The Effects of Amino Acid Replacements of Glycine 121 on Transmembrane Helix 3 of Rhodopsin*  

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Rhodopsin is a member of a family of G protein-coupled receptors with seven transmembrane (TM) helices. In rhodopsin, Gly121 is a highly conserved amino acid residue near the middle of TM helix 3. TM helix 3 is known to be involved in chromophore-protein interactions and contains the chromophore Schiff base counterion at position 113. We prepared a set of seven single amino acid replacement mutants of rhodopsin at position 121 (G121A, Ser, Thr, Val, Ile, Leu, and Trp) and control mutants with replacements of Gly114 or Ala117. The mutant opsins were expressed in COS cells and reconstituted with either 11-cis-retinal, the ground-state chromophore of rhodopsin, or all-trans-retinal, the isomer formed upon receptor photoactivation. The replacement of Gly121 resulted in a relative reversal in the selectivity of the opsin apoprotein for reconstitution with 11-cis-retinal over all-trans-retinal in COS cell membranes. The mutant pigments also were found to be thermally unstable to varying degrees and reactive to hydroxylamine in the dark. In addition, the size of the residue substituted at position 121 correlated directly to the degree of blue-shift in the $\lambda_{\text{max}}$ value of the pigment. These results suggest that Gly121 is an important and specific component of the 11-cis-retinal binding pocket in rhodopsin.

Rhodopsin, the photoreceptor molecule of the retinal rod cell, is a member of the family of G protein-coupled seven transmembrane (TM)1 helix receptors (1). The photoactive chromophore of rhodopsin is 11-cis-retinal, which is covalently bound in the interior of the protein as a protonated Schiff base. Photoisomerization of retinal causes receptor activation. Recently, a projection map of the TM helices has been obtained with 6-Å resolution in the plane of the membrane using electron microscopy on two-dimensional crystals of rhodopsin (2, 3). A number of biochemical and biophysical studies indicate that TM helix 3 is crucial for the activation mechanism of rhodopsin. One critical residue on TM helix 3 is Glu113, which serves as the counterion to the protonated retinylidene Schiff base (4–6).

Deprotonation of the Schiff base (7, 8) and protonation of Glu113 (9, 10) occur in the formation of metarhodopsin II and are important in forming light-activated rhodopsin. Three conserved residues are located at the cytoplasmic end of TM helix 3, Glu113, Arg120, and Tyr122, which are involved in transducin binding (11, 12). A conserved cysteine residue, Cys187, is located at the intradiscal end of TM helix 3 and forms a disulfide link to Cys187 (13). In addition, changes in the environments of Glu113 and Trp126 have been shown to occur upon formation of metarhodopsin II (14, 15).

In order to understand the mechanisms of spectral tuning and receptor photoactivation in the visual pigments, it is vital to gain additional information about the specific protein-protein and chromophore-protein contacts that define the chromophore-binding pocket in the ground state and how these contacts are affected by chromophore isomerization. There have been several models for how the retinal-protonated Schiff base interacts with Glu113 on TM helix 3 (16–18). In the most recent model, NMR constraints were used to position the chromophore in the interior of rhodopsin, which resulted in retinal situated between TM helices 3 and 6 with the $\beta$-ionone ring oriented toward TM helix 5 (19, 20). This binding site model suggests close interactions of the retinal with Gly121, which is strictly conserved in all visual pigments (21).

We show that site-directed mutation of Gly121 causes a decrease in thermal stability of the resulting pigment and an increase in reactivity with hydroxylamine. In addition, the size of the residue substituted at position 121 correlates remarkably well to the degree of blue-shift in the $\lambda_{\text{max}}$ value of the pigment. Furthermore, replacement of Gly121 results in a relative change of opsin selectivity for reconstitution with 11-cis-retinal over all-trans-retinal. In the following article (22), we show that the phenotypes of the Gly121 mutants described in this report can be reverted by the mutation of a specific residue, Phe261, on TM helix 6.

EXPERIMENTAL PROCEDURES  

Materials—Sources of reagents and materials have been previously reported (4, 15, 23, 24).

Construction of Rhodopsin Mutant Genes—Site-directed mutagenesis was performed using restriction fragment replacement (25) in a synthetic gene of rhodopsin (26), which had been cloned into the expression vector as described previously (27). All TM helix 3 mutant genes were generated by substituting a 63-base pair SpeI-RsaI restriction fragment with a synthetic duplex containing the desired codon alteration. The nucleotide sequences of all cloned synthetic duplexes were confirmed by the chain terminator method for DNA sequencing of purified plasmid DNA using [$^{35}$S]dATP or $[^{32}$P]dATP.

Expression and Preparation of Rhodopsin Mutants—Opsin genes were expressed in COS-1 cells as described previously (23, 27, 28) except that a lipofection procedure was employed in place of the DEAE-dextran transfection procedure. The cells were harvested and treated either with 11-cis-retinal followed by pigment purification in dodecyl maltoside as described (4) or were subjected to membrane preparation as described without chromophore regeneration (29). The pellet of COS...
cell membranes from a single 10-cm plate was resuspended in 0.5 ml of buffer and stored at −80 °C in 80-μl aliquots.

UV-visible Absorption Spectroscopy of Mutant Pigments—Spectroscopy was performed on a λ-19 Perkin-Elmer spectrophotometer at 25 °C on purified samples unless otherwise specified. The molar extinction coefficient (ε) of each mutant was determined by an acid denaturation method (4). Each ε value was calculated from the formula, ε = (A / Amax) 1/cm, where A was the absorbance at the λmax value, Amax was determined at 440 nm after acid denaturation, and εmax was the molar extinction of rhodopsin (4.27 × 10^5 M−1 cm−1). The spectral ratio, which is used as a measure of pigment yield and stability, is defined as Amax divided by A at the visible λmax value after correction for any difference of the mutant pigment ε values as described above.

Transducin Activation Assay—A radionucleotide filter-binding assay, which monitors the light-dependent guanine nucleotide exchange by transducin, was carried out at 10 °C as described previously (30). For pigment samples purified in dodecyl maltoside, pigment concentration was determined before the assay by visible spectroscopy according to the ε values given in Tables I and II. The assay mixture consisted of 4 μM transducin, 3.0 mM purified pigment, and 20 μM [35S]GTPγS in 50 μl of assay buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 4 mM MgCl2, 1 mM dithiothreitol). The activity assays of mutant opsins in COS cell membranes were performed as follows. Membrane aliquots were thawed at room temperature, and 1 μl of 11-cis-retinal (7.5 mM) or all-trans-retinal (4.7 mM) ethanol solution was incubated with 30 μl of membrane suspension in the dark at room temperature for 1 h before assay. The activity assays refer to any basal activity of a mutant in the absence of chromophore. Light activity refers to light-dependent transducin activation caused by samples incubated with 11-cis-retinal. All-trans-retinal activity refers to the light-independent transducin activation caused by opsins samples incubated with all-trans-retinal.

Action of Mutant Pigments with Hydroxylamine—The rates of hydroxylamine reaction with mutant pigments were determined in darkness at pH 7.0 as described previously (31). The conditions for the reaction at 20 °C were 25 mM hydroxylamine, 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 0.1% dodecyl maltoside.

Regeneration Kinetics of Mutant Opsin G121L with Retinals in Membranes—The regeneration time courses of mutant G121L with 11-cis-retinal or all-trans-retinal in COS cell membranes was measured by monitoring the initial rate of transducin activation at various periods after the addition of retinal to membrane aliquots. Retinal, 11-cis or all-trans (2 μM of 25 μM ethanol solution), was mixed with 60 μl of COS cell membrane suspension containing mutant opsin G121L in darkness and incubated at room temperature. At various times after retinal addition, aliquots (5 μl) were removed and immediately assayed by the filter-binding method to determine transducin activity. Samples regenerated with 11-cis-retinal were illuminated to start the reaction. Samples regenerated with all-trans-retinal were assayed under dim red light. The initial rate (pmol of GTP·γ·S bound per min) of the transducin activation determined from a linear regression analysis was then plotted as a function of the time of incubation. Activities at time equals 0 were opsin activity without added chromophore. The experimental data were fitted by an exponential-rise function of the form, y = a(1 − exp(−bx)) + c.

RESULTS

Spectral Properties of TM Helix 3 Mutants—Mutant opsin genes were prepared with single amino acid replacements at one of three sites in TM helix 3 of bovine rhodopsin, Gly114, Ala117, and Gly121 (Fig. 1). Each of the mutant opsin genes was transiently transfected into COS-1 cells. Each expressed opsin was regenerated with the 11-cis-retinal chromophore, and the mutant pigment was purified after solubilization with dodecyl maltoside detergent according to an established immunoaffinity absorption procedure (4, 31, 32).

The set of Gly121 mutants studied in detail included G121A, G121S, G121T, G121V, G121I, G121L, and G121W. The UV-visible spectrum of each of these mutants after purification in dodecyl maltoside is shown in Fig. 2. The spectral properties, including λmax value, molar extinction coefficient (ε), and spectral ratio (absorbance at 280 nm versus absorbance at the visible λmax value), were determined by UV-visible spectroscopy for each purified mutant. The results of the spectral properties of the Gly121 mutants are summarized in Table I. Replacement of Gly121 by alanine or serine had little effect on the spectral properties of the resulting mutants when compared with those of rhodopsin. Mutant pigment G121T displayed a blue-shifted λmax value (483 nm) and an increased spectral ratio (1.4) since acid treatment did not shift the 380 nm peak to 440 nm (not shown). The G121W mutant did not form a stable pigment under the conditions of the purification. However, the λmax value of mutant G121W can be inferred to be 461 nm as described in Table I. The blue shift of the visible λmax values induced by mutation at the 121-position was linearly correlated.
Rhodopsin Helix 3 Mutants

Spectral properties of the Gly121 mutant pigments

The mutant pigments were prepared in dodecyl maltoside as described previously (23, 31, 50). Values are given as means ± S.E. (n). Rho, rhodopsin; NA, not applicable.

| Pigment   | λ<sub>max</sub> (nm) | ε<sub>280</sub> × 10<sup>3</sup> M<sup>−1</sup> cm<sup>−1</sup> | Spectral ratio<sup>b</sup> | Hydroxylamine reactivity<sup>c</sup> (t<sub>1/2</sub>) min |
|-----------|----------------------|-----------------|--------------------------|------------------|
| Rho       | 500                  | 42.7            | 1.73 ± 0.04 (6)           | >600<sup>d</sup>  |
| G121A     | 498 ± 0.7 (4)        | 44 ± 0.9 (4)    | 1.82 ± 0.07 (4)           | 175 ± 53 (3)     |
| G121S     | 497 ± 0.4 (4)        | 43 ± 1.8 (3)    | 1.8 ± 0.1 (2)             | 43 ± 2 (3)       |
| G121T     | 483 ± 0.6 (5)        | 48 ± 4 (2)      | 2.5 ± 0.1 (2)             | 9.2 ± 1.7 (4)    |
| G121V     | 477 ± 0.5 (6)        | 42 ± 3 (2)      | 1.82 ± 0.04 (4)           | 2.6 ± 0.8 (3)    |
| G121I     | 475 ± 2.2 (5)        | NA              | 4.9 ± 0.9 (4)             | <0.5             |
| G121L     | 475 ± 0.3 (7)        | 40 ± 2 (3)      | 8.2 ± 1.1 (4)             | 6.4 ± 0.4 (3)    |
| G121W     | 461                  | NA              | NA                       | NA               |
| G121L/F261A|         | 0.3(7) 40       | 1.1(4) 6.4               |                  |
|          | G121T/F261A         | 2.2(5) NA       | 3(2) 1.82               |                  |
|          | G121V/F261A         | 4(2) 2.5        | 4(2) 2.5                 |                  |
|          | G121I/F261A         | 0.9(4) 1.82     | 1.8(3) 1.8               |                  |
|          | G121L/F261A         | 0.6(5) 42       | 0.1(2) 43                |                  |
|          | G121W/F261A         | 0.7(4) 44       | 0.07(4) 175              |                  |

<sup>a</sup> Molar extinction coefficient (ε) was determined as described under “Experimental Procedures.”
<sup>b</sup> Spectral ratio is the ratio between the protein absorption and the pigment absorption as determined by the formula A<sub>280</sub>/A<sub>λ<sub>max</sub></sub> × ε<sub>280</sub>.
<sup>c</sup> Decay of visible absorbance in the presence of 25 mM hydroxylamine at 20 °C was determined (Fig. 4). A half-time of decay was calculated from the best fit to a single-exponential decay function.
<sup>d</sup> Rhodopsin pigment was stable during up to 2 h of hydroxylamine treatment.

The ε value of G121I 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 (Fig. 2).

Mutant G121W did not form a stable pigment in dodecyl maltoside upon incubation with 11-cis-retinal (Fig. 2). A λ<sub>max</sub> value of mutant pigment G121L/F261A from that of G121L and adding the result to the λ<sub>max</sub> value of mutant G121W/F261A (all values in wave numbers) (22). The λ<sub>max</sub> value of pigment G121W can also be estimated from the linear correlation between the λ<sub>max</sub> value and the size of the side chain at position 121 (excluding G121W) (Fig. 3). This approach results in a λ<sub>max</sub> value of 465 nm for G121W.

with the volume of the side chain introduced (Fig. 3).

All of the Gly<sup>121</sup> mutant opsins except G121W were expressed in transiently transfected COS cells at approximately the same levels as rhodopsin. G121W expression was about 70 ± 4% of that of rhodopsin as judged by the A<sub>280</sub> value of immunoaffinity purified material from COS cells solubilized in dodecyl maltoside.

Sets of mutants with single amino acid replacements at Gly<sup>114</sup> or Ala<sup>117</sup> were prepared as controls for the Gly<sup>121</sup> mutants. Both Gly<sup>114</sup> and Ala<sup>117</sup> are predicted to be on the same helical face and are two and one helix turns toward the intradiscal end of TM helix 3, respectively (Fig. 1B). These residues should also be in close proximity to the retinal chromophore. In fact, a carboxylic acid group replacement at position 117 has been shown to be able to substitute for the Schiff base countereion of position 121 and reactivity is shown in Fig. 4. Each of the Ala<sup>117</sup> single replacement mutants, which included A117G, A117V, A117I, A117M, and A117W, formed a stable pigment with a spectral ratio similar to that of rhodopsin prepared under identical conditions. The λ<sub>max</sub> values of these mutants were also close to 500 nm, ranging from a slight blue shift to 496 nm for A117G to a slight red shift to 504 nm for A117W (Table II). Whereas bulky side chain replacements were not well tolerated at positions 114 or 121, at position 117 even the replacement of Ala<sup>117</sup> by a tryptophan resulted in a stable pigment.

Reactivity of Mutant Pigments with Hydroxylamine—The Schiff base in rhodopsin is remarkably stable in the presence of hydroxylamine in darkness but reacts rapidly upon illumination. Amino acid replacements that affect the Schiff base environment have been shown to decrease the stability of mutant pigments in the presence of hydroxylamine (4, 28). For example, mutant pigment E115Q was shown to react with hydroxylamine even in the dark (4). Each of the TM helix 3 mutant pigments described above was treated with hydroxylamine in darkness to probe Schiff base stability (Fig. 4). Rhodopsin did not react with hydroxylamine under the conditions of the experiment during 60 min in the dark. Each of the Gly<sup>121</sup> mutant pigments reacted with hydroxylamine in the dark. However, the relative reactivity among the mutant pigments varied substantially. An inverse correlation between residue size at position 121 and reactivity is shown in Fig. 4. G121A was moderately reactive, whereas G121V reacted rapidly. Mutant pigment G121I reacted too rapidly to measure accurately. The experimental data points fit well to a single-exponential decay function. According to these values, mutant pigment G121L reacted with hydroxylamine at least 100-fold more rapidly than rhodopsin. Mutant pigment G121W was even more reactive than G121L. The remaining Gly<sup>121</sup> mutant pigments displayed in-
membranes. These experiments were carried out to determine whether or not any of the mutant opsins was constitutively active (37). None of the Gly121 mutant opsins displayed significant constitutive activity (Table III). Under the same assay conditions, mutant E113A, which is known to be constitutively active, displayed greater than 20% activity (22).

Activation of Gly121 Mutant Opsins by All-trans-retinal in Membranes—Certain mutant opsins, and even native opsin to a lesser extent, are able to be activated directly by all-trans-retinal in the dark (4, 38, 39). This activity depends on the ability of all-trans-retinal to enter the chromophore-binding pocket of the opsin or mutant opsin (40). Transducin activation by Gly121 mutant opsins in COS cell membranes incubated with all-trans-retinal was determined. The time course of GTPγS binding is shown for rhodopsin, G121A, G121V, and G121L (Fig. 5), and the relative rates are listed in Table III. Incubation with all-trans-retinal enhanced transducin activity depending on the amino acid replacement at position 121. The activity of each of the seven Gly121 mutants in membranes incubated with all-trans-retinal was normalized to the percentage of its respective activity in light after regeneration with 11-cis-retinal of aliquots of the same membrane samples (Fig. 6). Even though none of the mutant opsins displayed constitutive activity, they were able to bind all-trans-retinal in membranes as judged by the relative amounts of transducin activity shown in Fig. 6. The transducin activation rates were assayed after incubation with all-trans-retinal for 1 h, which was shown to be a sufficient time for retinal binding to reach equilibrium (Fig. 7).

Regeneration Kinetics of Mutant Opin G121L in Membranes—The time course of the regeneration of 11-cis-retinal into G121L in COS cell membranes was monitored by monitoring the initial rate of light-dependent transducin activity as a function of time after the addition of 11-cis-retinal (Fig. 7). The kinetics of 11-cis-retinal regeneration with the G121L apoprotein followed an exponential rise with a rate constant of 0.04 min⁻¹. This rate constant is ~30 times smaller than the kinetics observed with native opsin. The kinetics of reconstitution of all-trans-retinal into G121L were also monitored (Fig. 7). The increase in activity as a function of time after the addition of all-trans-retinal to membranes containing G121L apoprotein can be approximated as a single-exponential rise with a rate constant of 1.1 min⁻¹. All-trans-retinal enters the chromophore-binding pocket to form an active species with a rate constant of approximately 30 times greater than that for 11-cis-retinal. In COS cell membranes, all-trans-retinal reconstitution with G121L reached completion almost immediately (within 2 min). In contrast, the half-time of reconstitution of G121L with 11-cis-retinal was approximately 19 min. Although at saturation, the level of activity of the all-trans-retinal reconstituted G121L mutant was lower (56%) than that of the 11-cis-retinal reconstituted G121L mutant after photolysis, it was

TABLE II
Spectral properties of helix 3 control mutants

| Pigment | λ<sub>max</sub> (nm) | ε (× 10<sup>3</sup> M⁻<sup>1</sup> cm⁻<sup>1</sup>) | Spectral ratio | Hydroxylamine reactivity (t<sub>1/2</sub>) |
|---------|---------------------|-----------------------------|----------------|----------------------------------|
| Rho     | 500                 | 42.7                        | 1.73 ± 0.04 (6) | >600                            |
| G114A*  | 497 ± 0.6 (4)       | 39.2 ± 1.3 (2)              | 1.59 ± 0.03 (2) | 172 (1)                         |
| A117G   | 496 ± 0.2 (5)       | 41.3 ± 1.2 (3)              | 1.74 (1)       | >600                            |
| A117V   | 498 ± 0.5 (5)       | 42.4 ± 1.9 (3)              | 1.75 ± 0.06 (2) | >600                            |
| A117I   | 500 ± 0.5 (4)       | 41.1 ± 1.9 (3)              | 1.73 (1)       | >600                            |
| A117M   | 502 ± 0.7 (3)       | 41.8 ± 1.3 (3)              | 1.76(1)        | >600                            |
| A117W   | 504 ± 0.7 (3)       | 39.3 ± 0.8 (3)              | 1.75 (1)       | 19 ± 2 (2)                      |

* Mutants G114V, Ile, Met, and Trp were expressed at very low levels and did not reconstitute in the presence of 11-cis-retinal to form pigments.

FIG. 4. Rates of hydroxylamine reaction with mutant pigments in darkness. Absorbance at the λ<sub>max</sub> value of each mutant pigment is scaled to 1.0 at time equals 0, before the addition of hydroxylamine. The abscissa values represent the time after addition of hydroxylamine. The symbols represent experimental data points as relative absorption values normalized to their absorption at time equals 0. The curves represent the single exponential decay fits to the data. Rhodopsin (Rho) did not react with hydroxylamine under these conditions in darkness during the 60 min of the experiment, and the line represents the linear regression fit derived from the data points. Pigment mutant G121L reacted too rapidly to be measured accurately (i.e. t<sub>1/2</sub> < 0.5 min) and is not shown. The half-time of decay (t<sub>1/2</sub>) values for each mutant are presented in Table I.

Intermediate reactivities that generally increased as the size of the residue at position 121 increased from alanine to leucine. The half-time of decay (t<sub>1/2</sub>) values for each Gly<sup>121</sup> mutant are presented in Table I.

Transducin Activation by Purified Gly<sup>121</sup> Mutants—The ability of each of the Gly<sup>121</sup> mutants to catalyze guanine nucleotide exchange by transducin was assayed under a variety of conditions using a filter-binding assay method (30). 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 are presented in Table III relative to that of rhodopsin prepared and assayed in parallel. The level of light-dependent activity in Table III represents the value normalized for the amount of pigment based on visible spectroscopy and molar extinction as presented in Table I. Three of the mutant pigments were able to activate transducin at an approximately normal level. Three mutant pigments were as much as 15% defective in light-dependent transducin-activating ability in solution, G121S, G121T, and G121V.

Characterization of Gly<sup>121</sup> Mutant Opsin Activity in Membranes—The Gly<sup>121</sup> mutant opsins were assayed in COS cell membranes. These experiments were carried out to determine whether or not any of the mutant opsins was constitutively active (37). None of the Gly<sup>121</sup> mutant opsins displayed significant constitutive activity (Table III). Under the same assay conditions, mutant E113A, which is known to be constitutively active, displayed greater than 20% activity (22).

Activation of Gly<sup>121</sup> Mutant Opsins by All-trans-retinal in Membranes—Certain mutant opsins, and even native opsin to a lesser extent, are able to be activated directly by all-trans-retinal in the dark (4, 38, 39). This activity depends on the ability of all-trans-retinal to enter the chromophore-binding pocket of the opsin or mutant opsin (40). Transducin activation by Gly<sup>121</sup> mutant opsins in COS cell membranes incubated with all-trans-retinal was determined. The time course of GTPγS binding is shown for rhodopsin, G121A, G121V, and G121L (Fig. 5), and the relative rates are listed in Table III. Incubation with all-trans-retinal enhanced transducin activity depending on the amino acid replacement at position 121. The activity of each of the seven Gly<sup>121</sup> mutants in membranes incubated with all-trans-retinal was normalized to the percentage of its respective activity in light after regeneration with 11-cis-retinal of aliquots of the same membrane samples (Fig. 6). Even though none of the mutant opsins displayed constitutive activity, they were able to bind all-trans-retinal in membranes as judged by the relative amounts of transducin activity shown in Fig. 6. The transducin activation rates were assayed after incubation with all-trans-retinal for 1 h, which was shown to be a sufficient time for retinal binding to reach equilibrium (Fig. 7).

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**DISCUSSION**

The orientations and assignments of the seven TM helices of rhodopsin have been proposed based on electron microscopy of rhodopsin (2) and sequence alignment and comparison of relevant biochemical and mutagenesis studies from a large number of G protein-coupled receptors (41). These studies have provided a low resolution model for the receptor and a conceptual framework for considering specific residues in the primary structure for mutagenesis studies. Information about the structure of the retinal chromophore in rhodopsin and how it is situated with respect to the seven-helical bundle has also come from chemical, biophysical, and molecular biological approaches (16, 17, 19, 42–48). The salient point to emerge from these studies has been the model of a neutral chromophore-binding pocket (4, 6, 49) with significant chromophore-opsin interactions arising from amino acid residues on TM helices 3 and 6 (4–6, 15, 50). In addition, NMR studies using isotopically labeled retinals in combination with molecular orbital calcula-

**Fig. 5. Transducin activation by Gly\(^{121}\) mutant pigments in COS cell membranes incubated with all-trans-retinal.** COS cell membranes containing the mutant opsin were prepared from transfected COS cells. 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 after the addition of membranes to the assay mixture. *rho*, rhodopsin. The data for all mutants are presented in Table III.

**Fig. 6. Transducin activation by Gly\(^{121}\) mutant pigments in COS cell membranes incubated with all-trans-retinal.** The activity of each Gly\(^{121}\) mutant in membranes incubated with all-trans-retinal is normalized to the percentage of its respective light-dependent activity after regeneration with 11-cis-retinal. The mean and standard error of at least three independent measurements are represented. *Rho*, rhodopsin. The data for all mutants are presented in Table III.

| Membranes | Opsin | All-trans-retinal | 11-cis-retinal (light) | Dodecyl maltoside, 11-cis-retinal (light) |
|-----------|-------|-----------------|------------------------|----------------------------------|
|           | \(\%^a\) | \(\%^a\) | \(\%^b\) | \(\%^c\) |
| Rho       | 0.9 ± 0.2 (5)   | 14 ± 5 (4)   | 100                    | 100     |
| G121A     | 1.6 ± 0.3 (3)   | 26 ± 2 (3)   | 101 ± 2 (3)           | 101 ± 7 (3) |
| G121S     | 0.9 ± 0.2 (3)   | 47 ± 6 (3)   | 82 ± 1 (3)            | 78 ± 7 (3) |
| G121T     | 0.3 ± 0.1 (3)   | 21 ± 3 (3)   | 82 ± 18 (3)          | 80 ± 6 (3)  |
| G121V     | 0.4 ± 0.1 (3)   | 33 ± 5 (3)   | 94 ± 21 (3)          | 83 ± 5 (3)  |
| G121I     | 1.0 ± 0.4 (3)   | 50 ± 8 (3)   | 81 ± 14 (3)          | 90 ± 1 (3)  |
| G121L     | 1.9 ± 0.3 (3)   | 56 ± 9 (5)   | 99 ± 12 (5)          | 100 ± 2 (3) |
| G121W     | 2.3 ± 0.4 (3)   | 47 ± 6 (6)   | 76 ± 12 (6)          | NA       |

\(^a\) The opsin and all-trans activities, which were measured in COS cell membrane preparations at room temperature, are presented as percentage of the activity of each mutant measured in aliquot of the same preparations after incubation with 11-cis-retinal and illumination. Values are given as mean ± S.E. (n). None of the mutant pigments was constitutively active.

\(^b\) 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 sample containing 0.5 mg/ml total protein.

\(^c\) 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 in a 100 \(\mu\)l reaction. The amount of pigment is determined its absorption at \(\lambda_{max}\) and the \(\varepsilon\) value given in Table I. NA, not applicable.

Significantly higher than that of rhodopsin (14%) measured in parallel (Table III).
fitted to an exponential-rise function of the form, all-transducin activation in darkness at a given time after the addition of hydroxylamine and its size dependence indicates that the Gly121

respectively.

c

c

c

c

After 11-cis-retinal addition bands of purified G121V, G121I, and G121L mutants decayed at least 1000-fold more rapidly than rhodopsin under identical conditions in the dark. In addition, it should be noted that the mutants with branched carbon chains at the 121-position (G121V and G121I) were less stable than G121L, even when the side chain volumes were smaller or comparable. The reactivity of a Schiff base with hydroxylamine is affected by the intrinsic pH of the Schiff base as well as its accessibility to solvent and to reactant. The general result of dark reactivity of every Gly121 replacement mutant with hydroxylamine and its size dependence indicates that the Gly121

mutant pigments are unstable due to a sterically induced perturbation of chromophore-opsin structure, which alters the Schiff base environment.

The progressive blue-shift of the \( \lambda_{\text{max}} \) values in Gly121 mutants with bulkier side chains is consistent with the idea that a steric perturbation at position 121 can disrupt the normal interaction between the retinal chromophore and its counterion at Glu113. This conclusion is not unreasonable since Glu113 is located approximately two and one-half helix turns below Gly121 and given the unusual geometry of the Glu113-chromophore ion pair (18). The altered counterion-chromophore interaction can presumably render the Schiff base more accessible to the solvent and to hydroxylamine in the dark and also change its pH (4, 17). Therefore, given the fact that Gly121 mutant opsins are able to bind 11-cis-retinal and to form functional pigments in membranes, we conclude that the instability of the Gly121 mutant pigments purified in detergent is a result of greater susceptibility of the protonated Schiff base linkage to hydrolysis.

Bovine opsin may become constitutively active secondary to mutation of a number of individual amino acid residues, including Glu113, Glu134, and Lys296 (37, 51), which have also been shown to be involved in the mechanism of rhodopsin photoactivation (40). The mutation presumably perturbs the equilibrium of the apoprotein population between inactive and active conformations and shifts it toward the active state (37, 52). This model explains the increased binding of all-trans-retinal, the equivalent of an agonist ligand in the biogenic amine receptor, to the constitutively active mutant opsins and the inhibition of the constitutive activity by the binding of the antagonist 11-cis-retinal. However, it has been shown recently that the opsin-all-trans-retinal complex activates transducin by a different mechanism than that of the native photolyzed pigment (39).

A unique and interesting phenotype of Gly121 mutant opsins (in membranes) is the significant binding of all-trans-retinal and the activation of transducin in the dark (Fig. 5), in the absence of constitutive activity. When the activity in the presence of all-trans-retinal is presented as a percentage of the light-dependent activity after 11-cis-retinal incubation, it is clear that the mutant opsins G121S, G121I, G121L, and G121W (presumably in their inactive conformations) have a significant defect in their abilities to discriminate between the two isomeric forms of the chromophore. Only the G121S mutant does not fit a general trend of increasing activity with side chain size, possibly due to the presence of a polar hydroxy group.

Taken together, the results of the spectral studies of the Gly121 mutants purified in dodecyl maltoside and the activity studies carried out in membranes strongly suggest that the replacement of the conserved glycine by any residue tested affects the architecture of the chromophore-binding pocket. The effects include a decrease in pigment stability and a loss of strict preference for the 11-cis-retinal isomer over all-trans-retinal. The effects are generally progressive with the size of the residue substituted for Gly121. This size effect is most striking in the case of the data presented in Fig. 3. The behavior of the Gly121 mutants is specific and is not observed with mutations at positions Gly114 or Ala117.

These results imply that Gly121 plays an important role in defining the conformation and specificity of the chromophore-binding pocket in rhodopsin. Considering the strong conservation of a glycine residue at position 121 in all visual pigments, it is intriguing to speculate that a glycine at this position creates a packing space for either the chromophore or for another amino acid side chain that contributes to the integrity of...
the retinal-binding pocket structure. Glycine residues are common in transmembrane helices (53, 54) and are known to be critical residues that allow good van der Waals contacts in helix-helix packing (55–57). It is also interesting to note that Gly121 is one of several residues with small side chains on TM helix 3 that is highly conserved. In particular, Gly114 and Ala117 lie on the same face of TM helix 3 as Gly121. However, substitution of Ala117 with residues having larger side chains does not significantly influence the binding of 11-cis-retinal or the other properties tested. In the following paper, the interactions of Gly121 are further explored by introducing second-site mutations (22).

The present results may also be reconciled with previous findings in a variety of other G protein-coupled receptors. In the human D2 dopamine receptor, the substituted-cysteine accessibility method was employed to map the ligand-binding-site crevice (58, 59). Several residues on TM helix 3 were shown to be the most reactive to methanethiosulfonate derivatives and were assigned to face a water-accessible crevice, Val111, Asp114, Val115, and Cys118. These four residues, which correspond to Gly114, Ala117, Thr118, and Gly121 in rhodopsin, form a cluster at the extracellular half of TM helix 3. In the 5-HT2A receptor, Ser159, which corresponds to Gly121 in rhodopsin, was found to interact with its ligand (60). In the human NK-1 receptor, Val118, also equivalent to Gly121 in rhodopsin, has been identified as a key residue to confer species selectivity for several non-peptide antagonists (61–63). The residue on TM helix 3 at a position corresponding to Gly121 in rhodopsin plays a role in regulating the specificity and affinity of ligand binding in many G protein-coupled receptors.

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REFERENCES

1. Strader, C. D., Fong, T. M., Tota, M. R. & Underwood, D. (1994) Annu. Rev. Biochem. 63, 101–132.
2. Schertler, C. F. X., Villa, C. & Henderson, R. (1993) Nature 362, 770–772.
3. Schertler, C. F. X. & Hargrave, P. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11578–11582.
4. Sakmar, T. P., Franke, R. R. & Khorana, H. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8309–8313.
5. Nathans, J. (1990) Biochemistry 29, 9746–9752.
6. Zhukovsky, E. A. & Oprian, D. D. (1989) Science 246, 928–930.
7. Longstaff, C., Calhoon, R. D. & Rando, R. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4209–4213.
8. Smith, S. O., de Groot, H., Gebhard, R. & Lugtenburg, J. (1992) Photochem. Photobiol. 56, 2119–2122.
9. Fahmy, K., Siebert, F. & Sakmar, T. P. (1994) Biochemistry 33, 13700–13705.
10. Jäger, F., Fahmy, K., Sakmar, T. P. & Siebert, F. (1994) Biochemistry 33, 10874–10882.
11. Fahmy, K. & Sakmar, T. P. (1995) Biochemistry 34, 7229–7236.
12. Franke, R. R., König, B., Sakmar, T. P., Khorana, H. G. & Hofmann, K. P. (1990) Science 247, 123–125.
13. Karmik, S. S., Sakmar, T. P., Chen, H. B. & Khorana, H. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8459–8463.
14. Fahmy, K., Jäger, F., Beck, M., Sakmar, T. P. & Siebert, F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10206–10210.
15. Lin, S. W. & Sakmar, T. P. (1996) Biochemistry 35, 11149–11159.
16. Birge, R. R. (1990) Biochem. Biophys. Acta 1016, 293–327.
17. Gat, Y. & Sheves, M. (1993) J. Am. Chem. Soc. 115, 3772–3773.
18. Han, M., DeDecker, B. S. & Smith, S. O., (1993) Biochemistry 32, 1425–1432.
19. Han, M. & Smith, S. O. (1995) Biochemistry 34, 1425–1432.
20. Han, M. & Smith, S. O. (1995) Biochemistry 34, 1425–1432.