Slow Binding of Retinal to Rhodopsin Mutants G90D and T94D†

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ABSTRACT: In an examination of the effect of three rhodopsin night blindness mutations on the rate of association of 11-*cis*-retinal with opsin, one of the mutations (G90D) was found to slow the rate of reaction by more than 80-fold. This effect does not appear to be general to night blindness mutations as the two other mutants (A292E and T94I) were not found to bind retinal with slowed kinetics. However, T94D was similar to G90D in that the rate of retinal binding was dramatically slowed. Gly90 and Thr94 are both located in the active site of the protein close to the Schiff base counterion Glu113. Thus, the slow kinetics of Schiff base formation appear to correlate with the introduction of a negative charge close to the Schiff base counterion, suggesting a possible role for Glu113 as a catalytic base in this reaction. Consistent with this model, the E113Q mutant was also found to bind retinal more slowly than the wild type.

The visual pigment rhodopsin is composed of the apoprotein opsin and an 11-*cis*-retinal chromophore covalently attached to the protein by means of a Schiff base linkage to Lys296 in the seventh transmembrane helix (*1*). Upon absorption of light, the 11-*cis*-retinylidene chromophore isomerizes to the all-*trans* form which initiates a cascade of events culminating ultimately in the active intermediate metarhodopsin II (MII)[1] (*2*, *3*). Following decay of MII, the chromophore dissociates from the protein to produce free all-*trans*-retinal and opsin. Regeneration of rhodopsin and resetting of the dark state of the photoreceptor cell require that a new molecule of 11-*cis*-retinal bind to the opsin. Despite the prominence of retinal binding and Schiff base formation in the chemical events comprising the visual process, this reaction has received relatively little attention since the early studies of Morton and Pitt (*4*, *5*).

Our laboratory has recently become interested in this reaction not only because of its importance to rhodopsin but also for what it might tell us about ligand binding to other G protein-coupled receptors. Our early experiments focused on a group of three rhodopsin mutants known to cause night blindness in humans (*6*−*14*). We noted that one of the mutations (G90D) resulted in a dramatic slowing of the rate of Schiff base formation with the chromophore. While the other two night blindness mutants A292E and T94I were found to bind retinal with rapid kinetics, a mutation in which Thr94 was changed to Asp also resulted in dramatic slowing of the rate of Schiff base formation in the protein. We present these findings here and suggest a model in which the effects of the Asp substitutions at positions 90 and 94 are mediated through the Schiff base counterion Glu113 (*15*−*17*).

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[1] Abbreviations: PBS, phosphate-buffered saline; MII, metarhodopsin II; DDM, β-D-dodecyl maltoside.

EXPERIMENTAL PROCEDURES

Materials. 11-*cis*-Retinal was synthesized and purified according to standard procedures (*18*). β-D-Dodecyl maltoside (DDM) was from Calbiochem (La Jolla, CA). The anti-rhodopsin monoclonal antibody 1D4 (*19*, *20*) was purified from hybridoma culture medium (National Cell Culture Center, Minneapolis, MN) by ion exchange chromatography on DE-52 (Sigma, St. Louis, MO). The antibody was then coupled to a Sepharose 4B solid support using previously described methods (*21*). Peptide I (DEASTTVSKTETSQVAPA) was purchased from American Peptide Co., Inc. (Santa Clara, CA).

Mutagenesis of the Opsin Gene and Expression of Mutants. The opsin mutants were constructed from a synthetic opsin gene in a pMT3-based vector (*22*), and cassette mutagenesis was used to create mutations in the gene as previously described (*16*, *23*). The opsin gene and mutants were transiently transfected into COS cells by the DEAE-dextran method (*24*) using either 2 μg of DNA/100 mm plate or 10 μg of DNA/150 mm plate.

Reconstitution and Purification of Receptors. Protein from transfected COS cells was reconstituted and purified at 4 °C as previously described (*25*, *26*). Briefly, 72 h after transfection, cells were harvested and washed with 10 mM phosphate buffer (pH 7.0) containing 150 mM NaCl (PBS). Cells were then solubilized in PBS containing 1% (w/v) DDM with 1 mM phenylmethanesulfonyl fluoride, and the protein was allowed to bind to the 1D4-Sepharose 4B column. The bound protein was washed first with 2 mM sodium phosphate buffer (pH 6.4) containing 0.1% (w/v) DDM and 150 mM NaCl, and then with 2 mM sodium phosphate buffer (pH 6.4) containing 0.1% (w/v) DDM (*25*). The protein was eluted at room temperature with 0.18 mg/mL Peptide I in 2 mM sodium phosphate buffer (pH 6.4) containing 0.1% (w/v) DDM and 7.5 mM NaCl. For experiments using stopped-flow spectrophotometry, all of the conditions were the same except the wash and elution steps were performed with 0.02% (w/v) DDM.

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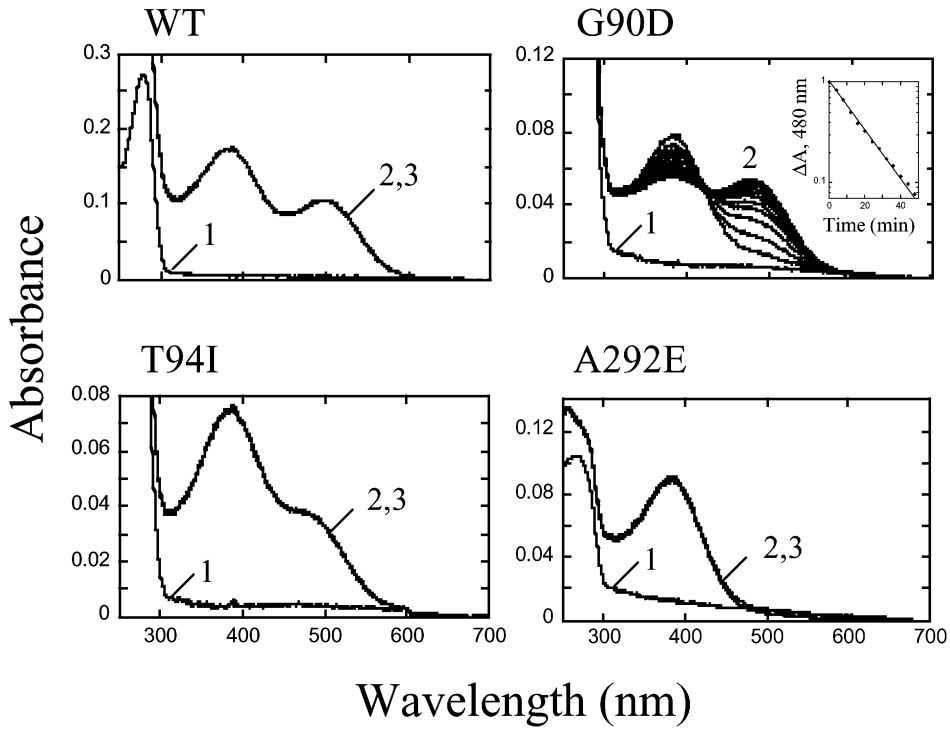

FIGURE 1: Binding of retinal to wild-type and mutant opsins. Mutants are as indicated above each panel. Three absorption spectra are shown for the wild type (WT), T94I, and A292E: (1) spectrum for the purified opsin, (2) spectrum immediately following addition of 11-*cis*-retinal, and (3) spectrum 4 min after addition of 11-*cis*-retinal. For G90D, the spectrum of purified opsin was first recorded (1). 11-*cis*-Retinal was then added, and subsequent spectra were recorded every 4 min (2). The inset shows a semilogarithmic plot of ΔA at 480 nm vs time for an increase in absorbance of the long-wavelength maximum following binding of retinal and formation of the protonated Schiff base. The solid line was generated using a k_{obs} of 0.054 min^{-1} (see Experimental Procedures for details).

Spectrophotometry and Data Analysis. UV−visible absorption spectra were recorded on a Hitachi model U-3210 spectrophotometer adapted for darkroom use by the manufacturer. Data were acquired with the aid of a Gateway 2000 computer using Spectra Calc software (Galactic Industries Corp.). All spectra were from samples with a path length of 1.0 cm, and the temperature was 25 °C.

Stopped-flow spectrophotometry was performed using an Applied Photophysics SX18.MV kinetic spectrophotometer. The optical path length of the sample was 1 cm; the final concentration of opsin was 0.5 μM, and the temperature was 25 °C.

Kinetic data were analyzed using KaleidaGraph version 3.08d. Fits to semilogarithmic plots were carried out using single exponentials and rate constants as reported in the figure legends. Except for the data in Figure 3

$$\Delta A = (A_f - A)/(A_f - A_i)$$

where A is the absorbance at any time t, A_i is the initial absorbance, and A_f is the final absorbance. In Figure 3, the data have not been normalized, and

$$\Delta A = A_f - A$$

Detergent Concentration. Conventional spectrophotometry experiments with the Hitachi spectrophotometer were performed with a DDM concentration of 0.1% (w/v). Stopped-flow spectrophotometry experiments were performed with a DDM concentration of 0.02% (w/v). The observed rate constants for the reaction of retinal with opsin are highly dependent on detergent concentration; an increase in detergent concentration results in a decrease in reaction rate (G. Xie and D. D. Oprian, unpublished results). As a consequence, rate constants for the two conditions should not be directly compared.

RESULTS

Wild-type opsin denatures rapidly in detergent solutions at neutral pH. At pH 6.4 and 4 °C, it exhibits limited stability and, if used shortly after purification, can be analyzed for the reaction with the 11-*cis*-retinal ligand. Under these conditions, as is shown in Figure 1, wild-type opsin binds 11-*cis*-retinal (4 μM) rapidly, and the binding reaction is complete within the dead time of the experiment (~1 min) as judged by the increase in long-wavelength absorbance with a maximum at 500 nm corresponding to formation of the protonated Schiff base chromophore. In stark contrast, the night blindness mutant G90D binds retinal very slowly, with a half-life of 12 min under similar conditions (Figure 1).

Slow binding of retinal appears not to be a property shared with the other night blindness mutants T94I and A292E. As is shown in Figure 1, T94I is similar to the wild-type protein in that it binds retinal within the 1 min dead time. While A292E opsin was too unstable to survive detergent solubilization and purification under these conditions and did not form a pigment with retinal (Figure 1), the Ala292 → Glu mutation could be examined in the context of a N2C,D282C mutant background which is known to significantly increase the stability of the apoprotein opsin (*18*). As shown in Figure 2, A292E opsin is stable in the N2C,D282C background, it generates a pigment with added 11-*cis*-retinal, and the reaction with retinal is complete within the 1 min dead time

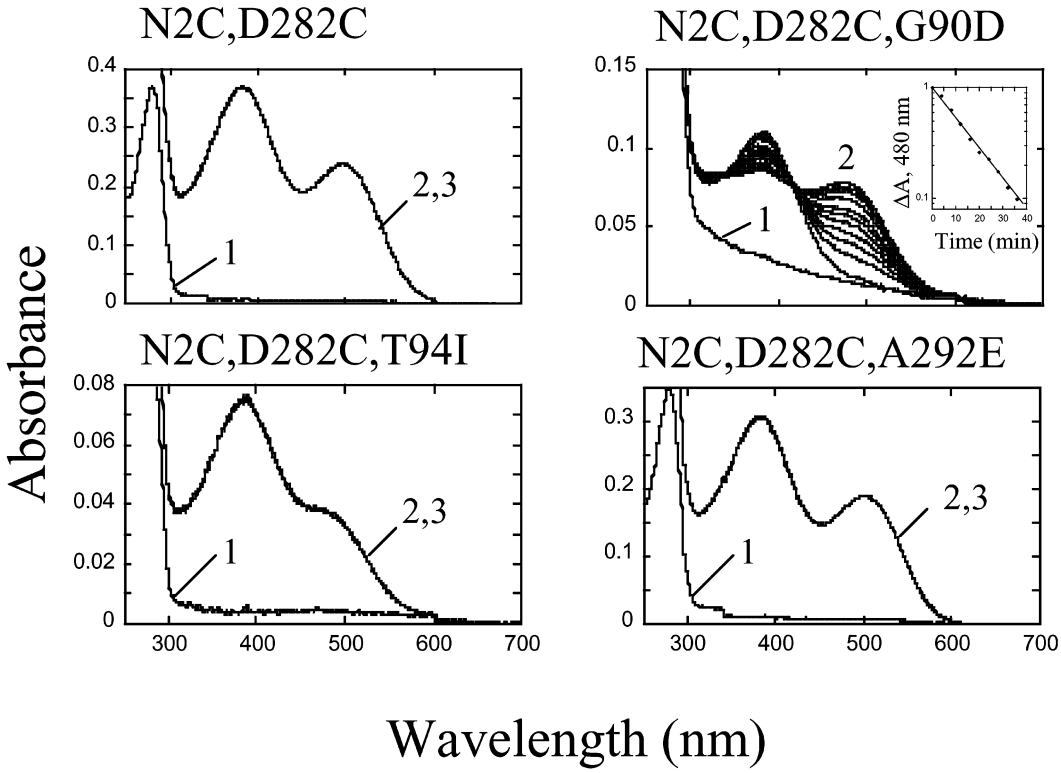

FIGURE 2: Binding of retinal to N2C,D282C mutant opsins. Mutants are as indicated above each panel. Three absorption spectra are shown for N2C,D282C, N2C,D282C,T94I, and N2C,D282C,A292E: (1) spectrum for the purified opsin, (2) spectrum immediately following addition of 11-*cis*-retinal, and (3) spectrum 4 min after addition of 11-*cis*-retinal. For N2C,D282C,G90D, the spectrum of purified opsin was first recorded (1). 11-*cis*-Retinal was then added, and subsequent spectra were recorded every 4 min (2). The inset shows a semilogarithmic plot of ΔA at 480 nm vs time for an increase in absorbance of the long-wavelength maximum following binding of retinal and formation of the protonated Schiff base. The solid line was generated using a k_{obs} of 0.059 min^{-1} (see Experimental Procedures for details).

of the experiment. Binding of retinal to wild-type, G90D, and T94I in the context of the N2C,D282C mutation is also shown in Figure 2 as a control. It is evident that the N2C,D282C background has little if any effect on the binding reaction. In fact, the pseudo-first-order rate constant for binding of retinal to the G90D mutant is essentially identical in the wild-type and N2C,D282C backgrounds ($k_{obs} = 0.054$ min^{-1} for G90D; $k_{obs} = 0.059$ min^{-1} for N2C,D282C,G90D). Thus, G90D appears to be unique among the rhodopsin night blindness mutants in binding retinal slowly.

To better assess the effect of the G90D mutation on the rate of binding, we examined the reaction by stopped-flow spectrophotometry. While the rapid mixing and short dead time of the stopped-flow spectrophotometer were clearly not needed to follow the absorbance change for the reaction of the G90D mutant, they were needed for the much faster reaction of the wild-type protein. As is shown in Figure 3, reaction of 11-*cis*-retinal with wild-type opsin (N2C,D282C background) is dependent on the concentration of retinal in the range of 2−8 μM and is nearly complete within 10 s of mixing at all concentrations that were tested. The reaction is essentially irreversible and proceeds with a second-order rate constant of 1.24×10^5 M^{-1} s^{-1}. Reaction of the G90D mutant is also dependent on retinal concentration, but the reaction is 80 times slower ($k = 1.5 \times 10^3$ M^{-1} s^{-1}) than with the wild-type protein (Figure 3).

In addition to following the increase in absorbance at 500 nm (480 nm for the G90D mutant), we also monitored the absorbance decrease at 380 nm. While the increase in absorbance at 500 nm reflects formation of the protonated Schiff base of the chromophore, the absorbance decrease at 380 nm reflects the loss of the retinal aldehyde and can be useful for determination of the rate-limiting step in the overall reaction. As is shown in Figure 4, the decrease at 380 nm is concomitant with the increase at 500 nm (480 nm for the G90D mutant). Therefore, the loss of aldehyde takes place with exactly the same rate as does formation of the protonated Schiff base.

As was shown above, the T94I mutant does not exhibit the slow binding characteristics of G90D. However, given the spatial proximity of Thr94 and Gly90 in the crystal structure of wild-type rhodopsin, we wondered whether a mutant with an Asp at position 94 might behave like G90D and display slow kinetics for the reaction with retinal. As is shown in Figure 5, the T94D mutant does indeed bind retinal slowly with an observed pseudo-first-order rate constant under these conditions of 0.135 min^{-1} in the wild-type background and 0.130 min^{-1} in the N2C,D282C background.

We also tested the counterion mutant E113Q for kinetics of retinal binding. As is shown in Figure 6, E113Q binds retinal with a pseudo-first-order rate constant of 0.45 min^{-1} (pH 6.0, 0.1% DDM, and 150 mM NaCl). This is on the order of the reaction rate for the G90D or T94D mutant (retinal concentration is roughly 4-fold higher in the E113Q experiment).

DISCUSSION

We have shown here that the rhodopsin night blindness mutant G90D (*9, 11−14*) has a previously unreported phenotype of slow binding of the 11-*cis*-retinal chromophore.

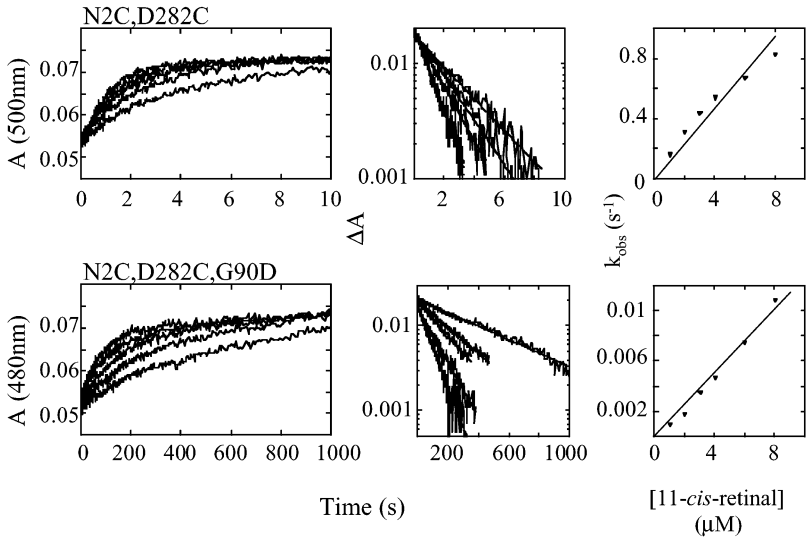

FIGURE 3: Kinetics of retinal binding to wild-type (N2C,D282C) and G90D (N2C,D282C,G90D) opsins using stopped-flow spectrophotometry. Mutants are as indicated. The top and bottom panels of the left-most column show a series of traces for the change in absorbance, *A*, with time at several different concentrations of 11-*cis*-retinal. The retinal concentration, in order of increasing absorbance at 5 s for N2C,D282C and 100 s for N2C,D282C,G90D, was 2, 3, 4, 6, and 8 μM (final concentrations). Middle panels are semilogarithmic plots of ΔA vs time for each trace shown in the panel to the left. Straight lines from simulations are superimposed over each data set to show the goodness of fit for determination of k_{obs}. Right-most panels show a plot of k_{obs} vs retinal concentration. The second-order rate constants derived from the slopes of the solid lines are 1.24×10^5 and 1.5×10^3 M^{-1} s^{-1} for N2C,D282C and N2C,D282C,G90D, respectively (see Experimental Procedures for details).

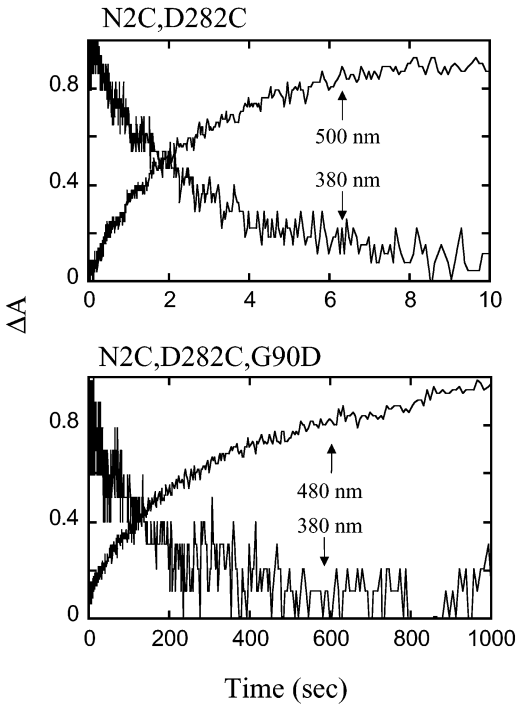

FIGURE 4: Binding of retinal to wild-type (N2C,D282C) and G90D (N2C,D282C,G90D) opsins. Each panel shows a comparison of the stopped-flow time course for the absorbance change at long wavelengths (500 nm for N2C,D282C and 480 nm for N2C,D282C,G90D), due to formation of the protonated Schiff base, with that at 380 nm, due primarily to the disappearance of free retinal. Opsins are as indicated above each panel, and the retinal concentration was 3 μM.

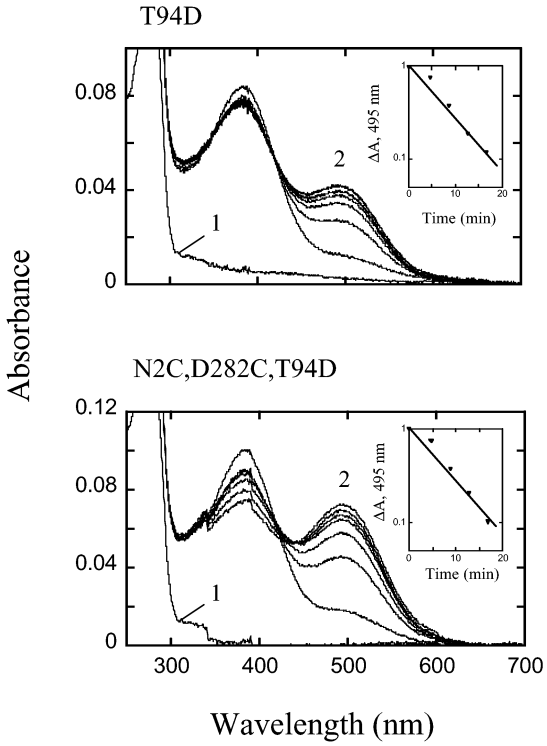

FIGURE 5: Binding of retinal to T94D and N2C,D282C,T94D opsins. Mutants are as indicated. For each panel, the spectrum of purified opsin was first recorded (1). 11-*cis*-Retinal (2−3 equiv) was then added, and subsequent spectra were recorded every 4 min (2). The inset shows a semilogarithmic plot of ΔA at 495 nm vs time for an increase in absorbance of the long-wavelength maximum following binding of retinal and formation of the protonated Schiff base. The solid line was generated using k_{obs} values of 0.135 min^{-1} for T94D and 0.130 min^{-1} for N2C,D282C,T94D (see Experimental Procedures for details).

The second-order rate constant for binding retinal is on the order of 80-fold slower in the mutant than in the wild-type protein. We do not know at this time whether the slow binding contributes to the symptoms of night blindness in individuals with the disease, but it is clear that this is not a general characteristic of night blindness mutations as the two other rhodopsin night blindness mutants, T94I (*6*, *27*) and A292E (*7*), do not bind retinal slowly. Therefore, G90D appears to be unique among the night blindness mutants with

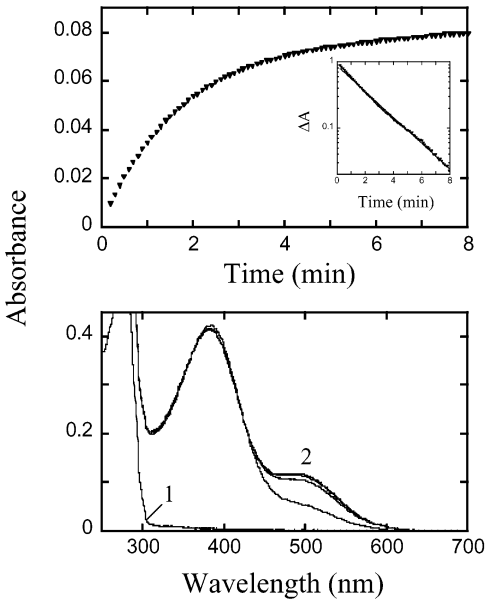

FIGURE 6: Binding of retinal to E113Q (N2C,D282C,E113Q) opsin. In the top panel is shown a time course for the absorbance change at 500 nm following addition of 11-*cis*-retinal (final concentration of ~10 μM) to the mutant opsin. The inset shows a semilogarithmic plot of ΔA vs time following binding of retinal and formation of the protonated Schiff base. The solid line was generated using a k_{obs} of 0.45 min^{-1} (see Experimental Procedures for details). For the bottom panel, the spectrum of purified E113Q opsin was first recorded (1). 11-*cis*-Retinal was then added, and subsequent spectra were recorded every 4 min (2).

regard to this property. It also appears unlikely that the slow binding would contribute to the symptoms of night blindness. While G90D opsin is constitutively active (*9*, *10*) and would be expected to remain in the opsin form longer than the wild type in a patient with the disease, the normal turn-off mechanisms involving rhodopsin kinase and arrestin (*28*) should be fully functional in these patients [unlike patients with recessive forms of the disease which involve null mutations in rhodopsin kinase and arrestin (*8*, *29*−*31*)]. Therefore, although the G90D opsin would be expected to accumulate to higher steady state levels in afflicted photoreceptor cells, the protein would be in inactive complexes of the phosphorylated opsin and arrestin, as is the case for the retinitis pigmentosa mutant K296E (*32*, *33*) and mutants of RPE65 (*34*, *35*).

We also showed that while the mutant T94I does not bind retinal slowly, the related mutant T94D does. Therefore, substitution of carboxylic acid side chains at position 90 or 94 dramatically slows the rate at which retinal binds to opsin, but a carboxylic acid residue at position 292 (in the mutant A292E) does not. All three residues are known to be close to the salt bridge between Glu113 and Lys296 in the active site of the protein where retinal binds. What then could be the difference between the position 90 and 94 mutants, which bind retinal slowly, and the position 292 mutant which does not? At this time, we cannot provide an unequivocal answer to this question; however, we can speculate about what might be the underlying difference. To do so, we first consider what is known about the chemistry of Schiff base formation and the structure of the active site of rhodopsin.

Formation of the Schiff base linkage in rhodopsin requires attack of lone pair electrons from an unprotonated, neutral amine of the Lys296 side chain on the aldehyde of 11-*cis*-retinal to form a carbinolamine intermediate. While the protonation state of Lys296 in opsin has never been determined directly, we can make a reasonable guess that it is predominantly in the protonated ammonium ion state for the following reasons.

(1) While the microenvironment of the Lys side chain may perturb the pK_a, it seems unlikely that it would do so to substantially lower the pK_a in opsin. The best known example of an active site Lys with a lowered pK_a is provided by acetoacetate decarboxylase (*36*, *37*). Acetoacetate decarboxylase utilizes rapid Schiff base formation as a step in its catalytic cycle, and the pK_a of the active site Lys in acetoacetate decarboxylase has been measured to be ~6, whereas an unperturbed pK_a of a Lys side chain free in solution is ~10.5 (*36*, *37*). The perturbed pK_a in acetoacetate decarboxylase results from an unfavorable electrostatic interaction with the side chain of an adjacent Lys residue in the protein. There are no other Lys or Arg residues close to Lys296 in the tertiary structure of rhodopsin (*38*, *39*). Furthermore, the pK_a of the Schiff base nitrogen in rhodopsin is known to be at least 11−12 and has been estimated to be as high as 16 (*40*). While the structures of rhodopsin and opsin may differ, existing FTIR evidence suggests that these differences are small (*41*). Therefore, it is likely that the environment of the Lys296 side chain nitrogen in opsin is not radically different from that in rhodopsin, and on this basis, we expect the Lys296 pK_a to be higher, if perturbed at all, than that of the free amino acid.

(2) More direct evidence comes from titration of a 2-hydroxy-5-nitrobenzylamine reporter group attached to the active site Lys in opsin (*42*). The pK_a of the amine was found to be more alkaline than could be determined in the experiments, and the pK_a of the phenolic oxygen was found to be 7.8, perturbed from 5.9 in model compounds. Both results are consistent with the presence of a negative charge at the active site and not with an environment that would lower the pK_a of the Lys296 amine (*42*).

(3) Finally, there is a group of activating mutations in rhodopsin (including the night blindness mutations presented here) that appear to promote the activated state by disrupting a salt bridge between the active site Glu113 and Lys296 (*10*). Accordingly, the Lys296 side chain in opsin is predicted to contain a positively charged ammonium ion.

In sum, the preceding arguments present a reasonable case for the expectation that the Lys296 nitrogen in opsin is protonated and does not contain lone pair electrons available for nucleophilic attack on the aldehyde of 11-*cis*-retinal. Therefore, a catalytic base is required for this reaction. We also know that the decrease in absorbance at 380 nm is concomitant with the increase at 500 nm for this reaction in both wild-type rhodopsin and the G90D mutant. This indicates that nucleophilic attack, or some step preceding nucleophilic attack, is rate-limiting, and it seems likely that nucleophilic attack itself is the rate-limiting step. If so, a likely candidate for the catalytic base is the Schiff base counterion Glu113. As can be seen in Figure 7, both Gly90 and Thr94 are close to Glu113 in the tertiary structure of rhodopsin, whereas Ala292 is located closer to Lys296. Therefore, carboxylate substitutions at positions 90 and 94 would be expected to have a greater impact on Glu113 than would a similar substitution at position 292, thus providing an account of the difference between substitutions at positions

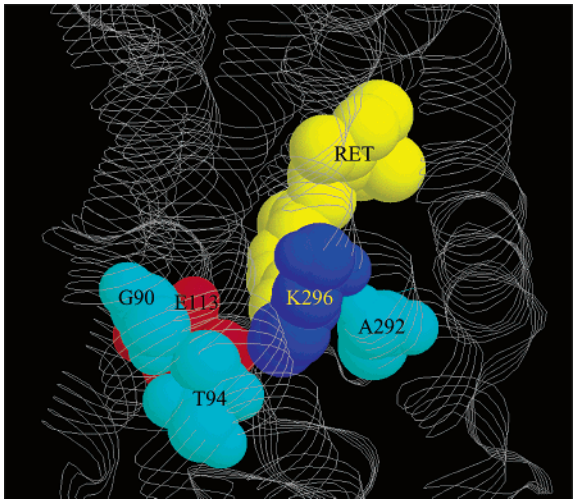

FIGURE 7: Diagram of bovine rhodopsin showing an expanded view of the active site. 11-*cis*-Retinal (RET), Lys296 (K296), the Schiff base counterion Glu113 (E113), and positions harboring congenital night blindness mutations are space-filled. The rhodopsin coordinates are from Protein Data Bank entry 1L9H (*38*). Cα−Cα distances are 3.99 Å for residues 90 and 113, 5.81 Å for residues 94 and 113, and 11.6 Å for residues 292 and 113.

90 and 94 and that at position 292. The introduction of a negative charge at either position 90 or 94 would be expected to result in protonation of Glu113, destroying its effectiveness as a catalytic base in the reaction of Lys296 with retinal, whereas a similar change at position 292 might not. In support of this model, FTIR experiments of Siebert and co-workers have shown that the Glu113 side chain in G90D mutant rhodopsin is indeed protonated (*13*). We have also shown here that Schiff base formation is slowed in the E113Q mutant opsin, although it should be noted that the E113Q mutation is likely to have an effect on the protonation state of Lys296 in the ground state as well and, therefore, the effect of the mutation is difficult to predict a priori.

In conclusion, the dramatic effect of the G90D and T94D mutations on slowing Schiff base formation in opsin appears to be best understood at this time in terms of a direct effect on the protonation state of Glu113 which appears to function as a catalytic base in the rate-limiting nucleophilic addition of Lys296 to retinal.

Finally, we note that Khorana and co-workers have described mutations of Trp265 in the sixth transmembrane helix which also dramatically slow Schiff base formation from 11-*cis*-retinal and the mutant opsins (*43*). These mutations are located near the β-ionone ring of retinal and likely perturb the reaction by a mechanism different from that of the mutations described here, perhaps by slowing noncovalent association of retinal with the protein. Further dissection of the reaction mechanism for the Trp265 mutants will be of great interest.

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