Spectroscopic Evidence for Altered Chromophore−Protein Interactions in Low-Temperature Photoproducts of the Visual Pigment Responsible for Congenital Night Blindness†

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

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

Received June 21, 1996; Revised Manuscript Received October 1, 1996⊗

ABSTRACT: The replacement of Gly^{90} by Asp in human rhodopsin causes congenital night blindness. It has been suggested that the molecular origin for the trait is an altered electrostatic environment of the protonated retinal Schiff base chromophore. We have investigated the corresponding recombinant bovine rhodopsin mutant G90D, as well as the related mutants E113A and G90D/E113A, using spectroscopy at low temperature. This allows the assessment of chromophore−protein interactions under conditions where conformational changes are mainly restricted to the retinal-binding site. Each of the mutant pigments formed bathorhodopsin- and isorhodopsin-like intermediates, but the concomitant visible absorption changes reflected differences in the electrostatic environment of the protonated Schiff base in each pigment. Fourier transform infrared-difference spectroscopy revealed effects on the chromophore fingerprint and hydrogen-out-of-plane vibrational modes, which were indicative of the removal of an electrostatic perturbation near C_{12} of the retinal chromophore in all three mutants. A comparison of the UV−visible and infrared-difference spectra of the mutant pigments strongly suggests that Glu^{113} is stably protonated in G90D. The corresponding carbonyl-stretching mode is assigned to a band at 1727 cm^{-1}. In contrast to the case in native bathorhodopsin, the *all-trans*-retinal chromophores in the primary photoproducts of the mutant pigments are essentially relaxed. The peptide carbonyl vibrational changes in mutants G90D and G90D/E113A suggest that this is due to a more flexible retinal-binding site. Therefore, the steric strain exerted on the chromophore in native bathorhodopsin may be caused by electrostatic forces that specifically involve glutamate 113.

Dim-light vision depends upon the molecular properties of the photoreceptor rhodopsin in the outer segment disk membranes of the retinal rod cells. The 11-*cis*-retinal chromophore of rhodopsin is covalently bound via a protonated Schiff base (PSB[1]) to Lys^{296} of the opsin apoprotein. The positive charge of the PSB is stabilized by the side chain carboxylate of Glu^{113} on transmembrane (TM) helix 3 of the receptor (Nathans, 1990; Sakmar et al., 1989; Zhukovsky & Oprian, 1989). In native rhodopsin, photoisomerization of the chromophore to *all-trans*-retinal results in deprotonation of the PSB and concomitant neutralization of Glu^{113}, which are key events in the formation of metarhodopsin II (MII) (Jäger et al., 1994) and the active receptor state, R* (Emeis et al., 1982; Kibelbek et al., 1991). Recombinant rhodopsins that adopt a MII-like conformation without deprotonation of the Schiff base have been described (Fahmy et al., 1994; Rao et al., 1994; Zvyaga et al., 1996). Among this class of mutant pigments, a glycine to aspartic acid replacement in TM helix 2 was shown to cause a form of congenital night blindness in humans (Rao et al., 1994; Sieving et al., 1995).

Two models for the molecular pathophysiology of the disease have been suggested. One model assumes breakage of a salt bridge between Lys^{296} and Glu^{113} in favor of a new electrostatic interaction between the lysine and Asp^{90} to explain the constitutive activity of the mutant opsin in the absence of a chromophore observed *in vitro* (Cohen et al., 1992; Rao et al., 1994). A high signaling level of the rod cell in the dark would explain the physiology of affected individuals (Rao et al., 1994). Another model suggests a lowered energy barrier for thermal isomerization of the chromophore in the holoprotein, thus causing basal activity in the dark, which raises the threshold for light perception (Sieving et al., 1995). Previous biochemical and spectroscopic data obtained with mutant pigment G90D suggested that Asp^{90} competes with Glu^{113} for electrostatic interaction with the PSB (Rao et al., 1994; Zvyaga et al., 1996).

In the present study, the location of the Schiff base counterion in mutant G90D is studied by visible and Fourier transform infrared (FTIR) spectroscopy at low temperature. The investigation of the primary photoproduct formation by visible absorption spectroscopy was motivated by the expected influence of the electrostatic environment of the PSB on the chromophore absorption (Birge et al., 1988; Blatz et al., 1972). Two photon absorption experiments have indicated that the retinal-binding site in native rhodopsin is neutral (Birge et al., 1985). Thus, a potential extra negative charge can either entirely substitute for the counterion Glu^{113}

† K.F. was supported by DFG Grant Fa 248/2-1. T.P.S. is an Associate Investigator of The Howard Hughes Medical Institute, and T.A.Z. was supported in part by NIH Training Grant EY 07138.

* Address correspondence to this author.

‡ Albert-Ludwigs-Universität.

§ The Howard Hughes Medical Institute, Rockefeller University.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: con A, concanavalin A; FTIR, Fourier transform infrared; HOOP, hydrogen-out-of-plane; MII, metarhodopsin II; PSB, protonated Schiff base; TM, transmembrane.

S0006-2960(96)01486-9 CCC: $12.00

or contribute an external point charge (Honig et al., 1979). In any case, a distinct effect on the visible absorption properties was expected if the newly introduced Asp^{90} has an ionized side chain.

The main purpose of the FTIR experiments was to characterize the protonation states of carboxylic acid groups in the dark state of mutant pigment G90D. In contrast to our previous study on G90D carried out at 5 °C, it was expected that at low temperature a putative C=O stretching vibration of protonated Asp^{90} or Glu^{113} would be better observable because absorption changes of other carboxylic acid groups are minimized. A corresponding carbonyl-stretching vibration not present in rhodopsin can be identified and assigned to Glu^{113}, in agreement with previous FTIR results (Zvyaga et al., 1996). We show that G90D replacement affects well-characterized vibrations of the 11-*cis*-retinal chromophore in a way which strongly supports the neutral state of Glu^{113}. Finally, it is shown how, given reasonable assumptions about the mutant pigment photoisomerization, altered chromophore−protein interactions in the batho-photoproduct of G90D can be explained by the lack of a negative charge on Glu^{113} as well.

MATERIALS AND METHODS

Construction and Preparation of Rhodopsin Mutants. The preparation of mutant opsins E113A, G90D, and G90D/E113A were previously reported (Sakmar et al., 1991; Zvyaga et al., 1996). Opsin genes were expressed in COS-1 cells, reconstituted with 11-*cis*-retinal, and purified as described (Franke et al., 1988, 1992; Zvyaga et al., 1994).

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 G90D, E113A, and G90D/E113A were purified for FTIR studies as reported (Fahmy et al., 1993). About 1 nmol of recombinant or con A-purified rhodopsin was dried under a stream of N_2 on a BaF_2 window. All samples contained 200 nmol of potassium phosphate buffer, pH 5. For mutant E113A, 200 nmol of NaCl was added to stabilize a protonated Schiff base pigment. H_2O/D_2O exchange was carried out at room temperature as reported (Fahmy et al., 1993) with the exception that the sample was previously converted to a stable iso-photoproduct at −185 °C. The iso-photoproduct was then recooled to −185 °C and converted to batho, thus causing an iso/batho difference spectrum in the presence of D_2O. This procedure allowed us to obtain H_2O and D_2O spectra from an identical sample, thereby saving sample material.

FTIR-Difference Spectroscopy. FTIR-difference spectra were obtained with a Bruker IFS-28 instrument with a liquid N_2-cooled MCT detector. Spectral resolution was 2 cm^{-1}. Interferograms ($n = 4096$) were averaged before and after illumination of the sample, and the difference spectra were calculated from the transformed single-channel spectra (Ganter et al., 1990). Photostationary mixtures of pigment states favoring the batho-intermediates were obtained from the recombinant pigments at −185 °C by irradiation with wavelengths between 420 and 450 nm (GG 420 and SWP 450 filters, Schott). The iso-photoproducts were enriched by illuminating the previously formed intermediates at the same temperature with wavelengths above 550 nm (OG 550 filter, Schott). Subsequent repetitions of these illumination conditions were used to generate batho/iso-difference spectra. For con A-purified rhodopsin, the filter combinations GG 420 and SWP 500, or OG 570, were used to enrich the photostationary mixtures for batho- and isorhodopsin, respectively. The different choice of filters for mutants versus rhodopsin takes into account the blue shift of the visible absorption of the dark states of the mutant pigments. Using a 150 W projector lamp and fiber optics, an illumination time of 30 s was sufficient in all cases for maximal photoproduct formation.

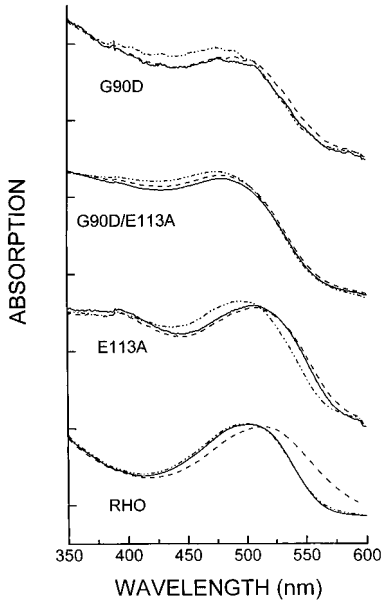

FIGURE 1: Absorption spectra of hydrated films of rhodopsin and mutant pigments in the presence of dodecyl maltoside detergent recorded at −185 °C. The samples contained 200-fold excess of phosphate buffer, pH 5. In the sample of E113A, 200 nmol of NaCl was added in order to stabilize the PSB by an inorganic chloride counterion. Illumination was performed as described in Materials and Methods. Solid lines: dark absorption. Dashed lines: batho-intermediates. Dotted lines: iso-intermediates. One ordinate unit corresponds to 20, 85, 30, and 270 mOD (from top to bottom). The extinction coefficients for the ground states of the pigments are 37 000, 32 000, 29 000, and 42 700 M^{-1} cm^{-1} for G90D, G90D/E113A, E113A, and rhodopsin, respectively (Zvyaga et al., 1993, 1995).

UV−Visible Absorption Spectroscopy. Spectroscopy was carried out on a Perkin-Elmer Lambda-17 instrument under conditions identical with those used for infrared spectroscopy with the exception that one-half the amount of recombinant material was used. Batho/iso absorption differences were recorded from four successive photoconversions, and the spectra were coadded to increase the signal-to-noise ratio. Data present in Figures 1 and 2 were not smoothed.

RESULTS

UV−Visible Spectroscopy of Mutant Pigments. Recombinant opsins with the amino acid replacements G90D, G90D/E113A, and E113A were expressed, regenerated with 11-*cis*-retinal, and purified in detergent as described (Zvyaga et al., 1996; Fahmy et al., 1993). Low-temperature visible absorption spectra from hydrated films of these mutants and native rhodopsin were recorded in order to characterize λ_{max} shifts of the mutant photoproducts versus those of rhodopsin. Figure 1 shows the absorption spectra measured in the dark and after either short- or long-wavelength illumination of hydrated films at −185 °C. Due to increased light scattering

in the film samples, the absolute wavelengths cannot be precisely determined, but the apparent peak positions are consistent with the previously reported λ_{max} values (Zvyaga et al., 1996). Upon illumination with blue light, all three recombinant pigments form batho-like intermediates, designated batho-intermediates or batho-photoproducts, as opposed to bathorhodopsin formed from native rhodopsin.[2] However, the bathochromic shifts are considerably smaller than those observed for rhodopsin. The accompanying visible absorption changes are about one-eighth of those usually observed. This is not due to partial photoconversion since photostationary mixtures of photoproducts and dark states are obtained after 15 s for each pigment and the compositions of these mixtures do not change upon prolonged illumination (10 min). Thus, a significantly lower quantum efficiency of batho formation cannot explain the reduced magnitude of absorption changes. In addition, bandpass filters were chosen to account for the blue-shifted visible absorption maxima of the mutant pigments (see Materials and Methods). Therefore, the small absorption changes during primary photoproduct formation must be caused by small intrinsic λ_{max} shifts in the mutants and/or increased quantum efficiency of iso-photoproduct formation from the mutant batho-intermediates. The latter possibility may explain the lack of clear absorption decreases in the "blue" part of the dark-state absorption band and the slight increase in this spectral range observed in mutant G90D/E113A. In any case, a larger fraction of iso-photoproducts in the photostationary mixture of the mutants versus that of rhodopsin may be present.

Similarly, the iso-intermediates obtained from the mutants with long-wavelength illumination differ from isorhodopsin. Again, the cutoff filter used for the conversion of the primary photoproducts to the iso-intermediates was selected according to the smaller λ_{max} value of the mutant batho-intermediates relative to that of bathorhodopsin. The batho-intermediates of G90D and G90D/E113A form iso-intermediates, which exhibit an increased absorption below 500 nm, rather than a clear band shift toward the blue because no significant absorption decrease above 500 nm is observed. This contrasts with the photoreaction of the E113A batho-intermediate, which forms an iso-photoproduct distinctly blue-shifted relative to both the batho-form and the dark state absorption. This property markedly distinguishes mutant E113A from rhodopsin, which displays a λ_{max} value only 5 nm larger than that of isorhodopsin. In spite of the spectral alterations described above, reversibility of the batho to iso photoconversion is retained in the three recombinant pigments.

The absorption changes of repeated batho/iso photoconversions are displayed in Figure 2. The positive and negative lobes of the traces for G90D and G90D/E113A are of similar size, whereas rhodopsin shows about 2 times as large a band for the disappearance of bathorhodopsin as compared with the formation of isorhodopsin. This observation also suggests that the spectral properties of the chromophores in the two photointermediates of the mutants are qualitatively different. The spacing of the positive and negative band centers is about 80 nm and is thus comparable to that of the normal batho/iso-difference spectrum, suggesting that the mutant photoproducts may exhibit a blue shift similar to that

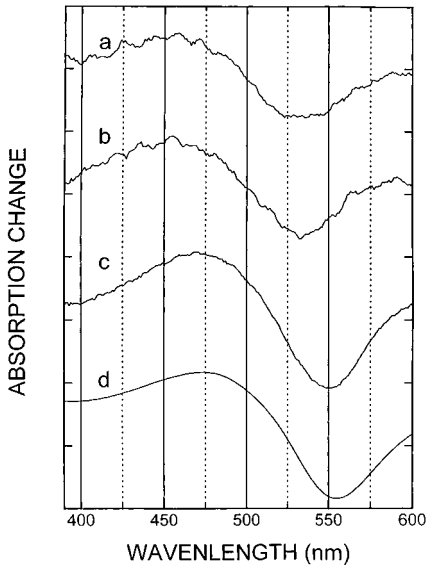

FIGURE 2: Absorption changes during batho- to iso-photoproduct transition. Four successive conversions were added to increase the signal-to-noise ratio for the absorption differences of the recombinant pigments: (a) G90D, (b) G90D/E113A, (c) E113A, and (d) rhodopsin. Conditions are as in the legend to Figure 1. One ordinate unit corresponds to 5 (a and b), 6 (c), and 75 (d) mOD.

of batho/isorhodopsin. However, comparison of the difference spectra of G90D and G90D/E113A with the absorbance spectra in Figure 1 shows that batho- and iso-photoproducts deviate from the corresponding rhodopsin intermediates. Similarly, one may argue that except for a blue shift of 5 nm, the difference spectrum of E113A resembles that of rhodopsin. However, this similarity is due to the compensating effects of an unusually small red shift of the batho-intermediate and an unusually large blue shift of the iso-photoproduct relative to the dark state absorption.

One may also argue that the small visible absorption changes during the batho-photoproduct formation of the mutant pigments are due to a decreased photoreaction rate rather than to small intrinsic red shifts. However, the batho- as well as the iso-photoproducts are readily formed within 15 s as is the case for rhodopsin. The actual proof of a photoconversion in the mutants similar to that of rhodopsin is provided by the magnitude of the infrared absorption changes described below. In summary, the results of visible spectroscopy indicate that the batho- and iso-intermediates of the mutant pigments differ from the normal photoproducts in a manner more complicated than a mere offset in their λ_{max} values as measured for the dark state absorptions. The recombinant pigments in which the native counterion Glu^{113} was replaced by alanine have in common that the red shift upon batho formation is greatly reduced. Thus, the change in the electrostatic chromophore environment is reflected in alterations of the visible spectroscopic properties of the dark states and low-temperature photoproducts. In particular, mutants G90D and G90D/E113A exhibit similar spectral phenotypes, arguing for similar electrostatic and/or steric chromophore environments in spite of the fact that Glu^{113} is present in mutant G90D.

FTIR-Difference Spectroscopy of Mutant Pigment G90D Photoproduct Formation. Figure 3 shows FTIR-difference spectra of mutant G90D and rhodopsin obtained at −185 °C with short-wavelength illumination under conditions identical with those used for the formation of the batho-like

[2] The nomenclature is applied to the mutant iso-intermediates as well.

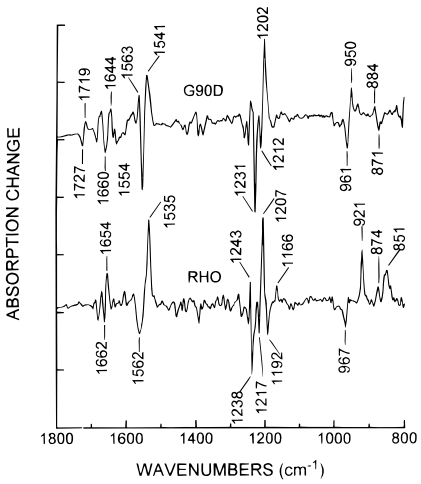

FIGURE 3: FTIR-difference spectra obtained by short-wavelength illumination of mutant G90D and rhodopsin. Absorption bands of the dark states are negative; those of the photoproducts are positive. Conditions are identical with the visible control experiments shown in Figure 1; 1 nmol of material was used, and one ordinate unit corresponds to 2 mOD.

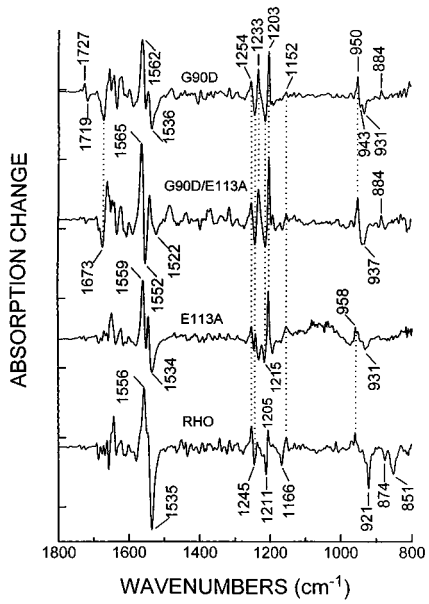

FIGURE 4: Batho/iso-difference spectra of mutant pigments and rhodopsin in H_2O recorded at −185 °C. Four successive photoconversions were added to a total of 4096 scans/trace. Absorption bands of the batho-intermediates are negative; those of the iso-intermediates are positive. One ordinate unit corresponds to 2, 1.2, 1.2, and 1.8 mOD (from top to bottom).

intermediates in Figures 1 and 2. The maximal absorption change of the fingerprint modes between 1150 and 1300 cm^{-1} in about 1 nmol of recombinant pigment is 6 mOD and thus is almost identical with that of 1 nmol of rhodopsin. Therefore, the amount of photoreacted chromophore is comparable in both pigments in spite of the small visible absorption changes described in the previous section. Based on the UV−visible absorption changes alone, only about one-eighth of the normal photoconversion would have been predicted. The significantly larger infrared absorption changes lend further support to changed intrinsic λ_{max} values of the photoproducts rather than reduced photoreaction efficiencies. The difference spectrum of bathorhodopsin is identical with that from washed ROS membranes (Bagley et al., 1985; DeGrip et al., 1988; Ganter et al., 1989; Kandori & Maeda, 1995). The spectrum is dominated by a difference band in the 1530−1560 cm^{-1} range where amide II and C═C stretching modes absorb. In spite of the potential overlap of both absorptions, red-shifted photoproducts of protonated retinal Schiff bases are often detected by a corresponding down shift of their strong C═C stretching mode. This is obvious for rhodopsin where the positive band at 1535 cm^{-1} is caused by the C═C stretching mode of bathorhodopsin as demonstrated by resonance Raman spectroscopy, which selects for chromophore vibrations only (Eyring & Mathies, 1979; Oseroff & Callender, 1974). Similarly, the 1541 cm^{-1} band of the mutant photoproduct is likely to partially reflect formation of the batho-intermediate identified by visible spectroscopy. Based on the correlation between the visible absorption and the C═C stretching frequency of polyenes (Doukas et al., 1978; Rimai et al., 1973), the higher frequency of the mutant photoproduct band can be explained by the smaller λ_{max} value of the mutant batho-intermediate. Another intense band of the batho-intermediate is found at 1207 cm^{-1} (i.e., in a range typical of C−C stretches coupled to C−H bending modes). This band is shifted to 1202 cm^{-1} in the mutant photoproduct, which again agrees with the formation of an intermediate with a smaller λ_{max} value than that of bathorhodopsin, since a lower degree of the π-electron delocalization can be inferred from the blue-shifted visible absorption. A larger downshift can be observed for the "C_{12}−C_{13}" stretch of the dark state at 1238 cm^{-1}, which in the mutant is found at 1231 cm^{-1}. This has been reported earlier (Fahmy et al., 1994; Jäger et al., 1994; Zvyaga et al., 1996) and will be discussed in more detail below. In essence, the frequency drop of this delocalized mode appears to be indicative of the neutralization of Glu^{113}.

As suggested by the visible control spectra, the iso-photoproduct contributes to the infrared absorption changes even under conditions of 450 nm band-pass illumination. This is shown by the fact that the intensity of the 1563 cm^{-1} band varies upon alteration of the wavelength of illumination (not shown). Therefore, this band reflects absorption of the blue-shifted mutant iso-intermediate.

The wavelength-dependent generation of mixtures of batho- and iso-intermediates is well-established for rhodopsin. The contribution of isorhodopsin is also mainly reflected in the C═C stretching frequency range because prominent fingerprint frequencies shift only little between batho- and isorhodopsin (Figure 4). Particularly, both photoproducts exhibit a strong absorption in the 1205−1210 cm^{-1} range caused by the C_{14}−C_{15} and C_8−C_9 stretching vibrations (Palings et al., 1987). Therefore, the strong positive band at 1202 cm^{-1} indicates that illumination of G90D forms an *all-trans* and 9-*cis* isomeric mixture in which the chromophores exhibit a lower degree of π-electron delocalization rather than unusual retinal isomers. A small band at ∼1154 cm^{-1} is typically observed in batho/iso-difference spectra and has been specifically assigned to the C_{10}−C_{11} stretching vibration of 9-*cis*-retinal in isorhodopsin. In addition, the stability of the low-temperature iso-photoproduct of G90D at room temperature (see Materials and Methods) parallels the behavior of normal isorhodopsin. Likewise, room temperature flash photolysis experiments agree with C_{11}═C_{12} double-bond isomerization as the primary event in G90D photoactivation leading to an *all-trans*-retinal-containing batho state which is less red-shifted than bathorhodopsin (Jäger et al., submitted). FTIR spectra of the transducin-

activating MII-like form of G90D also show chromophore protein interactions typically observed in the *all-trans*-retinal-containing native MII (Zvyaga et al., 1996). These results suggest that the isomerization pattern of the chromophore in G90D is identical with that in rhodopsin.

Regardless of the altered amounts of the two photoproducts formed, however, some spectral features of the mutant differ significantly from the native batho-difference spectrum and cannot be explained by the presence of an isoform. The coupled $C_{11}-H$, $C_{12}-H$ hydrogen-out-of-plane (HOOP) vibration of the 11-*cis* chromophore of rhodopsin at 967 cm^{-1} is shifted to 961 cm^{-1} in the dark state of the mutant as observed previously (Zvyaga et al., 1996). A mutational effect near C_{12} is also obvious for the batho-photoproduct as shown by the complete absence of the intense band at 921 cm^{-1}, which has been assigned to the 11-HOOP mode uncoupled from the 12-HOOP vibration in bathorhodopsin (Eyring et al., 1982; Palings et al., 1989). Instead, two new positive bands at 931 and 950 cm^{-1} are present. Since the visible controls show that a batho-intermediate is formed, the lack of the 921 cm^{-1} absorption indicates a different pattern of HOOP vibrations in the batho-photoproduct of G90D rather than the lack of such an intermediate. In contrast to rhodopsin, which does not show absorptions below 960 cm^{-1}, the dark state of G90D displays a negative band at 871 cm^{-1} that shifts to 884 cm^{-1} in the photoproduct(s). Since the infrared magnitude of HOOP mode absorption is increased upon single-bond twisting (Fahmy et al., 1991), the 11-*cis* chromophore of the dark state of G90D may be slightly distorted versus that of dark rhodopsin. Similarly, the lack of the intense HOOP vibrations between 840 and 880 cm^{-1} usually observed in the distorted *all-trans*-retinal chromophore of bathorhodopsin indicates that the chromophore is less distorted in the mutant photoproduct(s).

A distinct difference band at 1727 cm^{-1} (negative)/1719 cm^{-1} (positive) is visible in the mutant difference spectrum in a spectral range typical of C═O stretching frequencies. This band is sensitive to H_2O/D_2O exchange, which causes a frequency drop of 3 and 6 cm^{-1} in the negative and positive parts of the difference band, respectively (data not shown). Thus, the band can be assigned to the C═O stretching mode of a protonated carboxylic acid already present in the dark state of mutant G90D. Only very small absorbance changes between 1700 and 1800 cm^{-1} are observed upon formation of either batho- or isorhodopsin.

Batho/Iso FTIR-Difference Spectra of G90D, E113A, and G90D/E113A. A more detailed interpretation of spectral features in Figure 3 is impeded by the possible contributions of vibrations caused by the iso-photoproduct. In particular, it cannot be decided whether or not the 11-HOOP mode of the batho-intermediate has shifted from 921 to 950 cm^{-1} because isorhodopsin is also expected to absorb at this position. Therefore, we have based all further spectral comparisons among mutants and rhodopsin on batho/iso-difference spectra obtained by long-wavelength illumination of the previously formed batho-intermediates. The corresponding absorption changes offer the possibility to distinguish between iso- and batho-intermediates because the respective bands differ in sign in contrast to their superposition with positive sign when formed by illumination of the dark state. With the selected band-pass and cutoff filters, no appreciable change in the steady-state concentration of the initial state carrying the 11-*cis* isomer is observed (as shown by the lack of the 961 cm^{-1} vibration), but the signal-to-noise ratio is reduced. This is due to the large spectral overlap of the batho- and iso-photoproduct absorptions which prevents selective photoconversion of only one species in spite of proper wavelength selection. Therefore, the wavelength-dependent concentration changes of the batho- and iso-photoproduct are small although the total amount of initial photoproduct formation is about normal. This drawback can be compensated for by coaddition of difference spectra obtained by alternating short- and long-wavelength illuminations, which causes precisely reversed spectral changes (data not shown).

Carbonyl-Stretching Absorptions. Figure 4 shows the batho/iso difference spectra obtained with G90D, G90D/E113A, E113A, and rhodopsin by successive photoconversions between both intermediates. The C═O stretching vibration of a protonated carboxylic acid of the dark state of mutant G90D at 1727 cm^{-1} (Figure 3) is clearly visible in the iso-intermediate, whereas it absorbs at 1719 cm^{-1} in batho. This difference band disappears upon the additional replacement of Glu^{113} in G90D/E113A. Therefore, the band can be assigned to Glu^{113}, which must be protonated in the dark state of G90D as well as in its low-temperature photoproducts. Except for the 1719/1727 cm^{-1} difference band, the small absorption changes between 1700 and 1800 cm^{-1} usually observed during the batho/iso transition are reproduced in the difference spectra of the mutants.

Hydrogen-Out-of-Plane Vibrations. If the location of the counterion in the dark state of G90D is different from that in rhodopsin as suggested by the UV−visible spectra**,** the down shift of the "$C_{12}-C_{13}$" stretching mode, and the protonated state of Glu^{113}, it is expected that chromophore vibrations in the photoproducts should also be affected as a consequence of an altered immediate electrostatic environment of the PSB. Resonance Raman spectroscopy has indeed identified an influence of Glu^{113} replacement on the 11-HOOP mode of the *all-trans*-retinal-containing batho-intermediate of mutant E113A, which shifts from 920 to 934 cm^{-1} when chloride serves as counterion (Lin et al., 1992). A similar shift can be observed in the FTIR-difference spectrum of this mutant (Figure 4) in which the batho-photoproduct exhibits a band at 931 cm^{-1} (negative). Concomitantly, a band at 858 cm^{-1}, which can be discerned as a shoulder on the band at 851 cm^{-1} in bathorhodopsin and has been assigned to the 12-HOOP vibration, has vanished in the batho-intermediate of E113A. Both spectral alterations can be interpreted as increased coupling between the 11- and 12-HOOP modes in the mutant photoproduct (Lin et al., 1992). Apparently, the position of the infrared-active 11- and 12-HOOP modes can be related to the charge environment of the PSB in a similar manner as the resonance Raman bands.

Accordingly, the absence of a negative charge at position 113 is distinctly reflected in the frequencies of HOOP vibrations of the batho-photoproduct of G90D/E113A. The reproduced lack of a 858 cm^{-1} band and an upshift of the putative 11-HOOP frequency of G90D/E113A to 937−940 cm^{-1} may indicate slightly stronger coupling of the 11- and 12-HOOP modes than in E113A. In addition, the location or nature of the alternative counterion (Asp^{90} or chloride) seems to affect the magnitude of the frequency shift. Relying on this diagnostic value of the 11- and 12-HOOP modes in the mutant batho-intermediates, the FTIR-difference spectrum

of G90D strongly indicates that also in the batho-photoproduct no negative charge is present at position 113 because again the 921 cm^{-1} vibration has disappeared in favor of bands between 930 and 945 cm^{-1} and no 12-HOOP absorption is observed at 858 cm^{-1}. In contrast to the rho/batho-difference spectrum in Figure 3, it is now clear that the 11-HOOP frequency of the batho-intermediate has not shifted to 950 cm^{-1}. The absorption at this frequency is due to the iso-intermediate. Similarly, the 884 cm^{-1} band is not due to the batho-intermediate. Therefore, the batho/iso-difference spectra lack any batho-intermediate bands between 840 and 880 cm^{-1} that are of comparable size to those in native bathorhodopsin. The HOOP modes of the iso-intermediates are obviously less sensitive to the electrostatic alterations of the chromophore environment than those in bathorhodopsin. The frequency shift does not exceed 8 cm^{-1}, and no shift at all occurs in the iso-intermediate of E113A. However, the precise coincidence of the positive 950 cm^{-1} bands in mutants G90D and G90D/E113A versus the absorption at 958 cm^{-1} in mutant E113A and rhodopsin argues for similar chromophore-binding sites in the former two mutants.

Fingerprint Vibrations. The results from mutants E113A and G90D/E113A demonstrate that the HOOP vibrational modes near C_{12} of the *all-trans* chromophore in the batho-photoproducts are affected by the removal of the native counterion. It is thus interesting to investigate other chromophore vibrations that involve movements at or near C_{12} in order to draw conclusions about the chromophore environment in G90D. We have already pointed out that the $C_{12}-C_{13}$ stretching mode of the dark state of all three mutants is downshifted to 1231 cm^{-1} as previously observed in mutants with neutral amino acids at position 113. Since this mode is delocalized over the $C_{10}-C_{15}$ segment of the chromophore (Ganter et al., 1988 a,b; Palings et al., 1987), it may be sensitive to a variety of factors. In rhodopsin, no frequency shift of the $C_{12}-C_{13}$ stretching mode occurs during batho to iso photoconversion, which explains the lack of any difference band between 1230 and 1240 cm^{-1} in the batho/iso-difference spectra (Figure 4). In mutant E113A, the 1231 cm^{-1} (negative) vibration of batho shifts up to 1241 cm^{-1} (positive) in iso, thereby weakening the usually observed negative band at 1245 cm^{-1}.

Similarly, the frequencies of the putative $C_{12}-C_{13}$ stretching modes of the batho-intermediates of mutants G90D and G90D/E113A seem to differ from those of the iso-intermediates as well. The $C_{12}-C_{13}$ stretch is probably causing the 1233 cm^{-1} absorption in the iso-intermediates. The lower frequency as compared to the corresponding mode in the iso-intermediate of E113A leaves visible the negative band at 1245 cm^{-1} as usual. The appearance of difference bands attributable to the $C_{12}-C_{13}$ mode in the mutants shows that the removal of the native counterion affects the C_{12} environment differently in the batho- and iso-intermediates. Irrespective of the detailed mechanism by which the lack of a counterion at position 113 affects the C−C single-bond-stretching vibrations, the perfect coincidence of the difference bands of G90D and G90D/E113A further supports the neutral state of Glu^{113} in favor of a charged Asp^{90} side chain in mutant G90D. The same holds for the pronounced 1215 cm^{-1} (negative)/1203 cm^{-1} (positive) difference band, probably caused by the $C_{14}-C_{15}$ stretching mode usually observed at 1211/1205 cm^{-1}(Palings et al., 1987), which is observed in all three mutants.

Amide Vibrations. On the basis of the apparently similar chromophore-binding sites in mutants G90D and G90D/E113A, which is reflected in similar if not identical difference bands of chromophore vibrations between 800 and 1250 cm^{-1}, one would also expect similar protein conformational changes to occur in both mutants. This is supported by the band pattern between 1630 and 1680 cm^{-1} where almost identical difference bands typical of amide I absorption changes (predominantly peptide carbonyl-stretching vibrations) are observed for G90D and G90D/E113A. These changes are different from those occurring both in rhodopsin and in mutant E113A and exhibit larger band intensities. This is particularly obvious for the band at 1673 cm^{-1} in the batho-photoproducts of G90D and G90D/E113A. The absorption changes in the amide II spectral range between 1540 and 1570 cm^{-1} (predominantly peptide N−H bending coupled to C−N stretching) are expected to reproduce the same pattern of spectral similarities among the different pigments since they are caused by the identical conformational changes as the amide I absorption differences. However, this spectral region contains the largest deviations between the spectra of G90D and G90D/E113A. This is probably caused by different C═C stretching modes, which contribute to absorption changes in the same frequency range. Due to the difference in the visible absorption maximum, the C═C stretching frequency of the dark state of mutant G90D/E113A is expected to be higher than that of mutant G90D. This does not contradict the nearly identical fingerprint absorption changes because they are strongly coupled to the C−H bending modes, which renders them much less sensitive to the degree of bond conjugation than the C═C stretching modes (Fahmy & Siebert, 1990).

In summary, the FTIR-difference spectra reveal a protonated carboxylic acid in G90D, which is not present in rhodopsin and can be assigned to Glu^{113}. Peptide conformational changes during the batho/iso-intermediate transition are different from those in rhodopsin and more pronounced. In addition, well-characterized vibrations of the retinal chromophore in the batho- and iso-photoproduct of G90D are affected in a way which is quantitatively nearly identical with the mutational effects observed in the double mutant G90D/E113A. Qualitatively, these spectral deviations from rhodopsin resemble those of the single mutant E113A. The spectra of E113A and G90D/E113A strongly indicate that a perturbation near C_{12} of the retinal chromophore in bathorhodopsin is removed, thereby allowing partial coupling of the 11- and 12-HOOP modes in the batho-intermediates. This lends further support to the proposed interaction between Glu^{113} and the PSB chromophore near C_{12} in native rhodopsin and bathorhodopsin (Han & Smith, 1995a). The striking similarities of the spectral phenotypes of G90D and G90D/E113A with respect to this specific chromophore−protein interaction provide a strong argument for a neutral state of Glu^{113} in mutant G90D.

DISCUSSION

Spectroscopic Properties of Mutant Pigments. We have obtained low-temperature visible- and FTIR-difference spectra of detergent-solubilized rhodopsin and the rhodopsin mutants G90D, G90D/E113A, and E113A regenerated with 11-*cis*-retinal. In contrast to con A-purified rhodopsin, all three mutant pigments exhibit unusually small red shifts of the absorption maxima of their primary batho-photoproducts

at −185 °C. Likewise, the iso-photoproducts of the mutants differ from isorhodopsin. These deviations are not caused by a general blue shift of the photoproduct absorption similar to the blue-shifted absorption maxima of the dark states. Since the electrostatic environment of the PSB is an important determinant of the visible absorption maximum (Blatz et al., 1972) [for a theoretical treatment see Birge (1990) and Tallent et al. (1992)], the spectral deviations in the absorption maxima of different mutant pigment states from those of rhodopsin may roughly reflect different positions of the counterions relative to the retinal chromophore rather than grossly altered geometries of the chromophore-binding pockets. Based on this simplified model, the data suggest that the position of the counterion has changed in all three mutant pigments. This assertion is trivial for mutants E113A and G90D/E113A in which the native counterion Glu^{113} has been replaced by chloride or Asp^{90}, respectively. Surprisingly, however, the spectral properties of the mutant G90D are almost identical with those of G90D/E113A. Therefore, the data strongly indicate that also in mutant G90D the position of the counterion has changed even though Glu^{113} is still present. This conclusion is confirmed by the FTIR difference spectra which reveal a protonated carboxylic acid side chain of Glu^{113} in both the dark state of G90D and the low-temperature photoproducts.

In contrast to our previous study on MII formation by G90D (Zvyaga et al., 1996), the assignment of the new C═O stretching mode at 1727 cm^{-1} to Glu^{113} is markedly facilitated by the fact that at low temperature overlapping absorption changes of other internal carboxylic acid residues are negligible. However, Glu^{113} and Asp^{90} may be expected to compete electrostatically for ion pairing with the PSB. Therefore, an assignment of the C═O stretching band in the dark state of G90D to protonated Asp^{90}, rather than Glu^{113}, would also be compatible with the lack of this band in G90D/E113A if one assumes deprotonation of Asp^{90} as a consequence of the removal of a negative charge at Glu^{113} in the double mutant. These considerations render comparisons in other spectral regions indispensable for the verification of the band assignment to the carbonyl of protonated Glu^{113}.

Spectroscopic evidence has led to a picture of the retinal-binding site that places Glu^{113} near C_{12} of the retinal polyene (Han et al., 1993; Han & Smith, 1995a,b; Tallent et al., 1992). Neutralization of Glu^{113} can thus be expected to affect chromophore vibrations which involve C_{12} movement. We consider the unusually low $C_{12}-C_{13}$ stretching frequency of the 11-*cis* chromophore in the dark state of G90D highly indicative of the removal of a negative charge at position 113, whereas it appears that this mode is rather independent of the degree of π-electron delocalization. This is shown by the fact that previously studied mutant pigments with neutral amino acids at position 113 exhibit the same 7 cm^{-1} downshift of their $C_{12}-C_{13}$ stretching frequency (Fahmy et al., 1994; Jäger et al., 1994; Zvyaga et al., 1996). On the other hand, replacement of Glu^{113} by Asp in E113D does not shift the $C_{12}-C_{13}$ frequency but does induce a red shift of the λ_{max} to 510 nm (Jäger et al., 1994; Sakmar et al., 1991). Likewise, the mutant E122D exhibits the $C_{12}-C_{13}$ stretching mode at 1238 cm^{-1} but has a λ_{max} of 475 nm (Sakmar et al., 1989) close to that of G90D/E113A. Similarly, the mutant E122Q absorbs maximally at 480 nm (Sakmar et al., 1989) (i.e., close to the λ_{max} of mutant G90D), yet the $C_{12}-C_{13}$ stretching mode is not altered (Fahmy et al., 1993).

In a resonance Raman study on the nature of λ_{max} shifts, the diagnostic value of the fingerprint vibrations of 11-*cis*-retinal for the charge environment of the chromophore has also been employed (Lin et al., 1994). It was shown that the $C_{12}-C_{13}$ stretching frequency in iodopsin was downshifted versus that in rhodopsin by only ∼3 cm^{-1} in spite of the 70 nm red shift of the visible absorption, arguing against new protein perturbations associated with the larger opsin shift. The 2-fold larger frequency shift of the $C_{12}-C_{13}$ stretching mode observed here, as well as the complete consistency with other mutant data, allows the conclusion that a negative charge at position 113 has been removed in the dark state of G90D. Thus, we assign the deuterium-sensitive 1727 cm^{-1} band, which disappears upon additional replacement of Glu^{113} in mutant G90D, to the C═O stretch of protonated Glu^{113} rather than assuming an indirect effect from Asp^{90}. Consequently, the Gly^{90} to Asp replacement causes protonation of Glu^{113} via electrostatic competition for ion pairing with the PSB. This abolishes the negative charge of Glu^{113} usually present near C_{12} of the retinal chain and thus affects chromophore vibrations involving C_{12}.

This mechanism may also explain the downshift of the 11,12-HOOP of 11-*cis*-retinal in the dark state of G90D at 961 cm^{-1}. In addition, it rationalizes the general similarity if not coincidence of chromophore vibrational bands observed in the low-temperature photoproducts of G90D and G90D/E113A. Glu^{113} is already neutral in G90D, thereby rendering additional effects by the Glu^{113} to Ala replacement in G90D small. More specifically, the lack of the uncoupled 11-HOOP and 12-HOOP vibrations of bathorhodopsin at 921 and 858 cm^{-1}, respectively (Eyring et al., 1982; Palings et al., 1989) in the mutant batho-intermediates can be explained by the protonated state of Glu^{113} as well as by the appearance of new HOOP absorptions above 930 cm^{-1}. Since single-bond torsions have been shown to be insufficient to cause the unusual uncoupling of both HOOP modes in the *all-trans* chromophore of bathorhodopsin (Birge et al., 1988; Eyring et al., 1980; Warshel & Barboy, 1982), an electrostatic perturbation near C_{12} has been assumed (Palings et al., 1989).

In agreement with the model of the retinal-binding site and its neutral state (Birge et al., 1985), Glu^{113} is the only candidate for this perturbation. Protonation or replacement of this residue by a neutral amino acid is therefore expected to partially reintroduce coupling between both modes to a degree which may be limited by the remaining single-bond torsions in the *all-trans* chromophore. Resonance Raman spectra of E113A suggest that the $C_{12}-H$ wag is indeed less perturbed, allowing its frequency to shift upward. Thus, the $C_{12}-$wag may couple weakly with the C_{11}-H wag, pushing it up in frequency above 930 cm^{-1} (Lin et al., 1992). The batho/iso-difference spectra of E113A are entirely consistent with the resonance Raman data. The analogous spectral features in the batho/iso-difference spectrum of G90D strongly indicate that reintroduction of weak vibrational coupling in the batho-intermediate has occurred in G90D as well.

The simplest explanation for this deviation from normal bathorhodopsin is that the neutral state of Glu^{113} reduces the electrostatic perturbation of the chromophore similar to the replacement of this residue by alanine. Since the coupling strength of HOOP modes is roughly proportional to the bond order of the involved C═C double bond (Curry et al., 1985), the increased coupling versus bathorhodopsin argues for a

higher electron density in the $C_{11}=C_{12}$ double bond in all three recombinant pigments. This may be a direct consequence of the lack of the nearby negative charge of Glu^{113}. Simultaneously, single-bond torsions in the batho-photoproducts are reduced as evidenced by the lack of intense HOOP absorptions (Fahmy et al., 1991). This may indicate that relaxation to an almost planar *all-trans* geometry is possible in the mutants even at −185 °C. The larger amide I absorption changes in mutants G90D and G90D/E113A suggest that their chromophore-binding sites have become particularly flexible to relieve sterical strain on the chromophore usually generated upon bathorhodopsin formation. Apparently, the geometry of electrostatic interactions between the PSB chromophore and a localized counterion influences the partitioning of sterical strain between opsin and retinal.

The above evaluation of HOOP modes is based on the formation of a nominally *all-trans* chromophore in the batho-photoproduct of all G90D in spite of the altered electrostatic chromophore environment. This is strongly supported by the chromophore infrared absorption changes displayed in Figure 3 as well as by the additional evidence presented in the Results section. Likewise, resonance Raman data on E113A have shown that the lack of the native counterion does not interfere with the formation of the normal *all-trans* and 9-*cis*-retinal isomers in the low-temperature photoproducts (Lin et al., 1992). However, the native counterion Glu^{113} has been implicated in the mechanism of barrierless excited state isomerization in rhodopsin, which is thought to be responsible for the high quantum efficiency of MII formation (Tallent et al., 1992). It may therefore be expected that the efficiency of low-temperature photoproduct formation in G90D is reduced due to the altered location of the counterion at position 90. In a previous study on E113Q (Fahmy & Sakmar, 1993), we employed nonsaturating illumination conditions as contrasted by the short but saturating irradiation times applied here. It was found that the lack of the native counterion did not significantly reduce the efficiency of MII formation. In addition, photoisomerization was shown to be not necessarily linked to distinct UV−visible absorption changes. This parallels the results presented here which are consistent with a nearly normal photoreactivity of the G90D dark state in spite of small accompanying UV−visible absorption changes. Thus, the predominant effect of the changed counterion location in G90D is exerted on the visible absorption and on single-bond twists in the dark state and the photoproducts rather than on the photoisomerization process.

Possible Implications for Congenital Night Blindness. The low-temperature spectroscopic data from mutant G90D strongly indicate the occurrence of electrostatic competition between Asp^{90} and Glu^{113} for interaction with the PSB. The result is a neutral Glu^{113} side chain in the mutant G90D pigment. Consequently, the retinal-binding site in G90D stays neutral in spite of the introduction of a potentially extra negative charge. This supports the molecular model for constitutive activity of G90D opsin, which assumes breakage of a salt bridge between Glu^{113} and Lys^{296} (Cohen et al., 1992) in favor of an electrostatic interaction with Asp^{90} (Rao et al., 1994). However, the G90D opsin readily regenerates with 11-*cis*-retinal to form a pigment that is inactive in the dark and present in excess over the unregenerated molecules (Rao et al., 1994; Zvyaga et al., 1996). Thus, the properties of the regenerated holoprotein may contribute to the pathological trait as well. Our data show that the dark state of G90D carries a protonated side chain of Glu^{113} which is usually only formed in MII (Jäger et al., 1994). Thus an important feature of the transducin-activating receptor state is already anticipated in the dark. This partial similarity to MII may favor thermal transition of the dark state to an active conformation by either facilitating a structural change of a cytoplasmic domain or by facilitating thermal isomerization of the retinal chromophore. The electrostatic interaction between Glu^{113} and the PSB chromophore in native rhodopsin has been suggested to contribute to the energy barrier for thermal chromophore isomerization (Birge & Barlow, 1995). Thus it is reasonable to assume that the altered electrostatic environment of the chromophore in G90D may effect the rate of thermal *cis*/*trans* isomerization. The increased single-bond torsion deduced for the 11-*cis*-retinal chromophore of mutant G90D indicates destabilization of the dark state. The more relaxed geometry inferred for the batho-like photoproduct argues for stabilization of the isomeric state that ultimately leads to the active receptor conformation. Consequently, the energy gap between the 11-*cis* and *all-trans* chromophore states may be reduced in this mutant, and thermal isomerization to a MII-like form may thus occur in the dark. This would agree with the proposition that "dark−light" raises the threshold for dim-light perception in patients with congenital night blindness (Sieving et al., 1995). Models that implicate an extra negative charge in the retinal binding site of G90D as a molecular cause for the disease are not consistent with our results.

REFERENCES

Bagley, K. A., Balogh-Nair, V., Croteau, A. A., Dollinger, G., Ebrey, T. G., Eisenstein, L., Hong, M. K., Nakanishi, K., & Vittitow, J. (1985) *Biochemistry 24*, 6055−6071.

Birge, R. R. (1990) *Biochim. Biophys. Acta 1016*, 293−327.

Birge, R. R., & Barlow, R. B. (1995) *Biophys. Chem. 55*, 115−126.

Birge, R. R., Einterz, C. M., Knapp, H. M., & Murray, L. P. (1988) *Biophys. J. 53*, 367−385.

Birge, R. R., Murray, L. P., Pierce, B. M., Akita, H., Balogh-Nair, V., Findsen, L. A., & Nakanishi, K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4117−4121.

Blatz, P. E., Mohler, J. H., & Navangul, H. V. (1972) *Biochemistry 11*, 848−855.

Cohen, G. B., Oprian, D. D., & Robinson, P. R. (1992) *Biochemistry 31*, 12592−12601.

Curry, B., Palings, I., Broek, A. D., Pardoen, J. A., Lugtenburg, J., & Mathies, R. A. (1985) *Adv. Infrared Raman Spectrosc. 12*, 115−178.

DeGrip, W. J., Gray, D., Gillespie, J., Bovee, P. H. M., van den Berg, E. M. M., Lugtenburg, J., & Rothschild, K. J. (1988) *Photochem. Photobiol. 48*, 497−504.

Doukas, A. G., Aton, B., Callender, R. H., & Ebrey, T. G. (1978) *Biochemistry 17*, 2430−2435.

Emeis, D., Kühn, H., Reichert, J., & Hofmann, K. P. (1982) *FEBS Lett. 143*, 29−34.

Eyring, G., & Mathies, R. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 33−37.

Eyring, G., Curry, B., Mathies, R. A., Fransen, R., Palings, I., & Lugtenburg, J. (1980) *Biochemistry 19*, 2410−2418.

Eyring, G., Curry, B., Broek, A., Lugtenburg, J., & Mathies, R. (1982) *Biochemistry 21*, 384−393.

Fahmy, K., & Siebert, F. (1990) *Photochem. Photobiol. 51*, 459−464.

Fahmy, K., Siebert, F., & Tavan, P. (1991) *Biophys. J. 60*, 989−1001.

Fahmy, K., Jäger, F., Beck, M., Zvyaga, T. A., & Sakmar, T. P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10206−10210.

Fahmy, K., Siebert, F., & Sakmar, T. P. (1994) *Biochemistry 33*, 13700−13705.
Franke, R. R., Sakmar, T. P., Oprian, D. D., & Khorana, H. G. (1988) *J. Biol. Chem. 263*, 2119−2122.
Franke, R. R., Sakmar, T. P., Graham, R. M., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 14767−14774.
Ganter, U. M., Gärtner, W., & Siebert, F. (1988a) *Biochemistry 27*, 7480−7488.
Ganter, U. M., Schmid, E. D., & Siebert, F. (1988b) *Photochem. Photobiol. 2*, 417−426.
Ganter, U. M., Schmid, E. D., Perez-Sala, D., Rando, R. R., & Siebert, F. (1989) *Biochemistry 28*, 5954−5962.
Han, M., & Smith, S. O. (1995a) *Biochemistry 34*, 1425−1432.
Han, M., & Smith, S. O. (1995b) *Biophys.Chem. 56*, 23−29.
Han, M., DeDecker, B. S., & Smith, S. O. (1993) *Biophys. J. 65*, 899−906.
Honig, B., Dinur, U., Nakanishi, K., Balogh-Nair, V., Gawinowicz, M. A., Arnaboldi, M., & Motto, M. G. (1979) *J. Am. Chem. Soc. 101*, 7084−7086.
Jäger, F., Sakmar, T. P., & Siebert, F. (1993) in *Fifth international conference on the spectroscopy of biological molecules* (Theophanides, T., Anastassopoulou, J., & Fotopoulos, N., Eds.) pp 223−226, Kulwer Academic Publishers, Dordrecht, The Netherlands.
Jäger, F., Fahmy, K., Sakmar, T. P., & Siebert, F. (1994) *Biochemistry 33*, 10878−10882.
Jäger, S., Lewis, J. W., Zvyaga, T. A., Szundi, I., Sakmar, T. P., & Kliger, D. S. *Biochemistry* (submitted).
Kandori, H., & Maeda, A. (1995) *Biochemistry 34*, 14220−14229.
Kibelbek, J., Mitchell, D. C., Beach, J. M., & Litman, B. J. (1991) *Biochemistry 30*, 6761−6768.
König, B., Arendt, A., McDowell, J. H., Kahlert, M., Hargrave, P. A., & Hofmann, K. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6878−6882.
Lin, S. W., Sakmar, T. P., Franke, R. R., Khorana, H. G., & Mathies, R. A. (1992) *Biochemistry 31*, 5105−5111.
Lin, S. W., Imamoto, Y., Fukada, Y., Shichida, Y., Yoshizawa, T., & Mathies, R. A. (1994) *Biochemistry 33*, 2151−2160.
Nathans, J. (1990) *Biochemistry 29*, 937−942.
Oseroff, A. R., & Callender, R. H. (1974) *Biochemistry 13*, 4243−4248.
Palings, I., Pardoen, J. A., van den Berg, E., Winkel, C., Lugtenburg, J., & Mathies, R. A. (1987) *Biochemistry 26*, 2544−2556.
Palings, I., van den Berg, E., Lugtenburg, J., & Mathies, R. A. (1989) *Biochemistry 28*, 1498−1507.
Rao, V. R., Cohen, G. B., & Oprian, D. D. (1994) *Nature 367*, 639−642.
Rimai, L., Heyde, M. E., & Gill, D. (1973) *J. Am. Chem. Soc. 95*, 4493−4501.
Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309−8313.
Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3079−3083.
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.
Tallent, J. R., Hyde, E. W., Findsen, L. A., Fox, G. C., & Birge, R. R. (1992) *J. Am. Chem. Soc. 114*, 1581−1592.
Warshel, A., & Barboy, N. (1982) *J. Am. Chem. Soc. 104*, 1469−1476.
Zhukovsky, E. A., & Oprian, D. D. (1989) *Science 246*, 928−930.
Zvyaga, T. A., Min, K. C., Beck, M., & Sakmar, T. P. (1993) *J. Biol. Chem. 268*, 4661−4667; correction: (1994) *J. Biol. Chem. 269*, 13056.
Zvyaga, T. A., Fahmy, K., & Sakmar, T. P. (1994) *Biochemistry 33*, 9753−9761.
Zvyaga, T. A., Fahmy, K., Siebert, F., & Sakmar, T. P. (1996) *Biochemistry 35*, 7536−7545.

BI961486S