 μl of Pfu DNA polymerase (Stratagene). The cycling protocol was composed of 16 cycles at 96 °C for 1 min, followed by 50 °C for 1 min, and 72 °C for 14 min. Upon completion, the restriction enzyme DpnI was added to digest native rhodopsin DNA strands. Ten microliters of the PCR product was used to transform 125 μl of DH5α competent cells (−2 × 10^9 cells). After DNA extraction from selected clones, large plasmid preparations were isolated using the Wizard® Maxiprep DNA purification resin (Promega). All DNA sequences were verified by dideoxynucleotide chain-termination sequencing at the Molecular and Cellular Biology Core Facility (Dartmouth Medical School). Compensatory mutations for the L125R and A164V mutants were engineered by PCR mutagenesis as described above, using the L125R or A164V mutant as the template DNA. Isolation and confirmation of mutant DNA was performed as described above.

Expression and Purification of Rhodopsin and Rhodopsin Mutants—The procedure for the transient transfection of pMT4 vectors carrying the opsin genes in COS-1 cells has been modified from the previously described protocol (24). Cells were transiently transfected with vector containing wild-type or mutant rhodopsin DNA (20 μg/150-mm plate) in 10 ml of Dulbecco's modified Eagle's medium containing 0.1 mM Tris (pH 7.4) and 0.1 mM EDTA (5% DM, 50 μM nonapeptide corresponding to the C terminus rhodopsin sequence), and solubilization buffer (PBS, 1.0% w/v DM, 0.2 mM phenylmethylsulfonfyl fluoride, 5 mM ATP).

**EXPERIMENTAL PROCEDURES**

**Materials**—11-cis-retinal was a gift from Rosalie Crouch (Medical University of South Carolina) and NEI, National Institutes of Health. N-dodecyl-β-o-maltoside (DM) was purchased from Anatrace (Maumee, OH). Anti-rhodopsin-1D4 monoclonal antibody was a kind gift from Diane Perez (The Cleveland Clinic, Cleveland, OH) and was coupled to cyanogen bromide-activated-Sepharose 4B (Amersham Biosciences) as described (24). Goat-anti-mouse secondary antibody conjugated to the Alexa Fluor® 488 dye was purchased from Molecular Probes (Eugene, OR). The buffers used were: PBS buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH2PO4, 10 mM Na2HPO4, pH 7.2), rhodopsin elution buffer (PBS, 0.05% w/v DM, 0.05% w/v Triton X-100), and solubilization buffer (PBS, 1.0% w/v DM, 0.2 mM phenylmethylsulfonfyl fluoride, 5 mM ATP).

**Construction of Rhodopsin Gene Mutants**—Amino acid substitutions were introduced into the synthetic rhodopsin gene in the pMT4 expression vector (25) through PCR mutagenesis. Complementary sets of oligonucleotide primers (−30 bases each) were constructed using the change in codon 125 from (CTG (Leu) → CGG (Arg)) and in codon 164 from (GCT (Ala) → GTT (Val)). The reaction mixture contained 5 μl of 10X Pfu reaction buffer, 500 ng of wild-type construct, 500 ng of each primer, 0.2 mM dNTPs, 1 μl of Pfu DNA polymerase (Stratagene). The cycling protocol was composed of 16 cycles at 96 °C for 1 min, followed by 50 °C for 1 min, and 72 °C for 14 min. Upon completion, the restriction enzyme DpnI was added to digest native rhodopsin DNA strands.

**Microscopy**—Samples containing rhodopsin were used for immunostaining. Cells were fixed for 10 min in 4% formaldehyde in PBS at room temperature. Non-specific staining was blocked by incubation of the cells for 1 h in PBS containing 1% bovine serum albumin. goat-anti-mouse secondary antibody conjugated to Alexa Fluor® 488 dye and rhodopsin-1D4 mouse monoclonal antibody were used for immunostaining.

**Immunoblotting**—Rhodopsin protein samples were resolved on gel electrophoresis and transferred to nitrocellulose. Anti-rhodopsin-1D4 mouse monoclonal antibody was used for detection of rhodopsin (37°C), and horse-radish peroxidase was used as a secondary antibody. Detection was achieved by chemiluminescence using Amersham ECL detection reagents. Protein bands were quantitated using a FluorChem2 imaging system (Alpha Innotech Corp., San Leandro, CA) and AlphaEase FC software (Alpha Innotech Corp.).

**Ultraviolet-Visible Absorption Spectroscopy—UV-Vis absorption spectra were recorded using a PerkinElmer Life Sciences A-40 UV-Vis spectrophotometer. All spectra were obtained at 20 °C, using a bandwidth of 2 nm, a response time of 1 s, and a scan speed of 480 nm/min. Data were analyzed with the accompanying PerkinElmer UV WinLab program.

**Thermal Stability**—Rhodopsin thermal stability was determined by monitoring the decay of the 500 nm absorbance, at a constant temperature of 50 °C, using the UV-Vis absorption spectrophotometer. Absorption spectra were obtained at 5-min intervals until complete disappearance of the 500 nm absorbance. Absorption values at 500 nm (y-axis) were normalized against time (x-axis). Non-linear regression curve fitting to determine half-life (min) was performed using GraphPad Prism® and expressed as mean ± S.E. All means and S.E. were calculated from at least n = 3 separate experiments using separate protein preparations. Data were determined to be statistically significant using the unpaired t test (*p < 0.05; **p < 0.01; ***p < 0.001).

**Metarhodopsin II Formation**—Metarhodopsin II formation was measured as an increase in the 380-nm peak and loss of 500 nm absorption following illumination of rhodopsin protein. Protein samples were illuminated with a 150-watt fiber optic light (Fiber Lite A-200; Dolan-Jenner, Woburn, MA) equipped with a ~495-nm-long pass filter (Oriel) for either 10 s, 30 s, 1 min, 2 min, or 5 min (20 °C). Immediately after each period of illumination, a UV-Vis absorption spectrum was acquired to determine the amount of metarhodopsin II formation. Experiments determining metarhodopsin II formation were repeated at least three times.

**Metarhodopsin II Decay**—The rate of metarhodopsin II decay was...
measured after illumination of protein samples by following the rate of fluorescence increase (corresponding to retinal release). The protein samples contained 2 μg of protein in 200 μl of 2 mM NaH₂PO₄ (pH 6.0), containing 0.05% DM. Samples were bleached at 20 °C for 30 s, and the fluorescence increase was measured. The excitation and emission wavelengths were 295 nm (slit = 0.25 nm) and 330 nm (slit = 12 nm), respectively. Non-linear regression curve fitting was performed for each construct, and metarhodopsin II decay half-life (min) determined. All means and S.E. were calculated from at least n = 3 separate experiments using separate protein preparations. Data were determined to be statistically significant using the unpaired t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Western Blot Analysis of Rhodopsin and Rhodopsin Mutants—The presence of rhodopsin protein was determined through Western blot analysis, using a monoclonal antibody targeting the C terminus 1D4 epitope tag. Each eluted sample was subjected to 10% SDS-PAGE. The gel was transferred to a nitrocellulose membrane and blocked with 4% bovine serum albumin for 1 h, incubated with an anti-Rho-1D4 mouse monoclonal primary antibody (300 ng/ml) for 1 h, and stained with an Alexa Fluor® 488-conjugated goat anti-mouse secondary antibody (2 μg/ml) for 1 h. Cells were washed with PBS buffer and mounted on glass microscope slides with PBS. Cells were visualized with a Bio-Rad MRC-1024 krypton/argon laser confocal system with fluorescence excitation at 488 nm and detected with Photomultiplier detectors for three-color confocal imaging (Herbert C. Engler Cell Analysis Laboratory, Norris Cotton Cancer Center, Dartmouth Medical School, Lebanon, NH).

Computer Modeling—The rhodopsin crystal structure (Protein Data Base accession number 1HZX) (7) was visualized with the Swiss PDB Viewer computer program (GlaxoSmithKline, Geneva, Switzerland) (26). The native amino acid Leu125 was initially examined and residues within 5 angstroms were selected for mutagenesis, with a majority being converted to either Asp or Glu (Table I). Similarly, the native amino acid Ala164 was examined, and a number of residues on TM III, within 5 angstroms, were selected for mutagenesis to smaller residues. Energy minimization was performed with the native amino acids replaced by the mutant residues, using Swiss PDB Viewer. Of all the possible rotamers for each mutant residue (18 for Arg and 9 for Val), the most likely rotamer was selected as the one totaling the lowest score according to the equation: (score = (4 x clash (n) with backbone N-CA and N-C atoms) + (3 x clash (n) with backbone O atoms) + (2 x clash (n) with side-chain atoms) - H-bonds (n) - 4 x S-S-bonds (n)). Thus,
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Results

Initial analysis of the positions of RP mutations on the crystal structure of rhodopsin revealed distinct localization, such that transmembrane mutations could be divided into two major groups, one positioned around the Schiff base and the other in close proximity to the β-ionone ring of 11-cis-retinal. Beyond the transmembrane domain, a third major group of RP mutations was localized around the conserved disulfide bond and a fourth at the C-terminal tail. These results suggested that all four regions, the β-ionone ring, Schiff base, disulfide bond, and C-terminal tail, are essential for correct folding and cell surface expression. We further hypothesized that, within each region, distinct structural components (e.g., interhelical interactions, salt bridge) are present that, if disrupted, initiate instability and misfolding. Using the severely misfolded L125R (in TM III near the β-ionone ring), our initial intent was to establish the precise molecular defect initiated by the Arg substitution.

The L125R Rhodopsin RP Mutant Results in Severe Protein Misfolding—Rhodopsin mutant opsins were constructed, expressed, and purified in parallel with the wild-type receptor. The dark UV-Vis absorption spectrum (Fig. 2A) shows the formation of the characteristic wild-type rhodopsin chromophore with absorbance maxima at 500 and 280 nm, as well as the expected absorbance ratio A280:A500 = 2.0. The 280 nm absorbance is related to tryptophan and aromatic residues in the protein, whereas the 500 nm absorbance reflects 11-cis-retinal binding opsin. The RP mutant L125R shows no 500 nm absorbance and thus lacks chromophore formation (of eight residues in close proximity) resulted in an aggregate 40-kDa band (50 μM). SDS-PAGE analysis (1D4 monoclonal antibody) of wild-type rhodopsin (Fig. 2B) showed the characteristic 40-kDa band (Rho) with a heterogeneous glycosylation smear. In contrast, the L125R mutant (Fig. 2B), when transfected and purified in an identical manner to wild type, exhibited little protein and presented evidence of aggregation. In COS-1 cells expressing wild-type rhodopsin (Fig. 2C, part I), confocal microscopy showed that rhodopsin was transported to the cell membrane as expected. In contrast, cells expressing the L125R mutant (Fig. 2C, part II) retained opsin within the endoplasmic reticulum (ER) with no significant surface transport.

W126E, W126D, and E122L Rescue L125R Protein Misfolding—Fifteen double mutants (Table 1) were constructed in an attempt to stabilize the mutant in the hydrophobic transmembrane environment. Such stabilization would assist in determining the conformation of the mutant Arg residue (18 different conformers were possible). None of the attempted L125R double mutants exhibited detectable 500 nm absorbance (spectra not shown). However, upon SDS-PAGE analysis, only double mutants at the Trp126 position (L125R/W126D, L125R/W126E, L125R/W126G, and L125R/W126V) and at the Glu122 position (L125R/E122L) exhibited any significant improvement in protein stability and expression, compared with the L125R mutant (Fig. 2B). Additionally, complete rescue of expression and full glycosylation were apparent with the L125R/W126E and L125R/E122L double mutants. The smear observed with the rescuing double mutants was confirmed to be because of heterogeneous glycosylation through the expression of rhodopsin in the presence of tunicamycin, a glycosylation inhibitor (not shown), suggesting correct intracellular trafficking. The other 10 double mutants were identical to L125R, displaying no glycosylated rhodopsin and an aggregation of misfolded rhodopsin (not shown). Transport to the cell membrane was further confirmed through confocal microscopy (Fig. 2C), which depicted the L125R/W126E (III), and L125R/E122L (IV) rhodopsin proteins properly transported to the cell membrane. Although SDS-PAGE suggested that the L125R/W126V double mutant did improve protein expression (Fig. 2B), confocal microscopy showed a failure to reach the plasma membrane (Fig. 2C, IV).

L125R Disturbs a Critical Glu122–His211 (TM III to TM V) Salt Bridge—Upon confirming that only changes to Trp126 and Glu122 (of eight residues in close proximity) resulted in an improvement of the L125R mutant, we commenced crystal structure-based analysis (Swiss PDB) to identify the molecular basis for the compensation. There are 18 possible conformations for the Arg residue at position 125. The Swiss PDB Viewer was utilized to perform calculations (see "Experimental Procedures") to determine the stability of each conformer. Supporting our experimental data, the most favorable conformation positioned the Arg toward both Trp126 and Glu122 (Fig. 5B). This conformation (number 18/18) had a formula score of −6, the lowest among the 18 conformations, which scored in a range from −6 to +66, with +12 being the median score. Further crystal structure analysis revealed that Trp126 functioned in stabilizing the putative Glu122–His211 salt bridge, between TM III and TM V, respectively (Fig. 5A). Modeling of the L125R mutant (Fig. 5B) confirmed that Arg125 induces a steric clash with both Glu122 and Trp126 (purple lines). Modeling of the energy-minimized L125R/W126D (model not shown) and L125R/W126E (Fig. 5C) double mutants showed that the negatively charged residue at position 126 could partially stabilize Arg125 by hydrogen bonding. Another model, of L125R/E122L (Fig. 5D), showed that stable hydrogen bonding between Arg125 and His211 may restore the critical interaction between TM III and TM V. Additionally, the now hydrophobic Leu119 would be more stable within the hydrophobic binding pocket, with no steric hindrance to Arg125.

Mutations at Ala164 Decrease 11-cis-Retinal Binding, Thermal Stability, Metarhodopsin II Formation, and Metarhodopsin II Stability—Having analyzed the L125R mutation in detail, we wanted to examine another mutation in this region (A164V) to determine whether more than one mutation may be

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**Table 1: Retinitis Pigmentosa perturbs interhelical interactions**

| RP mutant and double mutants | RP mutant and double mutants |
|-----------------------------|-----------------------------|
| L125R (TMIII)               | A164V (TMIV)                |
| L125R (TMIII) + E122L (TMIII) | A164V (TMIV) + F115A (TMIII) |
| L125R (TMIII) + E122Q (TMIII) | A164V (TMIV) + L119A (TMIII) |
| L125R (TMIII) + W126D (TMIII) | A164V (TMIV) + E122D (TMIII) |
| L125R (TMIII) + W126G (TMIII) | A164V (TMIV) + W126A (TMIII) |
| L125R (TMIII) + W126V (TMIII) | A164V (TMIV) + W126L (TMIII) |

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Note: The table shows the mutations made, and transmembrane helical locations, in compensating for the defect that arises from the defined RP mutation. Mutations were based upon crystal structure analysis. Correction of functional defects as described under "Results" were assessed.
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The data show that the A164V mutant has a significantly decreased absorbance ratio A280/A500 compared to wild-type rhodopsin. This decrease is due to the mutation at position 164. The A164V mutant also exhibits a decreased half-life of thermal stability at 50 °C compared to wild-type and A164V rhodopsin. The mean ± S.E. for wild-type measured for A164V (p < 0.05)(Fig. 4F).

A164V Also Disrupts the Critical Glu122-His211 (TM III to TM V) Salt Bridge—Using the crystal structure of rhodopsin, the proximity of Ala164 to Leu119 and Ile123 was confirmed to 3.7 and 3.3 angstroms, respectively. Leu119 and Ile123 are adjacent to Glu122, which in turn forms the aforementioned salt bridge with His211. When examining a crystal structure model of the A164V mutant, we observed that disruption of the interhelical interaction near Leu119 and Ile123 by the RP mutant A164V reduces the distance between these residues (Fig. 5E). The mutant brings the side chain of 164 to a distance of 2.5 and 2.9 angstroms from the side chains of 119 and 123, respectively. This is closer than the wild-type distances of 3.7 and 3.3 angstroms, respectively. This reduction in distance, in turn, leads to steric hindrance between the two helices, ultimately altering the position of Glu122 and breaking the critical Glu122-His211 salt bridge. The L119A mutant increases the distance between the 119 and 164 side chains from 2.5 (mutant) to 4.0 (double mutant) angstroms (Fig. 5F). Likewise, the I123A mutant restores the 123–164 side-chain distance from 2.9 (mutant) to the wild-type-like distance of 3.3 (double mutant) angstroms (Fig. 5G). In contrast to L125R, the disruption for A164V is less severe, because some of this mutant rhodopsin still binds retinal. We have, however, reproduced the L125R phenotype (total lack of 11-cis-retinal binding) by combining the A164V RP mutant with the site-directed mutant W126A.
Retinitis pigmentosa is a severe retinal degenerative disease that ultimately leads to blindness. We are intrigued how a single missense mutation within 348 amino acids can lead to such severe consequences. Our goal in the current study is to determine the molecular basis for the initiation of the functional defects, using compensatory mutations in combination with crystal structure-based molecular modeling. Currently rhodopsin is the only G protein-coupled receptor with which we have such precise capabilities. Through these studies, we may develop fundamental insights to the growing list of diseases that arise from missense mutations in G protein-coupled receptors. For our studies we hypothesize that, within each region where RP mutations are found, distinct structural components (e.g. interhelical interactions, salt bridge) are present that, if disrupted, initiate instability and misfolding. Using the severely misfolded L125R and the partially misfolded A164V (in TM III and TM IV, respectively) near the β-ionone ring of 11-cis-retinal, our intent was to establish the precise molecular defects initiated by these substitutions, and in so doing identify a possible target for the development of structural-based pharmacological therapy.

**Improvement of Folding in the L125R Rhodopsin Mutant with Double Mutants at Positions Trp<sup>126</sup> and Glu<sup>122</sup>**—We have reconfirmed the severity of the L125R RP mutation with its total lack of 11-cis-retinal binding, poor expression, endoplasmic reticulum retention, and protein aggregation (14, 22, 23). Additional analysis of this RP mutant, utilizing the crystal structure of rhodopsin, revealed that the native Leu<sup>125</sup> was positioned within an extremely hydrophobic environment (within 5 angstroms from Trp<sup>126</sup>, Leu<sup>128</sup>, Phe<sup>212</sup>, Pro<sup>215</sup>, Leu<sup>216</sup>, and Phe<sup>261</sup>) that also contained two charged residues, Glu<sup>122</sup> and His<sup>211</sup> (Fig. 5A). Previous studies have determined that the large transmembrane hydrophobic residues (Phe<sup>208</sup>, His<sup>211</sup>, Phe<sup>212</sup>, Phe<sup>261</sup>, and Trp<sup>265</sup>) in this region stabilize binding of the β-ionone ring of 11-cis-retinal (20, 27). We hypothesized that the introduction of L125R into this highly hydrophobic region induces steric and electrostatic clashes with nearby hydrophobic residues, resulting in their shift away from native conformations, disturbing the β-ionone ring attachment and protein folding. Although none of the 15 double mutations improved 11-cis-retinal binding, immunoblotting and confocal microscopy showed clear improvements in protein expression, trafficking, and stability. All changes at position 126 exhibited significantly increased protein levels; however, only the L125R/W126E significantly rescued rhodopsin glycosylation and transport to the cell surface (Fig. 2, B and C). Introduction of a smaller residue at position 126 was responsible in part for the improvement in detectable protein and post-translational processing, by relieving steric hindrance; however, charge neutral-
Critical Glu122–His211 (TM III to TM V) Salt Bridge

Crystal Structure Analysis Reveals That L125R Disturbs a Critical Glu122–His211 (TM III to TM V) Salt Bridge—Crystal structure analysis allowed us to support the experimental data that were obtained and predict further mutations and outcomes. Swiss PDB Viewer was utilized to predict energy-minimized conformations for each rhodopsin mutant. Supporting our experimental data, the lowest energy conformation positioned the Arg toward Trp126 (Fig. 5B). This model supported our data, which showed that the L125R mutant was partially neutralized by W126E (Fig. 2, B and C). Further crystal structure analysis revealed that Trp126 functioned in stabilizing a putative Glu122–His211 salt bridge, between TM III and TM V, respectively (Fig. 5A). Previous studies have hypothesized that Glu122 and His211 may be hydrogen-bonded in rhodopsin, because this pair is conserved throughout the rod visual pigments (28, 29). Modeling of the L125R mutant (Fig. 5B) confirmed that Arg125 induces a steric clash with Glu122 (purple line). This would result in the charged glutamic acid being forced away from its native conformation, thus preventing its interaction with His211. The strength of this study is highlighted by our ability to predict and subsequently prove that a L125R/E122L double mutant (Fig. 5D) could significantly improve rhodopsin protein expression, glycosylation, and trafficking (Fig. 2, B and C). In the absence of our mutagenesis or modeling, one would predict that the L125R (a positive charge) could have been neutralized by the native, adjacent Glu122 (a negative charge). Our results were able to make the paradoxical prediction that neutralization of the Glu122 could actually lead to improvement. Only with the compensatory mutations, in combination with detailed crystal structure analysis, could these important insights be determined.

A crystal structure-based model of L125R/E122L (Fig. 5D) further confirmed our observations. E122L is able to stabilize the L125R mutant by allowing the formation of more stable hydrogen bonding between Arg125 and His211, without competition from Glu122. Additionally, the now hydrophobic Leu122 would be more stable within the hydrophobic 11-cis-retinal binding pocket than an unbound and charged Glu122. These models strongly supported our hypothesis and data and confirmed that the L125R mutant interfered with the Glu122–His211 salt bridge. As indicated by the rhodopsin crystal structure and our experimental data, the residues Glu122, Trp126, and His211 interact with one another in a complex and yet highly conserved hydrogen-bonding network, which is further stabilized by a number of surrounding hydrophobic residues. L125R, a serious rhodopsin RP mutant, interferes with this hydrogen bonding and with the surrounding hydrophobic residues, resulting in rhodopsin misfolding, inability to bind 11-cis-retinal, formation of an alternate Cys185–Cys187 disulfide
bond, and ultimately opsin degradation.

Fourier transform infrared spectroscopy has provided strong experimental evidence for the Glu\textsuperscript{212}-His\textsuperscript{311} interaction. Beck et al. (28) postulated that this interaction occurs via an additional "group X." The crystal structure in combination with our data supports Trp\textsubscript{126} as group X. Furthermore, a study by Lin and Sakmar (30) determined that the indole side chain of Trp\textsubscript{126} is hydrogen-bonded to another residue or group in the rhodopsin dark-state. Interestingly, the rhodopsin crystal structure depicts the hydroxyl group of the Glu\textsuperscript{212} side chain hydrogen-bonded to the backbone carboxyl of His\textsuperscript{311} (2.9 Å) and potentially with other residues at this position as well. The carbonyl from the Glu\textsuperscript{212} side chain, however, may accept a direct hydrogen bond from either the His\textsuperscript{211} imidazole (4.3 Å) or the Trp\textsubscript{126} indole (3.2 Å) side chains or a water-mediated hydrogen bond from either residue. Glu\textsuperscript{212} may form hydrogen bonds with either residue, depending on the pH of the local environment and the protonation state of His\textsuperscript{311}. Introduction of the L125R results in disruption of the interaction between TM III and TM V, preventing 11-cis-retinal binding.

The TM III-TM V Interhelical Interaction Is Also Critical for the A164V RP Mutation in TM IV—After the initial L125R studies, we postulated that this critical TM III-TM V interaction may play an important role with other RP mutations in this region. UV-Vis spectroscopy of A164V confirmed previously published observations that showed reduced 11-cis-retinal binding (Fig. 3A) (20, 31). Even more apparent were substitutions of Ala\textsuperscript{164} to Ile and Leu, supporting the exquisite mass sensitivity of this position. We also show that the A164V mutant disturbs the thermal stability of rhodopsin as well as metarhodopsin II formation and stability (Figs. 3C and 4). Two double mutations, side-chain reductions in TM III (A164V/L119A, and A164V/I123A), showed significant restoration of chromophore formation toward that of the wild-type receptor (Fig. 3B).

Furthermore, the A164V/L123A double mutant improved the defect in rhodopsin thermal stability and restored defects in metarhodopsin II formation and stability (Figs. 3C and 4, C and F). Thus, an important interhelical interaction had been disrupted between TM IV and TM III. The L125R causes disruption at position Trp\textsubscript{126} in addition to the Glu\textsuperscript{212}-His\textsuperscript{311} salt bridge. Similarly, if A164V (disrupts the salt bridge) is combined with W126A, which destroys the stabilizing effect of the tryptophan, complete misfolding occurs with no chromophore formation. Thus the interaction between Glu\textsuperscript{212}, His\textsuperscript{311} and Trp\textsubscript{126} is crucial for correct rhodopsin folding and function. These results again highlight the effectiveness of using mutagenesis and functional characterization, in conjunction with the crystal structure of rhodopsin and computer modeling, to determine the structural perturbations arising from the RP mutations and the requirements for normal rhodopsin folding.

Structural Basis for Abnormal Disulfide Bonding—With the initiating molecular defect having been established for the two RP mutants, we are now able to establish the etiology for formation of the abnormal Cys\textsuperscript{115}-Cys\textsuperscript{175} disulfide bond observed with both L125R and A164V (14). The structural basis for this loss arises from an alteration in the relative positions of Cys\textsuperscript{110} and Cys\textsuperscript{175}, the Cys\textsuperscript{110} being on the intradiscal side of TM III (containing Leu\textsuperscript{2}, Glu\textsuperscript{3}, Ile\textsuperscript{4}, and Leu\textsuperscript{5}) and the Cys\textsuperscript{175} being on the loop extending from TM IV (containing Ala\textsuperscript{164}) and TM V (containing His\textsuperscript{311}) (Fig. 1). This disulfide bond is critical, both in protein stability and expression (32, 33), with a default alternative bond (Cys\textsuperscript{122}-Cys\textsuperscript{185}) forming with misfolded protein (14, 15). These results also establish the structural basis for coupling between formation of the transmembrane domain and the intradiscal domain as previously described (20). The presence of the L125R or A164V mutant thus initiates a cascade of events at more distal sites leading to protein instability and misfolding.

CONCLUSIONS

It continues to intrigue us how a single amino acid change, within a 348-residue protein, can lead to protein misfolding, endoplasmic reticulum retention, rod cell death, retinal destruction, and ultimately blindness. Moreover, this is a recurrent theme among many disease-causing missense mutations in protein. We hypothesize that misfolding arises from a "domino effect", initiated by localized structural events, leading to more distant defects (e.g. formation of abnormal disulfide bond), protein misfolding, retention in the endoplasmic reticulum, and cell death. We reconfirmed the severity of the L125R and A164V mutants, both located near the β-ionone ring of 11-cis-retinal. Through the development of compensatory mutations and crystal structure-based computer modeling, we show that the charged Arg group directly interacts with adjacent residues (Trp\textsubscript{126}) and disrupts a critical interhelical interaction between TM III and TM V (Glu\textsuperscript{212},His\textsuperscript{311} interaction). Similarly, the increased size at 164 from Ala to Val results in altered conformations for interacting residues and disruption of the Glu\textsuperscript{212}-His\textsuperscript{311} interaction. In the native opsin, this interaction is adjacent to the β-ionone ring and is therefore critical in maintaining correct structural conformation of the 11-cis-retinal binding pocket. Thus, compensatory mutations, combined with crystal structure-based molecular modeling, are powerful tools in determining mutant conformation. A molecular understanding of the initial structural perturbations caused by point mutations in membrane proteins, in addition to providing insight into the functional defects, may assist in the development of ligands to stabilize the protein and prevent aggregation.

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REFERENCES

1. Sakmar, T. P. (1998) Prog. Nucleic Acids Res. Mol. Biol. 59, 1–34
2. Unger, V. M., Hargrave, P. A., Baldwin, J. M., and Schertler, G. F. (1997) Nature 389, 203–206
3. Altenbach, C., Klein-Seetharaman, J., Cai, K., Khorana, H. G., and Hubbell, W. L. (2001) Biochemistry 40, 15493–15500
4. Altenbach, C., Cai, K., Klein-Seetharaman, J., Khorana, H. G., and Hubbell, W. L. (2003) Biochemistry 42, 15483–15492
5. Klein-Seetharaman, J., Hwa, J., Cai, K., Altenbach, C., Hubbell, W. L., and Khorana, H. G. (2001) Biochemistry 40, 12472–12478
6. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
7. Teller, D. C., Okada, T., Behnke, C. A., Palczewski, K., and Stenkamp, R. E. (2001) Biochemistry 40, 7761–7772
8. Stejnovic, A., and Hwa, J. (2002) Recept. Channels 8, 33–50
9. Berson, E. L., Rosner, B., Sandberg, M. A., Hayes, K. C., Nicholson, B. W., Weigel-DeFranco, C., and Willett, W. L. (1995) Arch. Ophthalmol. 113, 1456–1459
10. Li, T., Sandberg, M. A., Pawlyk, B. S., Rosner, B., Hayes, K. C., Dryja, T. P., and Berson, E. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11923–11928
11. Noorwez, S. M., Kuksa, V., Imanishi, Y., Zhu, L., Filipek, S., Palczewski, K., and Kaushal, S. (2003) J. Biol. Chem. 278, 14442–14450
12. Sahibzada, S., Munro, P. M., Lauther, P. J., and Cheetham, E. M. (2002) J. Cell Sci. 115, 2907–2918
13. Karnik, S. S., Sakmar, T. P., Chen, H. B., and Khorana, H. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8459–8463
14. Hwa, J., Klein-Seetharaman, J., and Khorana, H. G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4872–4876
15. Hwa, J., Reeves, P. J., Klein-Seetharaman, J., Davidson, F., and Khorana, H. G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1932–1935
16. Bownds, D. (1967) Nature 216, 1178
17. Wang, J. K., McDowell, J. H., and Hargrave, P. A. (1980) Biochemistry 19, 5111–5117
18. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8309–8313
19. Nathans, J. (1990) Biochemistry 29, 9746–9752
20. Hwa, J., Garriga, P., Liu, X., and Khorana, H. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10571–10576
21. Kaushal, S., and Khorana, H. G. (1994) Biochemistry 33, 6121–6128
22. Garriga, P., Liu, X., and Khorana, H. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4560–4564
23. Andres, A., Kosoy, A., Garriga, P., and Manyosa, J. (2001) Eur. J. Biochem. 268, 5696–5704
24. Kaushal, S., Ridge, K. D., and Khorana, H. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4024–4028
25. Oprian, D. D., Molday, R. S., Kaufman, R. J., and Khorana, H. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8874–8878
26. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
27. Creemers, A. F., Kühne, S., Boeve-Geurts, P. H., DeGrip, W. J., Lugtenburg, J., and de Groot, H. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9101–9106
28. Beck, M., Sakmar, T. P., and Siebert, F. (1998) Biochemistry 37, 7630–7639
29. Shieh, T., Han, M., Sakmar, T. P., and Smith, S. O. (1997) J. Mol. Biol. 269, 373–384
30. Lin, S. W., and Sakmar, T. P. (1996) Biochemistry 35, 11149–11159
31. Reeves, P. J., Hwa, J., and Khorana, H. G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1927–1931
32. Karnik, S. S., and Khorana, H. G. (1990) J. Biol. Chem. 265, 17520–17524
33. Davidson, F. F., Loewen, P. C., and Khorana, H. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4029–4033