Characterization of the Mutant Visual Pigment Responsible for Congenital Night Blindness: A Biochemical and Fourier−Transform Infrared Spectroscopy Study †

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Characterization of the Mutant Visual Pigment Responsible for Congenital Night Blindness: A Biochemical and Fourier-Transform Infrared Spectroscopy Study†

Tatyana A. Zvyaga,‡,|| Karim Fahmy,§,|| Friedrich Siebert,§ and Thomas P. Sakmar*,‡

The Howard Hughes Medical Institute, Laboratory of Molecular Biology and Biochemistry, Rockefeller University, 1230 York Avenue, New York, New York 10021, and Institut für Biophysik und Strahlenbiologie, Albert-Ludwigs-Universität, Albertstrasse 23, 79104 Freiburg, Federal Republic of Germany

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ABSTRACT: A mutation in the gene for the rod photoreceptor molecule rhodopsin causes congenital night blindness. The mutation results in a replacement of Gly^{90} by an aspartic acid residue. Two molecular mechanisms have been proposed to explain the physiology of affected rod cells. One involves constitutive activity of the G90D mutant opsin [Rao, V. R., Cohen, G. B., & Oprian, D. D. (1994) *Nature 367,* 639−642]. A second involves increased photoreceptor noise caused by thermal isomerization of the G90D pigment chromophore [Sieving, P. A., Richards, J. E., Naarendorp, F., Bingham, E. L., Scott, K., & Alpern, M. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92,* 880−884]. Based on existing models of rhodopsin and *in vitro* biochemical studies of site-directed mutants, it appears likely that Gly^{90} is in the immediate proximity of the Schiff base chromophore linkage. We have studied in detail the mutant pigments G90D and G90D/E113A using biochemical and Fourier-transform infrared (FTIR) spectroscopic methods. The photoproduct of mutant pigment G90D, which absorbs maximally at 468 nm and contains a protonated Schiff base linkage, can activate transducin. However, the active photoproduct decays rapidly to opsin and free *all-trans*-retinal. FTIR studies of mutant G90D show that the dark state of the pigment has several structural features of metarhodopsin II, the active form of rhodopsin. These include a protonated carboxylic acid group at position Glu^{113} and increased hydrogen-bond strength of Asp^{83}. Additional results, which relate to the structure of the active G90D photoproduct, are also reported. Taken together, these results may be relevant to understanding the molecular mechanism of congenital night blindness caused by the G90D mutation in human rhodopsin.

Rhodopsin is the photoreceptor molecule of the retinal rod cell, which is responsible for dim-light vision. Rhodopsin contains an 11-*cis*-retinal chromophore, which is covalently bound to Lys^{296} of the opsin via a protonated Schiff base linkage. The positive charge of the retinal Schiff base is stabilized by a negative counterion provided by the side chain of Glu^{113} (Sakmar et al., 1989; Zhukovsky & Oprian, 1989; Nathans, 1990). Numerous mutations in the gene for rhodopsin have been linked to the disease autosomal dominant retinitis pigmentosa (ADRP) (Dryja et al., 1991; Humphries et al., 1992; Sung et al., 1991). A prominent symptom of ADRP is night blindness, which generally correlates with the degree of retinal degeneration and rod cell loss. One particular rhodopsin mutation, which results in a Gly^{90} to Asp replacement, has been shown to cause congenital night blindness in the absence of significant retinal degeneration (Sieving et al., 1995). The detailed elucidation of the molecular mechanism underlying congenital night blindness may lead to a better understanding of how the active conformation of rhodopsin is formed.

In a previous *in vitro* study, mutant pigment G90D was shown to activate transducin under conditions where a visible-absorbing photoproduct, i.e., a species with a protonated Schiff base, was predominantly formed (Rao et al., 1994). In the absence of chromophore, the mutant opsin also displayed constitutive activity. It was proposed that constitutive activity in the mutant opsin might be due to neutralization of Glu^{113} in favor of a new electrostatic interaction between a negative Asp^{90} carboxylate and the protonated Lys^{296} ϵ-amino group. Asp^{90} was shown to stabilize a protonated Schiff base in the mutant pigment G90D/E113Q (Rao et al., 1994). However, the identity of the counterion in mutant G90D was not determined. In addition, it was not determined whether or not the counterion was identical in both the dark state and the photoproduct of mutant pigment G90D.

We have carried out a detailed study of the Schiff base environments of the dark and active states of mutant pigment G90D. It is demonstrated that the protonated Schiff base photoproduct of G90D is compatible with a MII-like receptor conformation. The ability of the photoproduct to activate transducin decays rapidly and with the same time course as its Schiff base linkage. FTIR[1] -difference spectroscopy was employed to look for infrared absorption changes that could be assigned to Glu^{113} or Asp^{90} in mutant G90D. The key results show that the dark pigment has features of the active

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* Address correspondence to this author at: Box 284, Rockefeller University, 1230 York Ave., New York, NY 10021. Tel: 212-327-8288. FAX: 212-327-8370. E mail: sakmar@rockvax.rockefeller.edu.

‡ The Howard Hughes Medical Institute, Rockefeller University.

§ Albert-Ludwigs-Universität.

|| These authors contributed equally to the work presented.

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[1] Abbreviations: bp, base pairs; con A, concanavalin A; FTIR, Fourier-transform infrared; HOOP, hydrogen-out-of-plane; MII, metarhodopsin II; ROS, rod outer segment.

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MII conformation, including a neutral residue at position 113. Therefore, our data support the hypothesis of an electrostatic competition between Asp^{90} and Glu^{113} in mutant pigment G90D as a possible cause of congenital stationary night blindness.

MATERIALS AND METHODS

Construction and Preparation of Rhodopsin Mutants. Site-directed mutagenesis was performed using restriction fragment replacement (Lo et al., 1984) in a synthetic gene of rhodopsin (Ferretti et al., 1986), which had been cloned into the expression vector as described previously (Franke et al., 1988). Mutant G90D was prepared by replacement of a 70-bp *Bgl*II−*Rsr*II restriction fragment with a synthetic duplex containing the desired codon alteration. The construction of mutant E113A was previously described (Sakmar et al., 1991). The double mutant G90D/E113A was made by combining the 1371-bp *Avr*II−*Rsr*II restriction fragment of the plasmid containing the G90D mutation with the 4817-bp *Rsr*II−*Avr*II restriction fragment containing the E113A mutation. 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]-dATPαS. Opsin genes were expressed in COS-1 cells, and the corresponding pigments were reconstituted with 11-*cis*-retinal and purified following the procedures described (Franke et al., 1988, 1992; Zvyaga et al., 1994). Pigments were generally prepared in 100 mM NaCl/0.1% dodecyl maltoside detergent. In order to carry out experiments at different pH values, concentrated buffer solutions (10×) were added to give the desired final pH.

UV−Visible Absorption Spectroscopy and Photolysis of Mutant Pigments. Spectroscopy was performed on a Lambda-19 Perkin-Elmer spectrophotometer at 15 °C on purified samples as previously described (Zvyaga et al., 1994). Spectroscopy was also carried out under conditions identical to those used in the FTIR experiments described below using a λ-17 Perkin-Elmer instrument.

Transducin Activation Assay and Measurement of Activity Decay Rates. A radionucleotide filter-binding assay which monitors the light-dependent guanine nucleotide exchange by transducin was carried out as described previously (Min et al., 1993). Transducin activation rates were determined by linear regression analysis of the increasing amount of radioactivity retained on successive filters. The data acquired were used to calculate the corresponding decay rates using single exponential functions.

Measurement of Schiff Base Hydrolysis Rates. An acid denaturation method was used to monitor the rate of Schiff base hydrolysis after illumination of the recombinant pigments (Sakmar et al., 1989). After acid denaturation, all Schiff base-containing pigments are converted to a state that absorbs at 440 nm irrespective of the initial protonation state and absorption maximum.

Reaction of Mutant Pigments with Hydroxylamine. The rates of hydroxylamine reaction with mutant pigments were determined in darkness at pH 7.0 as previously described (Zvyaga et al., 1993).

Sample Preparation for FTIR Spectroscopy. Con A-purified ROS rhodopsin was prepared in 0.02% dodecyl maltoside as described (König et al., 1989). Mutant pigments with the amino acid replacements G90D and G90D/E113A were purified for FTIR studies as reported (Fahmy et al., 1993). About 2 nmol of recombinant or con A-purified rhodopsin was dried under a stream of nitrogen on a CaF_2 window for experiments at 0 °C. Control measurements showed that the hygroscopic nature of the concentrated detergent-solubilized sample ensured sufficient hydration for unperturbed MII formation. All samples contained 200 nmol of potassium buffer of pH 5. H_2O/D_2O exchange was carried out as reported (Fahmy et al., 1993).

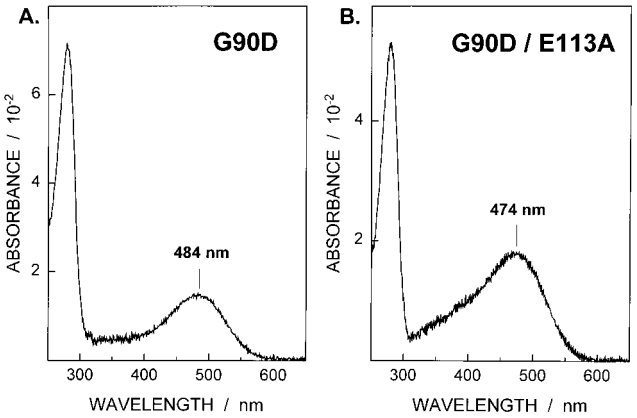

FIGURE 1: UV−visible absorption spectra of mutant pigments. Spectra of pigments G90D (A) and G90D/E113A (B) after regeneration with 11-*cis*-retinal and purification in dodecyl maltoside detergent buffer in darkness are shown. The λ_{max} values were 484 and 474 nm, respectively.

FTIR-Difference Spectroscopy. FTIR-difference spectra were obtained with a Bruker IFS-28 instrument with a liquid nitrogen-cooled MCT detector. Spectral resolution was 2 cm^{-1}. Interferograms were averaged ($n = 1024$) before and after illumination of the sample, and the difference spectra were calculated from the transformed single channel spectra (Ganter et al., 1990). Illumination at 0 °C was carried out by irradiation with wavelengths above 515 nm (OG-515 filter, Schott). Under these conditions, con A-purified rhodopsin forms MII.

RESULTS

Rhodopsin mutant G90D, which contains an amino acid replacement corresponding to the mutation identified to cause congenital night blindness, was constructed by site-directed mutagenesis, expressed, and purified in a detergent solution for biochemical and spectroscopic analysis. Mutant G90D/E113A was prepared in parallel as a control. Both mutants regenerated with 11-*cis*-retinal to form stable visible-absorbing pigments. The absorption maxima of the dark states of G90D and G90D/E113A were blue shifted versus native rhodopsin by 16 and 26 nm, respectively (Figure 1, panels A and B). Upon illumination, the mutant pigments G90D and G90D/E113A formed photoproducts that absorbed maximally at 468 and 454 nm, respectively (Figure 2, panels A and B), indicative of protonated Schiff base pigments similar to MI of rhodopsin. Illumination of con A-purified rhodopsin in detergent causes an absorption shift from 500 to 380 nm as a consequence of Schiff base deprotonation upon formation of the active receptor state MII (not shown). The λ_{max} values of the dark states of both mutants and their photoproducts were independent of pH over the range of 5−8 (not shown). Both pigment photoproducts activated transducin in response to light, although to lesser degrees than

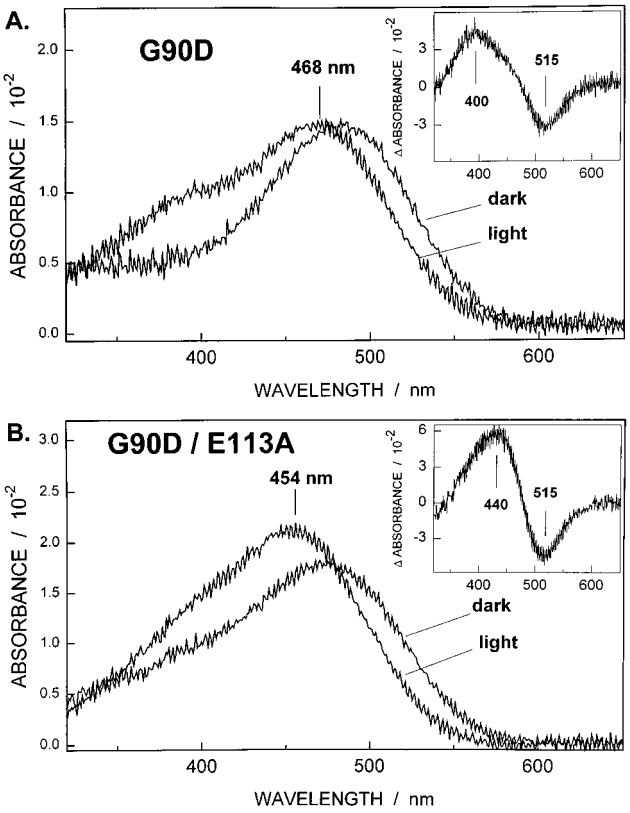

FIGURE 2: Photobleaching spectra of mutant pigments. Spectra of pigments G90D (A) and G90D/E113A (B) are shown before and after illumination for 10 s at 15 °C. A photobleaching difference spectrum is shown as an inset for each pigment. The light minus dark absorption differences are shown. The mutants formed primarily photoproducts with visible-absorbing λ_{max} values of 468 nm for G90D and 454 nm for G90D/E113A. A second smaller spectral component absorbing at about 380 nm was also detected. This 380 nm-absorbing species cannot be distinguished from free *all-trans*-retinal caused by Schiff base hydrolysis. Both mutant photoproducts eventually decayed to 380 nm due to Schiff base hydrolysis (see Table 2).

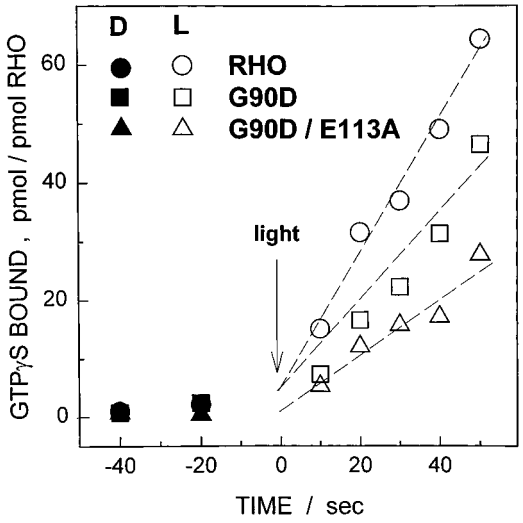

FIGURE 3: Transducin activation by mutant pigments. The ability of the mutant photoproducts to activate transducin was evaluated by a GTPγS filter-binding assay. The amount of GTPγS bound per pmol of pigment is plotted as a function of time. Closed symbols represent binding in the dark, while open symbols correspond to binding after illumination. Mutant G90D activated transducin at a slightly reduced level when compared with rhodopsin. G90D/E113A showed a more significant impairment in its ability to activate transducin. The spectral and biochemical properties of both mutants are presented quantitatively in Table 1.

rhodopsin (Figure 3). The relative activities with respect to rhodopsin were 0.59 and 0.43 for G90D and G90D/E113A, respectively. These data are evaluated and summarized in Table 1.

The protonated Schiff base photoproducts of the mutants decayed to 380 nm-absorbing species. The results of simultaneous measurements of visible absorbance changes, transducin activation rates, and Schiff base hydrolysis are presented in Table 2. The 468 and 454 nm absorptions of the photoproducts of G90D and G90D/E113A, respectively, decay essentially in parallel with the Schiff base as measured by acid denaturation. These data show that the 380 nm absorbance is due to generation of opsin and free retinal rather than to additional short wavelength-absorbing photoproducts with unprotonated Schiff bases. The decay time courses of transducin activation rates also correlate well with the decays of the visible-absorbing photoproducts of both mutants. Table 2 summarizes the time constants for the decays. For each mutant photoproduct, the decay half-lifetimes for the visible absorbance, the rate of transducin activation, and the Schiff base agree within 10−15% and are statistically identical. In contrast, a significant difference between the two mutant pigments is the nearly 4 times faster decay of the transducin activation rate of mutant G90D ($t_{1/2}$ = 3.9 min) versus that of mutant G90D/E113A ($t_{1/2}$ = 14.5 min) (see Table 2). The decay of mutant G90D activity is also significantly faster than that of rhodopsin measured under nearly identical conditions ($t_{1/2}$ = 18 min) (Sakmar et al., 1989).

The reactivity of hydroxylamine with the mutant pigments is shown in Figure 4. The experiment was carried out in darkness in order to probe the Schiff base accessibility to hydroxylamine. Under the conditions of the assay, rhodopsin does not react with hydroxylamine (Zvyaga et al., 1993). However, both mutants reacted with hydroxylamine in darkness. The decay of visible absorption followed first-order kinetics. The $t_{1/2}$ of the decay was 2.7 min for each mutant pigment.

The UV−visible photobleaching-difference spectra of hydrated films of the two mutants prepared for FTIR studies were measured (Figure 5). The difference spectrum of mutant G90D exhibits a positive lobe at 428 nm and a negative lobe corresponding to depletion of the dark-state absorbance at 513 nm. The difference spectrum of mutant G90D/E113A exhibits a positive lobe at 442 nm and a negative lobe at 515 nm. Taking the broadness of the absorption difference bands into account, the actual absorption maxima of the mutant photoproducts under the conditions of the FTIR measurements are in general agreement with the spectral changes recorded in solution (Figure 2). The shorter wavelength of maximal absorption increase upon formation of the G90D photoproduct in spite of its red-shifted absorption versus G90D/E113A in the dark (Figure 1) may be due to a small amount of a 380 nm-absorbing photoproduct as is also observed in the spectrum taken in solution (Figure 2). The lack of a significant absorption change at 380 nm upon photoproduct formation for both mutants demonstrates that, under the conditions of the FTIR experiment, predominantly protonated Schiff base photoproducts were stabilized.

FTIR-difference spectroscopy was used to probe the protein conformational state of each mutant. FTIR-difference

Table 1: Spectral and Biochemical Properties of Recombinant Pigments

pigment	λ_{max} value (nm) dark	photoproduct[b] 1	photoproduct[b] 2	ϵ[c] (M^{-1} cm^{-1})	activation rate[a] (pmol/(pmol·min)) (mean ± SE (*n*))	rel act.
rhodopsin	500	380		42,700	70 ± 1.8 (4)	1.00
G90D	484	468	380	37,000	41 ± 1.2 (4)	0.59
G90D/E113A	474	454	380	32,000	30 ± 0.8 (4)	0.43

[a] Figure 3 shows the results of a single GTPγS filter-binding experiment. [b] The λ_{max} value (nm) of the photoproduct after illumination as described in Materials and Methods is given. For the mutant pigments, a small amount of 380 nm-absorbing species cannot be distinguished from free *all-trans*-retinal caused by Schiff base hydrolysis. [c] Extinction coefficients (ϵ) were measured by the acid denaturation method (Sakmar et al., 1989).

Table 2: Comparison of Decay Kinetics of Mutant Pigments

pigment	half-time of decay ($t_{1/2}$) (min)[a] (mean ± SE (*n*)) photoproduct[b]	activity[c]	Schiff base[d]
G90D	3.9 ± 1.3 (3)	3.9 ± 0.9 (3)	4.2 ± 0.1 (2)
G90D/E113A	12.5 ± 0.4 (3)	14.5 ± 2.3 (5)	13.5 ± 1.7 (2)

[a] Decay experiments were carried out in which the photoproduct spectrum, the transducin-activating ability, and the presence of a Schiff base chromophore linkage were monitored simultaneously at pH 8.0 at 15 °C. A half-time of decay was extracted from the function that best fit the data for each independent experiment (see Materials and Methods). The average values of multiple experiments are presented. [b] The half-times of disappearance of the primary photoproducts are given: G90D − A_{468}; G90D/E113A − A_{454}. [c] Activity refers to the ability of the light-activated pigment to catalyze GTPγS binding by transducin. The half-times of decay are for activation rates as determined in filter-binding experiments as shown in Figure 3. [d] Schiff base decay was measured by the acid denaturation method (Sakmar et al., 1989).

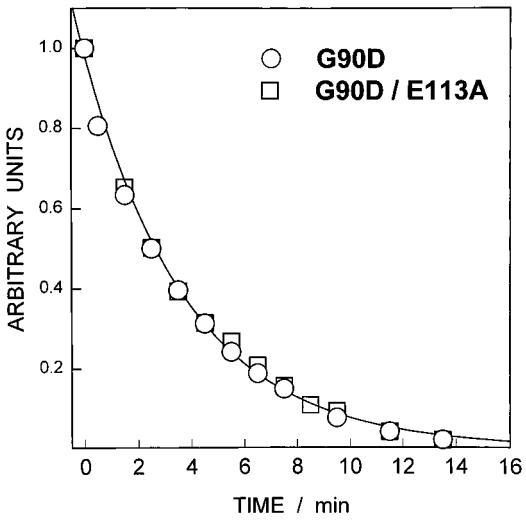

FIGURE 4: Rates of hydroxylamine reaction with mutant pigments in darkness. Arbitrary units of absorbance at 484 nm for mutant G90D and 474 nm for mutant G90D/E113A are scaled to equal 1.0 at time equals zero. The abscissa values represent the time after addition of hydroxylamine. The experiment was carried out with 100 mM hydroxylamine (pH 8.0) at 15 °C. The decay curves were identical for the two mutants and fit well to a single exponential decay function. The half-time of decay ($t_{1/2}$) was 2.7 min for each mutant. Rhodopsin did not react with hydroxylamine under these conditions in darkness.

spectra obtained in H_2O are shown in Figure 6 for mutant G90D (trace A), G90D/E113A (trace B), and con A-purified ROS rhodopsin (trace C). The most prominent band of the MII state (positive bands) is an amide I vibration (peptide carbonyl stretching mode) at 1644 cm^{-1}. It has not been assigned to any particular peptide bond(s) but is generally

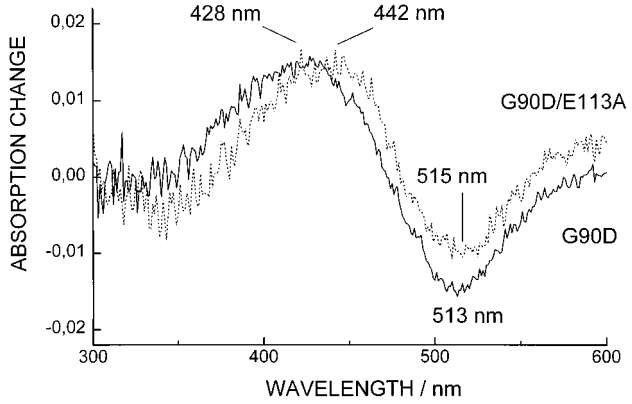

FIGURE 5: UV−visible absorption photobleaching difference spectra of hydrated films of mutant G90D (solid line) and G90D/E113A (dotted line). The samples were prepared as for FTIR spectroscopy experiments with the exception that half the usual amounts of pigment and phosphate buffer (pH 5) were used. The films were cooled to 0 °C in the spectrophotometer in the dark, and absorption spectra were recorded. After illumination (λ > 495 nm) for 90 s, spectra were again recorded. The light minus dark absorption differences are shown. Each of the mutant spectra was stable for at least 15 min after illumination.

displayed by the photoproducts of pigments that activate transducin (see Fahmy et al., 1995). In contrast, this band is not displayed by a mutant photoproduct which has been shown to be incapable of transducin activation (Fahmy et al., 1994), nor does this absorption change occur during formation of any earlier trapped photoproduct of rhodopsin. Therefore, this band is a highly sensitive marker band for the formation of the transducin-activating conformation of rhodopsin, or rhodopsin mutants. Clearly, this absorption is observed in the protonated Schiff base photoproducts of mutants G90D and G90D/E113A.

A negative absorption band of the dark state at 1626 cm^{-1} is also reproduced in the mutant difference spectra, but is weakened in favor of a negative absorption at 1618 cm^{-1}, which in rhodopsin only occurs as a shoulder (Figure 6). More pronounced deviations from the amide I absorption changes occur between 1690 and 1650 cm^{-1}. Dark rhodopsin has an intense absorption at 1656 cm^{-1}. The lack of this band in the difference spectra of both mutants may in part be due to an altered C═N stretching frequency which in the dark state of rhodopsin absorbs at this position. However, altered amide I modes of helical segments are likely to contribute to absorption changes at this frequency as well. A similar mutational effect has been described for mutant E113A (Jäger et al., 1994). In addition, the positive part of a characteristic Rho/MII-difference band at 1695 cm^{-1} (negative)/1687 cm^{-1} (positive) is almost abolished in the mutant spectra. Therefore, the observed deviations from

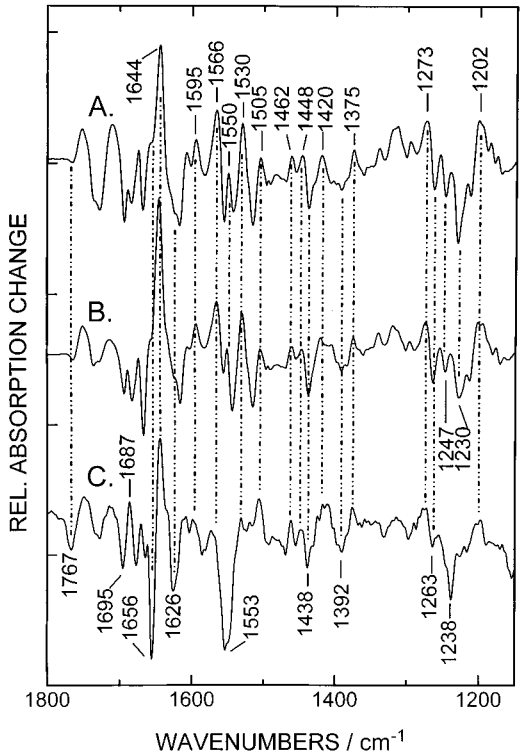

FIGURE 6: FTIR-difference spectra obtained at 0 °C after illumination. (A) Mutant G90D. (B) Mutant G90D/E113A. (C) Native rhodopsin. About 2 nmol of pigment was used in each experiment. All samples contained 200 nmol of phosphate buffer (pH 5) and were measured in the presence of H_2O. Under these conditions, rhodopsin forms the deprotonated Schiff base photoproduct MII. Photoproduct absorption bands point upward, whereas bands of the dark states point downward. The tick interval on the ordinate corresponds to 0.005 absorption units.

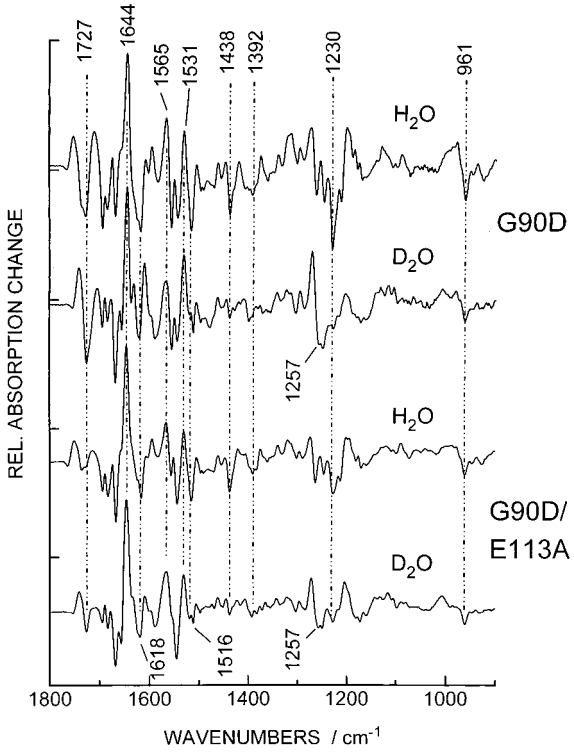

FIGURE 7: Comparison of FTIR-difference spectra of mutants G90D and G90D/E113A recorded at 0 °C in H_2O or D_2O. Conditions and conventions are as described in Figure 6.

rhodopsin must be due to the G90D replacement, which both pigments have in common, and not to the second replacement at Glu^{113}, which does not induce appreciable additional changes. Apparently, the Gly^{90} to Asp mutation affects the vibrational frequency of peptide carbonyl groups which are not part of an α-helical structure for which a C═O stretching mode at about 1650 cm^{-1} would be expected. In the amide II spectral range (1540−1560 cm^{-1}) both recombinant pigments show a 1550 cm^{-1} vibration not present in the MII-difference spectrum of rhodopsin. There is also a more structured appearance of the difference bands between 1530 and 1560 cm^{-1} for both mutants. The predominant alterations from rhodopsin are caused by the Gly^{90} to Asp replacement alone, since the additional Glu^{113} to Ala mutation affects relative peak intensities but does not induce distinct deviations from the difference spectrum of the single mutant. This is also the case for the negative band at 1516 cm^{-1} seen in the dark states of both mutants, but not in dark rhodopsin.

Outside the frequency range of the prominent amide II vibrations, a good correspondence of peak positions can be observed. Thus, a group of unassigned vibrations of amino acid side chains in the 1350−1500 cm^{-1} range are well reproduced among all the difference spectra. All dark states exhibit distinct absorptions at 1438 and 1392 cm^{-1}. The latter is flanked by absorption increases at about 1420 and 1375 cm^{-1}. This band pattern has been observed in the active protonated Schiff base photoproduct of mutant E113A/A117E, but not in its inactive state (Fahmy et al., 1994). The same holds for the doublet at 1448 cm^{-1}/1462 cm^{-1}, which is well reproduced in both mutant photoproducts.

Chromophore modes are observed between 1050 and 1300 cm^{-1} where the coupled C−C stretches and C−H bending vibrations of the retinal polyene chain absorb. Based on FTIR studies (Ganter et al., 1988a,b) and resonance Raman experiments of retinal analogue pigments (Palings et al., 1987), it is known that the prominent negative band at 1238 cm^{-1} in rhodopsin is a highly delocalized mode. It contains predominantly $C_{12}-C_{13}$ stretching character but also the $C_{10}-C_{11}$ stretching mode and the $C_{14}-C_{15}$ stretch coupled to the $C_{15}-H$ and N−H bending mode (Palings et al., 1987; Ganter et al., 1988a,b). This so-called "$C_{12}-C_{13}$" mode at 1238 cm^{-1} is sensitive to perturbations at the terminal part of the chromophore between C_{10} and the Schiff base nitrogen. Interestingly, the "$C_{12}-C_{13}$" stretch in mutant G90D/E113A, *as well as* in the single mutant G90D, is downshifted by 8 cm^{-1} as has been observed for mutants in which the counterion Glu^{113} had been replaced by Ala (Jäger et al., 1994; Fahmy et al., 1994).

In spite of the frequency shift of the "$C_{12}-C_{13}$" stretch, the delocalized nature of this mode, and particularly the involvement of the N−H bending vibration, is retained in the mutant pigments as shown by the distinct effect of H/D exchange on the fingerprint vibrations (Figure 7). The H/D exchange causes a loss of coupling between the N−H bending mode above 1300 cm^{-1} and the C−C stretches below 1300 cm^{-1}. The resulting typical upshift of the "$C_{12}-C_{13}$" stretch to 1257 cm^{-1} in D_2O indicates that the coupling between the involved coordinates has essentially not changed in the mutants versus rhodopsin, as has also been reported for mutant E113A (Jäger et al., 1994). In H_2O, the lower frequency of the "$C_{12}-C_{13}$" stretch in the mutants uncovers a small negative band at 1247 cm^{-1}, which in rhodopsin is discernible as a shoulder on the 1238 cm^{-1} band. Therefore,

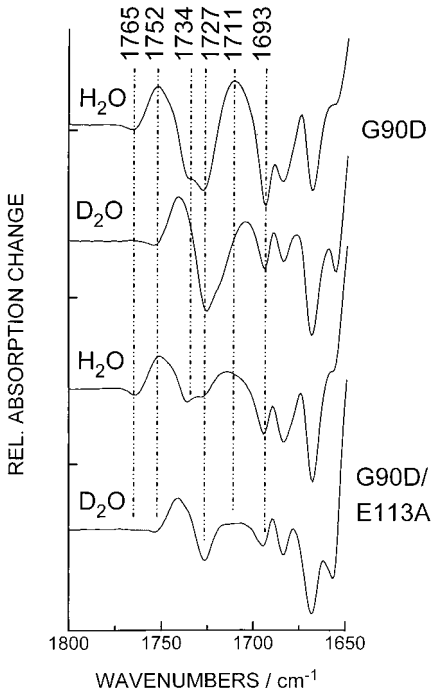

FIGURE 8: FTIR-absorption changes in the spectral range of the carbonyl-stretching vibrations of protonated carboxylic acid groups. Data are identical with those shown in Figure 7. Spectra are normalized with respect to the 1263 cm^{-1}/1270 cm^{-1} difference band (Figure 6).

this absorption is probably not caused by the mutations but is merely better resolved due to less overlap with the "$C_{12}-C_{13}$" stretch. Again, the additional Glu^{113} to Ala replacement in the G90D background alters the relative band intensities but does not cause additional band shifts.

Other fingerprint vibrations are hardly affected at all by the additional Glu^{113} to Ala mutation. In both mutant photoproducts, the intensities of the positive absorption bands at 1200 cm^{-1} are increased to levels usually observed only in MI. Due to the protonated Schiff bases in the mutant photoproducts (as in MI), such a similarity is expected and can be explained by the positive charge of the chromophore which increases the infrared absorption (Siebert & Mäntele, 1980). The 1263 cm^{-1} (negative)/1273 cm^{-1} (positive) difference bands are well reproduced in the spectra of both mutant pigments.

In addition to the fingerprint modes, the retinal chromophore causes hydrogen-out-of-plane (HOOP) vibrations below 1000 cm^{-1}. In the MII difference spectra, however, only the coupled $C_{11}-C_{12}$ HOOP of dark rhodopsin at 967 cm^{-1} has appreciable intensity (not shown). This band is downshifted in G90D by 6 cm^{-1}, and again no further influence on this band is exerted by the additional Glu^{113} to Ala replacement (Figure 7).

The C═O stretching modes of protonated carboxylic acid groups absorb between 1690 and 1800 cm^{-1}. This spectral range is shown for mutants G90D and G90D/E113A in Figure 8 for measurements in H_2O and D_2O. It has been shown that Asp^{83} is protonated but very weakly hydrogen bonded in dark rhodopsin and that hydrogen bonding increases slightly upon MII formation causing the 1767 cm^{-1} (negative)/1752 cm^{-1} (positive) band shift of the carbonyl stretching vibration (Fahmy et al., 1993; Rath et al., 1993). The appearance of this difference band is also a marker for the formation of a MII-like protein conformation (Klinger & Braiman, 1992). In the difference spectra of both mutants, a slightly downshifted negative band of reduced intensity is found at 1765 cm^{-1}. It is likely that, as in rhodopsin, the band is caused by Asp^{83}. Possible reasons for the reduced intensity of the C═O stretching mode of this residue will be discussed below. Since the photoproducts of both recombinant pigments also show the typical 1752 cm^{-1} band, part of this band can be assigned to protonated Asp^{83} in the mutant photoproducts. In MII, another contribution to the 1752 cm^{-1} absorption comes from Glu^{122}, which in dark rhodopsin absorbs at 1734 cm^{-1} and shifts to 1752 cm^{-1} in MII (Fahmy et al., 1993). The same band shift can be discerned in the difference spectra of the mutants, arguing for unperturbed changes in the hydrogen-bond strength of Glu^{122}. These IR absorption changes have been identified in detergent-solubilized recombinant rhodopsin. In reconstituted lipids, however, no upshift of the C═O stretching frequency of Glu^{122} has been observed (Rath et al., 1993; De Caluwé et al., 1995). Finally, a positive band at 1711 cm^{-1} is present in the photoproduct of G90D. It has been shown that the C═O stretching vibration of protonated Glu^{113} in MII absorbs at this position (Jäger et al., 1994). Therefore, Glu^{113} seems to be protonated in the photoproduct of G90D.

The main deviations from the MII-difference spectrum are the much more pronounced negative band in the 1725−1735 cm^{-1} range and the presence of a band at 1693 cm^{-1}. This indicates that additional C═O stretching vibrations of the dark state of mutant G90D contribute to the spectrum. The existence of C═O stretching modes of protonated carboxylic acid groups different from Asp^{83} and Glu^{122} is also supported by the band shifts in D_2O. For rhodopsin, the effects of H/D exchange on these bands have been described in detail (Ganter et al., 1988a, 1989). The 1734 cm^{-1} band shifts into a negative band at 1727 cm^{-1}, which has been assigned to an unusually high frequency peptide carbonyl stretching mode insensitive to isotope exchange. In mutant G90D, however, a shoulder at 1720 cm^{-1} is discernible in the 1727 cm^{-1} band measured in D_2O. Glu^{113} or Asp^{90} may cause the 1725−1735 cm^{-1} (H_2O) and the 1720 cm^{-1} (D_2O) absorption of the dark state, assuming that the D_2O sensitivity of the neighboring band of Glu^{122} at 1734 cm^{-1} has not changed in the mutant. This assumption can be validated by inspecting the difference spectra of the double mutant G90D/E113A. The major influence of the additional amino acid replacement at position 113 is indeed a distinct reduction of the negative 1725−1735 cm^{-1} absorption and a concomitant decrease of the 1711 cm^{-1} band. This shows that Glu^{113} causes a difference band at 1725−1735 cm^{-1} (negative)/1711 cm^{-1} (positive). Thus, Glu^{113} is at least partly protonated in the dark state as well as in the photoproduct of G90D.

When measured in D_2O, the shoulder at 1720 cm^{-1} in the negative band at 1727 cm^{-1} is absent in mutant G90D/E113A. It can be concluded that the 1720 cm^{-1} absorption is indeed solely due to deuterated Glu^{113} and not caused by a mutation-induced increase of the isotope shift of Glu^{122} from 1734 to 1720 cm^{-1}. The C═O stretch of deuterated Glu^{122} in the dark state of G90D/E113A is superimposed with the isotope-insensitive band at 1727 cm^{-1} as known from rhodopsin. As in the difference spectra of other mutants that lack a counterion at position 113, a residual absorption band around 1710 cm^{-1} is seen in the photoproduct of the double mutant. However, in contrast to previously studied recombinant pigments, a slight sensitivity to H/D exchange

is still observed in the broad positive absorption at 1710 cm^{-1}. Since Glu^{113} is absent in mutant G90D/E113A, we assign this absorption to the partial protonation of Asp^{90} upon photoactivation of G90D/E113A.

In addition to the unusual 1725−1735 cm^{-1} (negative)/1711 cm^{-1} (positive) difference band of protonated Glu^{113}, the single amino acid replacement in G90D causes a negative band at 1693 cm^{-1} which is not observed in rhodopsin. With the given assignments of Asp^{83}, Glu^{113}, and Glu^{122} to bands above 1700 cm^{-1}, we consider this band to be caused either by Asp^{90} (which in this case must be partly protonated in the dark state) or by an unusually high absorbing amide I mode. The D_2O sensitivity of this band argues for the Asp^{90} assignment. However, the isotope effect may as well be caused by overlap with the positive band at 1705 cm^{-1} downshifted from 1711 cm^{-1}. In the double mutant, not only a predominant effect above 1700 cm^{-1} is observed, but the 1693 cm^{-1} absorption is reduced as well and has lost isotope sensitivity.

To summarize the FTIR spectroscopy results presented in Figure 8, mutant G90D shows increased absorption of the dark state at 1725−1735 cm^{-1}, which can be assigned to the C═O stretching mode of an at least partially protonated Glu^{113}. Glu^{113} stays protonated after illumination and causes the absorption increase at 1711 cm^{-1}. In addition, an isotope-sensitive difference band at 1693 cm^{-1} (negative)/1711 cm^{-1} (positive) not present in difference spectra of rhodopsin is observed and may be caused by partially protonated Asp^{90}. In the double replacement mutant G90D/E113A, only a small positive band around 1710 cm^{-1} can be assigned to Asp^{90}. This residue is entirely ionized in the dark and undergoes only partial light-induced protonation.

DISCUSSION

The Gly^{90} to Asp mutation in human rhodopsin has been identified as the cause of congenital night blindness. The molecular pathophysiology underlying the trait is unclear. The aim of the current study was to subject the mutant pigment G90D to a detailed biochemical and spectroscopic characterization. Studies of mutant pigment G90D/E113A were carried out in parallel as a control. When regenerated with chromophore, mutant G90D formed a stable pigment with a λ_{max} value of 484 nm (Figure 1). This value is in good agreement with values of 480 and 483 nm reported previously (Kaushel & Khorana, 1994; Rao et al., 1994). The λ_{max} value of mutant pigment G90D/E113A (474 nm) also agrees well with that of a similar mutant, G90D/E113Q (472 nm) (Rao et al., 1994).

Both mutant pigments G90D and G90D/E113A formed relatively stable photoproducts at 15 °C with λ_{max} values of 468 and 454 nm, respectively (Figure 2). These λ_{max} values suggest the presence of a protonated Schiff base linkage, which decays to form opsin and free retinal without appreciable formation of a 380 nm-absorbing MII-like pigment (Table 2). The mutants were somewhat defective in their ability to activate transducin after illumination (Figure 3). For example, the rate of transducin activation by mutant pigment G90D was 59% of that of rhodopsin under identical conditions (Table 1). The ability of pigment G90D to activate transducin after illumination decayed over time. The rate of this activity decay was essentially identical to the decay of the photoproduct at 468 nm, and to the decay of the Schiff base bond (Table 2). Thus, the 468-nm photoproduct of mutant G90D, which contains a protonated Schiff base chromophore linkage, activates transducin as was previously suggested (Rao et al., 1994). The fact that a protonated Schiff base photoproduct can activate transducin contrasts with the situation found in rhodopsin (Kibelbek et al., 1991), but parallels previous reports on light-dependent transducin activation by the mutant pigment E113A/A117E (Fahmy et al., 1994; Zvyaga et al., 1994).

It can be concluded that the 454-nm photoproduct of the control mutant G90D/E113A also activates transducin (Table 2). The major biochemical difference between the G90D and G90D/E113A photoproducts is the nearly 4 times slower decay of activity for G90D/E113A as compared to G90D (Table 2). However, the salient result is that the G90D active photoproduct is significantly less stable ($t_{1/2}$ = 3.9 min) than that of either G90D/E113A ($t_{1/2}$ = 14.5 min) or rhodopsin ($t_{1/2}$ = 18 min) (Table 2; Sakmar et al., 1989).

Previous studies of mutant E113A/A117E helped to identify marker bands in the FTIR-difference spectrum of rhodopsin that appear to be characteristic of the active conformation, independent of the protonation state of the Schiff base (Fahmy et al., 1994). Based on these marker bands, it is suggested above that the light-induced absorption changes in the mutant pigments are consistent with the formation of MII-like photoproducts. The deviations from a typical MII-difference spectrum, which are caused by the introduction of an additional internal carboxylic acid group at position 90 in G90D, can be divided into two classes: (1) effects on absorption bands in the C═O stretching frequency range between 1800 and 1690 cm^{-1}, and (2) indirect mutational effects on peptide carbonyl and chromophore vibrations.

Effects on Absorption Bands in the C═O Stretching Frequency Range between 1800 and 1690 cm^{-1}. The proposed competition between Asp^{90} and Glu^{113} for electrostatic interaction with the protonated Schiff base in mutant G90D (Rao et al., 1994) would require Glu^{113} to exist in the protonated form. The protonation states of carboxylic acid groups can be investigated directly by inspection of the C═O stretching modes absorbing between 1690 and 1800 cm^{-1}. The major effect of the G90D replacement is the enhancement of an absorption decrease at 1725−1730 cm^{-1}, indicative of a protonated carboxylic acid group in the dark state of the mutant that is not present in rhodopsin. Furthermore, this group becomes perturbed after photoactivation of the mutant. A straightforward interpretation would be to assign this additional band to the new carboxylic acid introduced in the mutant, i.e., Asp^{90}. However, we have shown that this band, as well as the positive band at 1711 cm^{-1}, is largely reduced by the replacement of Glu^{113} by Ala. It will be shown below also that well characterized chromophore vibrations in the dark states of mutants G90D and G90D/E113A are indicative of the protonated state of Glu^{113}. Therefore, we assign the bands at 1725−1730 cm^{-1} (negative) and 1711 cm^{-1} (positive) to protonated Glu^{113} in the dark state and the photoproduct of G90D, respectively. Thus Asp^{90} must compete with Glu^{113} for electrostatic interaction with the protonated Schiff base, which allows Glu^{113} to be protonated.

In general, a replacement of Glu^{113} by a neutral amino acid residue greatly enhances the hydroxylamine reactivity of the mutant pigment in darkness (Sakmar et al., 1989;

Zvyaga et al., 1993, 1994). The fact that both mutants G90D and G90D/E113A react with hydroxylamine in darkness (Figure 4) also supports the conclusion that Glu^{113} is at least partially protonated in the dark state of mutant G90D. However, we cannot exclude that the electrostatic competition is incomplete. In the FTIR-difference spectrum of mutant G90D, Asp^{90} may be partly protonated and cause a difference band at 1693 cm^{-1} (negative)/1711 cm^{-1} (positive) as well. If this assignment is accepted, the disappearance of the 1693 cm^{-1} absorption in the double mutant can be explained by the complete replacement of the counterion function by Asp^{90} in the dark due to the lack of Glu^{113}. In the photoproduct of the double mutant, a small fraction of Asp^{90} becomes protonated and causes the small positive C═O stretching absorption at 1710 cm^{-1}. This band shows that, in addition to substituting for the counterion function of Glu^{113}, a small fraction of the Asp^{90} carboxylic acid side chains may also function as proton acceptor for the Schiff base proton in the G90D/E113A photoproduct. This interpretation is consistent with the light-induced formation of a small amount of 380-nm absorbance detected in solution and under conditions of the FTIR experiments.

Our interpretation of the spectral consequences of the amino acid replacements is based on the assumption that the carboxylic acid groups of Asp^{83} and Glu^{122} have not changed their previously identified frequencies measured for rhodopsin. This assumption seems valid because the peak positions of these residues, as well as the magnitude of their frequency shifts after H/D exchange, are retained in the difference spectrum of G90D after H/D exchange. However, the absorption decrease of Asp^{83} at 1767 cm^{-1} (negative) is less pronounced than usual. Difference bands are only observed if the physical environment of a residue is changed upon photoactivation. Therefore, the reduction of the Asp^{83} difference band indicates that the dark state of G90D already has some local conformational similarities with the light-activated form, which reduces the amplitude of further observable changes during complete formation of the active conformation. The fact that Asp^{83} lies on the same helix as the replaced glycine residue (helix 2) may support the view of a local mutationally induced conformational change. Furthermore, the normal appearance of the difference band of Glu^{122} on helix 3 argues for the absence of "long-range" effects of the G90D exchange.

Indirect Mutational Effects on Peptide Carbonyl and Chromophore Vibrations. Pronounced deviations from the normal MII-difference spectrum are found at 1656 cm^{-1}, where both mutants exhibit reduced negative bands. The C═N stretch of dark rhodopsin is found at this frequency, and the expected change in the Schiff base environment in the mutants may in part account for the difference. However, the effect is too large to be entirely due to an altered C═N stretching mode (for an estimate of the relative intensity of the C═N stretching mode of rhodopsin, see Siebert et al., 1983). Therefore, an amide I difference band must also be reduced. This may again reflect a partial anticipation of a local active conformation as proposed for the Asp^{83} environment and would support the concept of weighted additivity of local "off−on" transitions during photoactivation of rhodopsin (Fahmy et al., 1995).

The lack of a peptide carbonyl band of unusual high frequency at 1687 cm^{-1} parallels a situation described for Glu^{122} replacement (Fahmy et al., 1993), where a band at 1727 cm^{-1} was affected. The mutation was located adjacent to consecutive glycine residues, which are weak helix stabilizers, on transmembrane helix 3. Gly^{90} is also one out of a pair of consecutive glycine residues on transmembrane helix 2, and the same argument may apply for mutants G90D and G90D/E113A. The actual secondary structure may be less defined by intrahelical C═O···H−N hydrogen bonds than by neighboring amino acids. Thus, the corresponding peptide conformations may result in C═O stretching frequencies which differ from the usually observed 1650 cm^{-1} in α-helices.

The amide II spectral range between 1540 and 1560 cm^{-1} cannot be used as a monitor of the protein conformational change because of the overlap with the C═C stretching difference bands of the chromophore. Since protonated Schiff base photoproducts are formed by the mutant pigments, the C═C stretches are expected to have higher intensities and different frequencies when compared with the native MII state (Doukas et al., 1978; Rimai et al., 1973; Siebert & Mäntele, 1980). In particular, the changes in the C═C stretching modes are expected to be similar to those usually observed upon MI formation because this photoproduct also has a protonated Schiff base. This explains the more structured appearance of the difference bands between 1530 and 1560 cm^{-1} for both mutant pigments, which exhibit a 1550 cm^{-1} vibration not present in the MII-difference spectrum of rhodopsin. To the low frequency side of the amide II spectral range, a negative band at 1516 cm^{-1} not seen in rhodopsin is observed in the difference spectra of both mutants G90D and G90D/E113A. Such a band has previously been observed in the MII-difference spectrum of the single amino acid replacement mutant E113A (Jäger et al., 1994). Thus, the replacement of Gly^{90} by Asp appears to induce spectral changes which resemble those caused by neutralization of Glu^{113}.

Chromophore vibrations are also affected by the G90D replacement. The frequencies of the coupled C−C stretching and C−H bending vibrations between 1100 and 1250 cm^{-1} are downshifted by 2−7 cm^{-1}. However, the fingerprint difference bands appear generally similar to those of rhodopsin and show that the chromophore isomerization in both mutants is specifically 11-*cis* to *all-trans*. The small frequency shifts can be explained by the reduced delocalization of the retinal Schiff base π-electron system as manifested in the blue-shifted visible absorption maximum of mutant G90D, as well as that of mutant G90D/E113A. Hence, the alteration of bond strength between single and double bonds in the retinal polyene chain is expected to be more pronounced in the mutants than in rhodopsin. Correspondingly, single bond and double bond stretching frequencies should be decreased and increased, respectively.

The most pronounced effect found for the "C_{12}−C_{13}" stretching frequency of the dark states of the mutants merits special discussion. In rhodopsin, this delocalized mode is found at 1238 cm^{-1} and is sensitive to perturbations between C_{10} and the Schiff base, including H/D exchange of the Schiff base proton (Palings et al., 1987; Ganter et al., 1988a,b). The fact that the isotope sensitivity is conserved in both mutants shows that the normal mode character of the 1230 cm^{-1} vibration has essentially not changed, although the frequency has dropped by 8 cm^{-1}. Since the downshift is about 3 times larger than that of the other fingerprint modes, it seems likely to be caused by perturbations involving the

terminal half of the chromophore in addition to the reduced π-electron delocalization discussed above.

As to the nature of possible chromophore perturbations, a comparison with previously studied mutants is most revealing. The dark state of the mutant E113A, in which chloride served as inorganic counterion, showed a shift of the 1238 cm^{-1} absorption to 1230 cm^{-1} (Jäger et al., 1994; Lin et al., 1992), whereas the E113D replacement did not cause any band shift in the fingerprint range (Jäger et al., 1994). Therefore, the "$C_{12}-C_{13}$" frequency seems to be largely determined by the electrostatic chromophore environment, rather than by specific steric interactions. Likewise, a 1230 cm^{-1} band has been observed in mutants E113Q (Lin et al., 1992) and E113A/A117E (Fahmy et al., 1994). The 1238 to 1230 cm^{-1} band shift is consistent with a model of the chromophore-binding pocket in which the carboxylate of Glu^{113} is close to C_{12} of the chromophore (Birge et al., 1990; Han et al., 1993). An effect of the G90D replacement on the chromophore near C_{12} is further supported by the downshift of the $C_{11}-C_{12}$ HOOP from 967 to 961 cm^{-1}. Again, the double mutant exhibits the same spectral feature, suggesting that the effect in mutant G90D is exerted through neutralization of Glu^{113}. In summary, the most pronounced indirect effect of the G90D replacement on the chromophore fingerprint vibrations is on a band (1238 cm^{-1}), which is highly sensitive to the existence of a negative charge at position 113. This band is found in the dark states of mutants G90D and G90D/E113A at a frequency indicative of a neutral side chain at position 113.

Relevance to Congenital Night Blindness. A molecular mechanism was proposed for congenital night blindness based on the observation that the mutant G90D opsin displayed constitutive activity *in vitro* (Rao et al., 1994). According to the model, the Asp^{90} residue destabilizes a salt bridge between Glu^{113} and Lys^{296} (Cohen et al., 1993). The resulting opsin activity causes the rod cell to adapt to an "on" signal in darkness so that an increased dark-adapted threshold is observed. However, it was pointed out that this explanation may not be totally consistent with the finding of normal pigment density and a lack of significant retinal degeneration *in vivo* in individuals with night blindness caused by the G90D mutation (Sieving et al., 1995). The physiology of the rod function deficit was proposed to be most consistent with increased basal signaling from thermal isomerization of the mutant pigment (Sieving et al., 1995). Photoreceptor noise has been previously correlated to thermal isomerization of an unprotonated Schiff base chromophore (Barlow et al., 1993; Birge & Barlow, 1995).

Several biochemical and spectroscopic features of the G90D pigment from the present study are relevant. The 468-nm photoproduct of pigment G90D, which is slightly defective in its ability to activate transducin, decays rapidly. The ability of the photoproduct to activate transducin decays with the same time course. Although the primary photochemical isomerization was shown to be 11-*cis* to *all-trans*, secondary photochemical inactivation of the photoproduct by visible light could theoretically occur. FTIR studies show that the dark pigment has key features of the active MII conformation, including a neutral residue at position 113. In addition, Asp^{83} seems to be exposed to a protein environment in the dark which is similar to that in the active MII conformation (Fahmy et al., 1993; Rath et al., 1993). Similarly, an amide I absorption change usually occurring upon MII formation seems to be anticipated by the mutation. Thus, the pigment has some structural features of the "on state" even in darkness.

Although the Schiff base pK_a of pigment G90D is above 8, it may be significantly lower than that of rhodopsin because of the altered electrostatic environment of the Schiff base demonstrated by the FTIR studies. Thus, rate of thermal isomerization of the chromophore, and the resulting photoreceptor noise, might be expected to be increased for the G90D pigment. However, the FTIR evidence for electrostatic competition between Asp^{90} and Glu^{113} strongly argues for a neutral chromophore binding site as in rhodopsin, rather than a mutational introduction of an additional negative charge (Sieving et al., 1995). Low temperature FTIR studies of early G90D photoproducts will be helpful to elucidate relevant chromophore−opsin interactions in greater detail.

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