Functional Interaction of Transmembrane Helices 3 and 6 in Rhodopsin

REPLACEMENT OF PHENYLALANINE 261 BY ALANINE CAUSES REVERSION OF PHENOTYPE OF A GLYCINE 121 REPLACEMENT MUTANT

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Replacement of a highly conserved glycine residue on transmembrane (TM) helix 3 of bovine rhodopsin (Gly121) by amino acid residues with larger side chains causes a progressive blue-shift in the \( \lambda_{\text{max}} \) value of the pigment, a decrease in thermal stability, and an increase in reactivity with hydroxylamine. In addition, mutation of Gly121 causes a relative reversal in the selectivity of opsin for 11-cis-retinal over all-trans-retinal. It was suggested that Gly121 plays an important role in defining the 11-cis-retinal binding pocket of rhodopsin (Han, M., Lin, S. W., Smith, S. O., and Sakmar, T. P. (1996) J. Biol. Chem. 271, 32330–32336). Here, we combined the mutant opsin G121L with second site replacements of four different amino acid residues on TM helix 6: Met257, Val258, Phe261, and Trp265. We show that the loss of function phenotypes of the G121L mutant described above can be partially reverted specifically by the mutation of Phe261, a residue highly conserved in all G protein-coupled receptors. For example, the double-replacement mutant G121LF261A has spectral, chromophore-binding, and transducin-activating properties intermediate between those of G121L and rhodopsin. This rescue of the G121L defects did not occur with the other second site mutations tested. We conclude that specific portions of TM helices 3 and 6, which include Gly121 and Phe261, respectively, define the chromophore-binding pocket in rhodopsin. The results are placed in the context of a molecular graphics model of the TM domain of rhodopsin, which includes the retinal-binding pocket.

G protein-coupled receptors, including the visual pigment rhodopsin, have been studied extensively by site-directed mutagenesis. In many cases, the biochemical phenotype of a mutant receptor involves the loss of a particular function. Unfortunately, a loss of function can be related either directly or indirectly to a particular amino acid replacement. It is often difficult to distinguish between direct and indirect effects. As a result, such studies often do not produce significant structural or mechanistic insights. Certain strategies have evolved to circumvent the problem of interpreting loss of function phenotypes. These include the construction of chimeric receptors (1, 2), the use of biophysical methods to assay defective mutants (3, 4), and the use of genetic selection methods, when available.

In the preceding paper (5), we presented a study of a highly conserved glycine residue on transmembrane (TM) helix 3 of bovine rhodopsin (Gly121). It was suggested that Gly121 played a role in defining the 11-cis-retinal-binding pocket of rhodopsin. Here, we show that the loss of function phenotypes of the Gly121 mutants can be partially reverted by the mutation of Phe261 on TM helix 6. This effect is specific to Phe261 and does not occur with other residues on TM helix 6 (Met257, Val258, and Trp265). The results show that specific portions of TM helices 3 and 6 in rhodopsin comprise specific retinal-protein contacts that define the chromophore-binding pocket. The present results are consistent with a model of rhodopsin based on data from electron microscopy studies (6), NMR and two-photon spectroscopy measurements (7–9), and a comparative analysis of G protein-coupled receptors (10). A molecular graphics model of the transmembrane domain of rhodopsin is presented that illustrates the relative proximity of the retinal chromophore to Gly121 and Phe261. Since Phe261 is highly conserved among all G protein-coupled receptors (11), these results may be relevant to understanding the molecular mechanisms of activation of G protein-coupled receptors.

EXPERIMENTAL PROCEDURES

TM helix 6 mutant genes were generated by substituting a 45-base pair Miul-Ndel restriction fragment with a synthetic duplex containing the desired codon alteration(s). Mutant G121L was prepared as described (5). The TM helix 3/6 double mutants were assembled from the single mutants by ligation of the appropriate EcoRI-SpeI or SpeI-ApoI restriction fragments. The nucleotide sequences of all cloned synthetic duplexes were confirmed by the chain terminator method for DNA sequencing of purified plasmid DNA using \( ^{35} \text{S} \text{dATP} \). The expression, purification, spectroscopic characterization, and biochemical assays of the mutant pigments were carried out as previously reported (5).

RESULTS

Spectral Properties of Single-replacement TM Helix 6 Mutant Pigments—Mutant opsin genes were prepared with single amino acid replacements at one of four sites in TM helix 6 of bovine rhodopsin, Met257, Val258, Phe261, and Trp265. These positions were chosen based on molecular graphics models of

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1 The abbreviations used are: TM, transmembrane; GTPyS, guanosine 5′-O-(thiotriphosphate); dATP-S, deoxyadenosine 5′-O-(thiotriphosphate).
bovine opsin and chicken red cone opsin (12), which indicated that an amino acid with a bulky side chain such as leucine substituted for Gly121 on TM helix 3 might be in potential van der Waals contact with either Met257, Val258, Phe261, or Trp265. Four Phe261 mutants were characterized, F261A, F261T, F261V, and F261W (Table I). In addition, mutants M257A, V258A, and W265Y were studied (Table II). The spectral properties, including $\lambda_{\text{max}}$ value, molar extinction coefficient ($\epsilon$), and spectral ratio (absorbance at 280 nm versus absorbance at the visible $\lambda_{\text{max}}$ value), were determined by UV-visible spectroscopy for each purified mutant. The $\lambda_{\text{max}}$ values of the mutant pigments were all within 2 nm of the value for rhodopsin, except for that of W265Y (485 nm). The stability of the mutant pigments in dodecyl maltoside detergent as judged by spectral ratio were also essentially normal. Only the mutant pigment F261W was somewhat unstable or defective in its ability to reconstitute with 11-cis-retinal. The results reported previously concerning Trp265 mutants (13, 14) are in good agreement with the present work.

Reactivity of Single-replacement TM Helix 6 Mutant Pigments with Hydroxylamine—In contrast to the case of the Gly121 mutant pigment reported in the previous paper (5), which reacted with hydroxylamine in the dark, each of the above-mentioned TM helix 6 single-replacement mutants was stable in the presence of hydroxylamine. The hydroxylamine stability of each of the mutants tested was indistinguishable from that of rhodopsin assayed under identical conditions.

Second Site Replacement of Phe261 Caused Reversion of the Gly121 Mutant Spectral Phenotype—The effects of Gly121 replacements were presented in detail in the preceding paper (5). A progressive increase in the size of the side chain of the substituting residue resulted in a progressive blue shift of the pigment $\lambda_{\text{max}}$ value, a progressive loss of stability of pigment in dodecyl maltoside as represented by an increased spectral ratio, and reactivity to hydroxylamine in the dark. As shown in Fig. 1 and Table I, each of these spectral phenotypes was reverted by the introduction of a second site replacement at position Phe261 that reduced the size of the residue 261 side chain. For example, the G121L mutant reconstituted very poorly with 11-cis-retinal and displayed a $\lambda_{\text{max}}$ value of 475 nm. The F261A mutant was essentially normal in its ability to reconstitute with chromophore and displayed a normal $\lambda_{\text{max}}$ value of 500 nm. Remarkably, the double mutant G121L/F261A reconstituted with chromophore to the same extent as F261A and displayed a $\lambda_{\text{max}}$ Value of 482 nm (Fig. 1).

The ability of the second site mutation at position Phe261 to rescue the defect in the G121L mutant depended on the residue substituting for phenylalanine. The G121L/F261W mutant did not show a reversion, and the G121L/F261T and G121L/F261V mutants had intermediate reversion when compared with that of mutant G121L/F261A (Fig. 1 and Table I). The effect of the Gly121 mutation on hydroxylamine reactivity was only slightly rescued by the second site F261A mutation (Table I). The $t_1/2$ values of mutant pigments G121L and G121L/F261A were 6.4 and 11.1 min, respectively, as compared with $>600$ min for

![Fig. 1. UV-visible absorption spectra of recombinant pigments. The spectra of mutant pigment G121L and four double-replacement mutants with second site mutations at position Phe261 are shown. Samples were treated with 11-cis-retinal and purified in dodecyl maltoside detergent buffer in darkness. The spectra are normalized to the absorbance at 280 nm of rhodopsin. Replacement of Phe261 by progressively smaller residues restores the ability of Gly121 mutants to bind 11-cis-retinal. The G121L/F261A mutant displays a $\lambda_{\text{max}}$ value of 482 nm, which is intermediate between those of G121L (475 nm) and rhodopsin (500 nm). The spectral ratio of mutant pigment G121L/F261A is similar to that of F261A and rhodopsin (Table I). The spectral properties of all recombinant pigments described in this report are presented in Tables I and II.](image-url)

**Table I**

| Pigment            | $\lambda_{\text{max}}$ (nm) | $\epsilon$ (M$^{-1}$ cm$^{-1}$) | Spectral ratio | Hydroxylamine reactivity ($t_1/2$) (min) |
|--------------------|-----------------------------|---------------------------------|----------------|----------------------------------------|
| Rho                | 500                         | 42.7                            | 1.73 ± 0.04 (6) | $>600^c$                              |
| G121L              | 475 ± 0.3 (7)               | 40.5 ± 1.7 (3)                  | 8.2 ± 1.1 (4)  | 6.4 ± 0.4 (3)                          |
| F261A              | 500 ± 0.4 (6)               | 41.6 ± 1.7 (3)                  | 1.90 ± 0.08 (3)| $>600$                                |
| G121L/F261A        | 482 ± 0.3 (6)               | 37.7 ± 1.7 (3)                  | 1.83 ± 0.10 (3)| 11.1 ± 1.3 (3)                        |
| F261V              | 500 ± 0.3 (3)               | 37.6 ± 1.7 (2)                  | 1.62 ± 0.03 (2)| $>600$                                |
| G121L/F261V        | 476 ± 0.3 (3)               | 41.4 ± 3.4 (2)                  | 2.53 ± 0.11 (2)| 1.1 ± 0.2 (3)                         |
| F261W              | 502 ± 0.7 (3)               | 41.3 ± 1.8 (2)                  | 1.99 ± 0.01 (2)| $>600$                                |
| G121L/F261W        | 477 ± 0.9 (4)               | 34.9 ± 0.1 (2)                  | 2.6 ± 0.6 (2)  | 1.5 ± 0.4 (3)                         |
| G121L/F261W        | 501 ± 0.4 (6)               | 46.5 ± 0.8 (3)                  | 3.1 ± 0.4 (2)  | $>600$                                |
| G121L/F261W        | 478 ± 0.7 (3)               | 41.5 ± 4.8 (2)                  | 11 ± 4 (2)     | 11.6 ± 4.5 (3)                        |
| G121L              | 461                         | NA                             | NA             | NA                                     |
| G121L/F261A        | 467 ± 0.2 (0)               | 3.47 ± 0.05 (2)                 | 0.43 ± 0.01 (2)|                                        |

$^a$ Molar extinction coefficient ($\epsilon$) was determined as described (5, 16).

$^b$ Spectral ratio is the ratio between the protein absorption and the pigment absorption determined as described (5).

$^c$ Decay of visible absorbance in the presence of 25 mM hydroxylamine at 20 °C was determined (5). A half-time of decay was determined from the best fit to a single-exponential decay function.

$^d$ Rhodopsin pigment was stable during up to 2 h of hydroxylamine treatment.

$^e$ Mutant G121L did not form a stable pigment in dodecyl maltoside upon incubation with 11-cis-retinal (5). A $\lambda_{\text{max}}$ value for G121L was deduced by subtracting the $\lambda_{\text{max}}$ value of mutant pigment G121L/F261A from that of G121L and adding the result to the $\lambda_{\text{max}}$ value of mutant G121W/F261A (all values in wave numbers).

$^f$ The $\epsilon$ value of G121W/F261A could not be measured accurately due to the presence of free retinal, which is generated by gradual hydrolysis of Schiff base during the purification procedure (5).
rhodopsin. The second site rescue of the spectral properties of the G121L mutant was not observed with the M257A, V258A, or W265Y mutations (Table II). This result shows the high specificity of the second site reversion property of the 261-position.

**Transducin Activation by the Single- and Double-replacement Mutants**—The ability of each of the mutants to catalyze guanine nucleotide exchange by transducin was assayed under a variety of conditions using a filter-binding assay method. For each mutant pigment reconstituted with 11-cis-retinal and purified in dodecyl maltoside detergent, light-dependent transducin activity was measured in solution. The activity values in Tables III and IV are presented relative to that of rhodopsin prepared and assayed in parallel. It is noteworthy that the F261A and F261T mutant pigments showed significant defects in light-dependent transducin activation. As shown in Fig. 2, the defect in the ability of F261A to activate transducin in dodecyl maltoside could be rescued by the second site G121L mutation. The double mutant G121L/F261T showed an activity phenotype similar to that of G121L/F261V (Table III).

The mutant opsins were also assayed in COS cell membranes. The measurement of constitutive activity was required as a control to be able to evaluate the measurement of the abilities of the mutant opsins to be activated directly by all-trans-retinal in the dark (15, 16). Several of the mutant opsins showed constitutive activity. Of the single-replacement mutants studied, only M257A displayed more than 10% constitutive activity. However, several of the double-replacement mutants were significantly active as shown graphically in Figs. 3 and 4 and numerically in Tables III and IV. The highest levels of activity were displayed by the double mutants G121L/F261V and G121L/M257A, which showed constitutive activities of 48 and 46%, respectively. Other mutants with high levels of constitutive activity included G121L/F261T (15%) and E113A/G121L (35%). For comparison, under the same assay conditions, mutant E113A, which was previously reported to be constitutively active (17), displayed 21% activity (Table IV).

As reported in the preceding paper (5), the G121L mutant opsin did not display constitutive activity but did show an unusually high level of activity in the presence of all-trans-retinal. Mutant F261A was the same as native opsin in constitutive activity and activity when incubated with all-trans-retinal in membranes (Table III). However, the double mutant G121L/F261A was intermediate between the two single mutants in its ability to activate transducin in the presence of all-trans-retinal (Table III and Fig. 3). This result shows that the F261A substitution can partially rescue the phenotype of the G121L mutant in terms of the specificity of the chromophore-binding pocket for all-trans-retinal.

A detailed summary of the activities in membranes of each single- and double-replacement mutant is presented in Figs. 3 and 4. The first salient result is that the combination of the G121L mutation with a second mutation at one of several sites, including Glu113, Met257, or Phe261 (but not Trp265), resulted in a double mutant opsin with significant constitutive activity. The second salient result is that the property of the G121L mutant to bind all-trans-retinal in membranes, which was described in the preceding paper (5), could be partially prevented by a second site replacement of Phe261 by a residue with a smaller side chain (i.e., F261A). This effect is specific to the Phe261 position and was not observed with second site mutations at Glu113, Met257, Val258, or Trp265.

**DISCUSSION**

The substitution of bulkier side chains for Gly1221 resulted in (i) progressive blue shift of the pigment \( \lambda_{\text{max}} \), (ii) increased rate of hydroxylamine reactivity of the pigment in the dark, and (iii) a slower rate of reconstitution with 11-cis-retinal and higher affinity for all-trans-retinal binding by the mutant opsin (5). These results provided insight into the isomer specificity of the chromophore-binding pocket and the integrity of the native chromophore-counterion interaction. We carried out the present study to evaluate the possibility that inter-residue interactions mediate the conformation of the binding pocket. We in-

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**Table II**

Spectral properties of control mutant pigments

| Pigment    | \( \lambda_{\text{max}} \) (nm) | \( \epsilon \times 10^3 \, M^{-1} \, \text{cm}^{-1} \) | Spectral ratio | Hydroxylamine reactivity (t_{1/2} min) |
|------------|---------------------------------|---------------------------------|----------------|-------------------------------------|
| Rho        | 500                             | 12.7                            | 1.73 ± 0.04 (6) | >600                                |
| M257A      | 501 ± 0.2 (4)                   | 40.2 ± 0.2 (2)                  | 1.89 ± 0.01 (2) | >600                                |
| G121L/M257A| 473 ± 1.5 (2)                   | NA                             | 14 ± 7 (3)      | NA*                                 |
| V258A      | 500 ± 0.3 (3)                   | 41.0 ± 0.8 (2)                  | 1.79 ± 0.08 (3) | >600                                |
| G121L/V258A| 473 ± 0.0 (3)                   | 41.3 ± 1.3 (2)                  | 13 ± 8 (3)      | NA*                                 |
| W265Y      | 485 ± 0.9 (4)                   | 43.4 ± 1.6 (2)                  | 2.01 ± 0.18 (2) | >600                                |
| G121L/W265Y| 477 ± 1.5 (2)                   | NA                             | 31 ± 5 (2)      | NA*                                 |
| E113A/G121L| NA                             | NA                             | NA*             | NA*                                 |

a Unstable even in the absence of hydroxylamine.

b No detectable pigment was formed by this mutant opsin.

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**Fig. 2.** The activation of transducin by mutant pigments G121L, F261A, and G121L/F261. The ability of each mutant to activate transducin was evaluated by a GTP-\( S \)-filter-binding assay. The amount of GTP-\( S \) bound to a filter is plotted as a function of time. A, light-dependent activities of mutant pigments regenerated with 11-cis-retinal and purified in dodecyl maltoside (DM) buffer are shown. The reactions were initiated by illumination. B, transducin activation by mutant pigments in COS cell membranes incubated with all-trans-retinal (ATR). Under the conditions of the assay, mutant F261A or rhodopsin (not shown) did not significantly activate transducin in the presence of all-trans-retinal. However, all-trans-retinal was able to stimulate transducin activation by mutant G121L. In addition, the activity of double-replacement mutant G121L/F261A was significantly decreased from that of mutant G121L. Normalized data for all mutants tested are presented in Tables III and IV.
Helix-Helix Interactions in Rhodopsin

TABLE III

| Pigments | Membranes | Opsin<sup>a</sup> | All-trans-retinal<sup>b</sup> | 11-cis-Retinal/light<sup>c</sup> | Dedecyl maltoside, 11-cis-retinal/light<sup>d</sup> |
|----------|-----------|------------------|-------------------------------|---------------------------------|-----------------------------------------------|
| Rho      | 0.9 ± 0.2 (5) | 14 ± 5 (4)       | 100                           | 100                             |
| G121L    | 1.9 ± 0.3 (3) | 56 ± 9 (5)      | 99 ± 12 (5)                   | 100 ± 2 (3)                     |
| F261A    | 1.0 ± 0.2 (3) | 12 ± 4 (3)      | 79 ± 7 (3)                    | 40 ± 2 (3)                      |
| G121L/F261A | 9.4 ± 3.8 (3) | 35 ± 11 (3)    | 105 ± 14 (3)                  | 81 ± 8 (3)                      |
| F261T    | 1.9 ± 0.5 (3) | 23 ± 11 (3)     | 81 ± 16 (3)                   | 16 ± 3 (3)                      |
| G121L/F261T | 13 ± 2 (3) | 44 ± 13 (3)     | 119 ± 26 (6)                  | 80 ± 6 (3)                      |
| F261V    | 7.6 ± 1.6 (3) | 43 ± 11 (3)     | 99 ± 14 (3)                   | 88 ± 9 (3)                      |
| G121L/F261V | 48 ± 5 (3) | 64 ± 10 (3)     | 94 ± 4 (3)                    | 59 ± 2 (3)                      |
| F261W    | 0.2 ± 0.0 (3) | 3 ± 1 (3)       | 120 ± 11 (3)                  | 98 ± 5 (3)                      |
| G121L/F261W | 4.8 ± 1.2 (3) | 48 ± 5 (3)     | 86 ± 19 (6)                   | 103 ± 10 (3)                    |

<sup>a</sup> The opsins all-trans activities, which are measured in membranes, are presented as percentage of the activity of each mutant measured in membranes after incubation with 11-cis-retinal and illumination.

<sup>b</sup> Light activity is reported as the percent of the light activity of rhodopsin measured under the same conditions: 427 ± 61 (n = 12) pmol of GTP·S bound per min, for a membrane aliquot containing 0.5 mg/ml total protein.

<sup>c</sup> Pigments were purified in dodecyl maltoside buffer and assayed at 10 °C. The activity is reported as the percent of the light activity of rhodopsin measured under the same conditions: 135 ± 1 (n = 3) pmol of GTP·S bound per min per pmol of pigment. The amount of pigment is determined by spectroscopy and the ε value given in Table I.

Fig. 3. The activation of transducin by mutant opsin G121L and G121L in combination with second site mutations at position 261. COS cell membranes were prepared from transiently transfected COS cells. The ability of each mutant to activate transducin was evaluated by a GTP·S filter-binding assay. Membranes alone (open bars) and membranes incubated with all-trans-retinal (ATR) in the dark (hatched bars) were assayed. Open bars represent constitutive activity. Each value is normalized to the percentage of its respective activity in light after regeneration with 11-cis-retinal in membranes. The mean and standard error of at least three independent measurements are shown. The data for all mutants are presented numerically in Table III.

Fig. 4. The activation of transducin by selected single- and double-replacement mutant opsins. COS cell membranes were prepared from transiently transfected COS cells. The ability of each mutant to activate transducin was evaluated by a GTP·S filter-binding assay. Membranes alone (open bars) and membranes incubated with all-trans-retinal (ATR) in the dark (hatched bars) were assayed. Open bars represent constitutive activity. Each value is normalized to the percentage of its respective activity in light after regeneration with 11-cis-retinal in membranes. The mean and standard error of at least three independent measurements are represented. The data for all mutants are presented numerically in Table IV.

Val<sup>258</sup> is conserved in the rhodopsins but is not generally highly conserved among either vertebrate or invertebrate pigments. Phe<sup>261</sup> is highly conserved among all visual pigments (except in the red cone pigments where it is replaced by a tyrosine and in some invertebrate rhodopsins where it is replaced by a tryptophan) and among nearly all G protein-coupled receptors (11, 18). Trp<sup>265</sup> is also highly conserved among all visual pigments (except in the blue pigments where it is replaced by a tyrosine) and among most G protein-coupled receptors.

The G121L mutant was combined with second site replacements to create a set of eight double mutants: G121L/F261A, G121L/F261T, G121L/F261V, G121L/F261W, G121L/M257A, G121L/V258A, G121L/W265Y, and E113A/G121L. The UV-visible spectra of the purified Gly<sup>121</sup>/Phe<sup>261</sup> mutants are presented in Fig. 1. There is a trend to reversion of the blue-shifted λ<sub>max</sub> value and high spectral ratio of the G121L mutant as the side chain at position 261 becomes smaller in the double-re-

Produced a series of second site mutations with smaller side chains, which might be expected to reverse the phenotypes of the G121L mutant.

The amino acid residues Val<sup>157</sup>, Ile<sup>219</sup>, Met<sup>257</sup>, Val<sup>258</sup>, Phe<sup>261</sup>, and Trp<sup>265</sup> were identified for study based on molecular graphics models of bovine rhodopsin. The side chains of these amino acids were closest (within about 3 to 7 Å) to the side chain Leu<sup>121</sup> in a model of the G121L mutant. Interestingly, four of these six amino acid residues are located on TM helix 6. The two amino acid residues not located on TM helix 6 were Val<sup>157</sup> (TM helix 4) and Ile<sup>219</sup> (TM helix 5). Val<sup>157</sup> is not well conserved among visual pigments and was not evaluated in the present study. Ile<sup>219</sup> is conserved among vertebrate and invertebrate visual pigments. However, replacement of Ile<sup>219</sup> by amino acids with smaller side chains did not rescue the G121L phenotype (not shown). Met<sup>257</sup> is conserved among all vertebrate visual pigments and a majority of invertebrate pigments.
placement mutants. The thermal stabilities of these pigments were also markedly improved from that of the G121L mutant. This "rescue" of the G121L spectral phenotype was not seen with second site replacements at positions Met257, Val258, or Trp265 (Table I). Therefore, it can be concluded that a specific role of Glu113 in stabilizing the Schiff base (16, 19, 20) and residues mutated in the present study include Gly121, Met257, Val258, and these 2 helices. Favorable steric interactions are necessary for any increased steric interaction caused by the leucine residue at position 121. In this model, TM helices 3 and 6 form a significant portion of the chromophore-binding pocket, and Gly121 and Phe261 are in close proximity.


table

| Pigments | Membranes |
|----------|-----------|
|          | Opsin     | All-trans-retinal | 11-cis-Retinal/light | Dedecyl maltoside, 11-cis-retinal/light |
| Rho      | 0.9 ± 0.2(5) | 14 ± 5(4) | 100 | 100 |
| E113A    | 21 ± 6(3) | 58 ± 12(3) | 100 ± 16(3) | 28 |
| E113A/G121L | 35 ± 8(3) | 93 ± 9(3) | 96 ± 20(3) | 93 |
| M257A    | 10 ± 1(3) | 112 ± 16(3) | 102 ± 3(3) | 22 ± 4(3) |
| G121L/M257A | 46 ± 4(3) | 92 ± 12(3) | 91 ± 3(3) | 27 ± 4(3) |
| V258A    | 0.6 ± 0.1(3) | 13 ± 2(3) | 189 ± 27(3) | 86 ± 2(3) |
| G121L/V258A | 2.0 ± 0.3(3) | 54 ± 3(3) | 218 ± 57(3) | 103 ± 6(3) |
| W265Y    | 2.0 ± 0.8(3) | 10 ± 5(3) | 70 ± 8(3) | 82 ± 2(3) |
| G121L/W265Y | 3.9 ± 0.8(3) | 76 ± 11(3) | 89 ± 14(3) | 112 ± 10(3) |

* The activity of mutant pigment E113A was previously reported (21).
* No stable pigment was detectable in dodecyl maltoside detergent.
* A small amount of pigment could be detected from a photobleaching-difference spectrum. The concentration of the pigment was determined assuming ε = 42.7 × 10³ M⁻¹cm⁻¹.


diagram

FIG. 5. Molecular graphics model of the membrane-embedded domain of rhodopsin. The positions of the TM helices are based on the projection structure of the protein (23). The helix assignments are from a primary structure analysis of the G protein-coupled receptor family (10). TM helix 3 is tilted and buried within the bundle of transmembrane helices. The chromophore Schiff base bond is at Lys296. Glu113 serves as the counterion to the protonated retinal Schiff base and is situated near the extracellular border of TM helix 3. Amino acid residues mutated in the present study include Gly121, Met257, Val258, Phe261, and Trp265. Substitution of a bulky group at position 121 may move TM helix 3 in the plane of the membrane away from TM helix 6. An essential Cys132-Cys175 disulfide bond at the intradiscal surface of helix 3 (34) may act as the pivot point for this motion and restrict vertical translation of TM helix 3 relative to the other helices. In the double mutant G121L/F261A, a smaller alanine residue at position 261 on TM helix 6 may compensate for any increased steric interaction caused by the leucine residue at position 121. In this model, TM helices 3 and 6 form a significant portion of the chromophore-binding pocket, and Gly121 and Phe261 are in close proximity.
the helix assignments and rotational orientations suggested by Baldwin (10). TM helices 4, 6, and 7 are oriented roughly perpendicular to the membrane plane, while TM helices 1, 2, 3, and 5 are tilted. The most striking feature of this packing arrangement is that helix 3 is surrounded by several of the other helices. The retinal chromophore is covalently attached to Lys269 and lies in the interior of the helix bundle with the β-ionone ring oriented toward TM helix 5. Results from cross-linking studies are consistent with the Cg carbons of the ionone ring being near Trp265 and Leu266 (24, 25). Trp265 is highly conserved among all visual pigments, and its role in forming the retinal-binding site and in the photoactivation mechanism of rhodopsin has been previously discussed (13). The two residues of interest in this study, Gly121 and Phe261, are oriented toward the protein interior and appear to be near to each other and to the retinal chromophore.

We suggested in the preceding paper (5) that the small volume of Gly121 facilitated packing interactions with the retinal. We also demonstrated that the phenotypes exhibited by Gly121 mutants were not observed with mutations at Ala117, one helical turn away. The marked difference in sensitivity toward substitution at positions 117 and 121 indicates that the interaction is specific and suggests that the retinal chromophore is positioned above Ala117 in the interior of the protein.

Phe261 belongs to a sequence of conserved aromatic residues, (F/Y/W)XXX(W/Y)XXY, which lines the face of TM helix 6 oriented toward the protein interior (26). In rhodopsin, the sequence includes Phe261, Trp265, and Tyr268. The results of this study and others on Trp265 indicate that the aromatic residues reside near the chromophore and play a role in the binding-pocket structure. In bacteriorhodopsin, a cluster of aromatic residues, Trp182, Tyr185, and Trp189 on TM helix 6, and Trp86 on TM helix 3, also form a large part of the retinal-binding pocket. Moreover, similar tripeptide aromatic sequences present in the visual pigments are conserved in the biogenic amine class of G protein-coupled receptors (adrenergic, dopaminergic, serotonergic, etc.) (11). The phenyalanine at the equivalent 261-position in these receptors is thought to be involved in stabilizing the positive charge on the ligand via interactions with its π orbital (27).

Recent experiments suggest that the motions of TM helices 3 and 6 (13, 28, 35, 36) may be involved in the conformational change of rhodopsin to its active receptor state in metarhodopsin II. These motions are thought to provide a mechanism for changing the structure of the cytoplasmic loops connecting helices 3 and 4 and helices 5 and 6, which are known to provide sites for transducin activation (29). Interestingly, we observed that a small side chain (alanine or threonine) substituted at the 261-position produced a mutant pigment that showed consistently lower light-dependent transducin activity in detergent and in membranes. This defect was rescued by a larger side chain at the Gly121 position. In fact, mutant pigments with large side chains at either or both positions (G121L/F261W, G121L/F261A,T,V) displayed transducin activity levels that in membranes. This result provides evidence for the cooperative and specific roles of TM helices 3 and 6 in both forming the chromophore-binding pocket and activating rhodopsin.

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