Characterization of Rhodopsin Congenital Night Blindness Mutant T94I†

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ABSTRACT: The Thr94 → Ile mutation in the second transmembrane segment of rhodopsin has been reported to be associated with a congenital night blindness phenotype in a large Irish pedigree. Previously, two other known rhodopsin mutants that cause congenital night blindness, A292E and G90D, have been shown in vitro to constitutively activate the G protein transducin in the absence of a chromophore. The proposed mechanism of constitutive activation of these two mutants is an electrostatic disruption of the active site salt bridge between Glu113 and Lys296 that contributes to stabilization of the protein in the inactive state. Here, the T94I rhodopsin mutant is characterized and compared to the two other known rhodopsin night blindness mutants. The T94I mutant opsin is shown also to constitutively activate transducin. The T94I mutant pigment (with a bound 11-*cis*-retinal chromophore), like the other known rhodopsin night blindness mutants, is not active in the dark and has wild-type activity upon exposure to light. Similar to the Gly90 → Asp substitution, position 94 is close enough to the Schiff base nitrogen that an Asp at this position can functionally substitute for the Glu113 counterion. However, in contrast to the other night blindness mutants, the T94I MII intermediate decays with a half-life that is approximately 8-fold slower than in the wild-type MII intermediate. Thus, the one phenotype shared by all congenital night blindness mutants that is different from the wild-type protein is constitutive activation of the apoprotein.

Rhodopsin, the visual pigment found in vertebrate retinal rod cells, mediates vision under conditions of dim light intensity. It is composed of two parts: an apoprotein opsin and an 11-*cis*-retinal chromophore covalently attached to the protein via a protonated Schiff base linkage to the ϵ-amino group of Lys296 located in the seventh transmembrane helix. The positive charge on the Schiff base nitrogen is stabilized through an electrostatic interaction with the carboxylate on the counterion Glu113 (*1*−*3*), resulting in a highly perturbed pK_a for the Schiff base that has been estimated to be as high as 16 (*4*). Upon light activation, the 11-*cis*-retinal chromophore isomerizes to all-*trans*-retinal, initiating a series of conformational changes in the protein that result ultimately in the active intermediate metarhodopsin II (MII)[1] (*5*). MII is the only intermediate capable of activating the G protein transducin (*6*). To set the receptor back to the dark state, the chromophore hydrolyzes and dissociates from the protein, and a new molecule of 11-*cis*-retinal binds to the vacant active site.

Mutations of several different amino acid residues in rhodopsin result in constitutive activation of the apoprotein, or opsin, form of rhodopsin (*7*, *8*). That is, these mutants are capable of activating transducin in the absence of the chromophore. Of particular interest to our laboratory has been a group of mutations involving four amino acid residues in the active site of the protein: Gly90, Glu113, Ala292, and Lys296 (*8*−*12*). Mutations at these four positions are thought to constitutively activate opsin by a common mechanism of action; they all disrupt a salt bridge between Glu113 and Lys296 that helps constrain the protein in an inactive conformation. These mutations are of interest not only because they provide insight into the mechanism of activation of the receptor but also because they are found in two different human diseases of the retina. Mutations of Lys296 (K296E and K296M) have been shown to cause retinitis pigmentosa (RP) (*13*), while mutations of Gly90 and Ala292 (G90D and A292E, respectively) have been shown to cause congenital stationary night blindness (*11*, *14*). RP is a devastating degenerative disease resulting in complete blindness as a consequence of the loss of both rod and cone photoreceptor cells. Congenital stationary night blindness is, in comparison, a much less severe phenotype resulting only in the loss of rod-mediated vision under dim light conditions. While the mechanism by which the Lys296 mutations cause RP is currently unknown (*15*, *16*), the constitutively active phenotype of the G90D and A292E mutants provides a reasonable explanation of the underlying molecular dysfunction in night blindness (*8*, *10*, *11*).

Recently, al-Jandal et al. (*17*) have reported a third rhodopsin mutant, T94I, responsible for causing night blindness in an Irish family. While the Thr94 → Ile mutation has not been previously characterized biochemically, it has been proposed to constitutively activate the protein in a manner similar to that found for the other night blindness mutations (*17*). The location of the Thr94 → Ile substitution in the second transmembrane helix is close to Gly90 (Figure

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[1] Abbreviations: MII, metarhodopsin II; DDM, β-D-dodecyl maltoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline.

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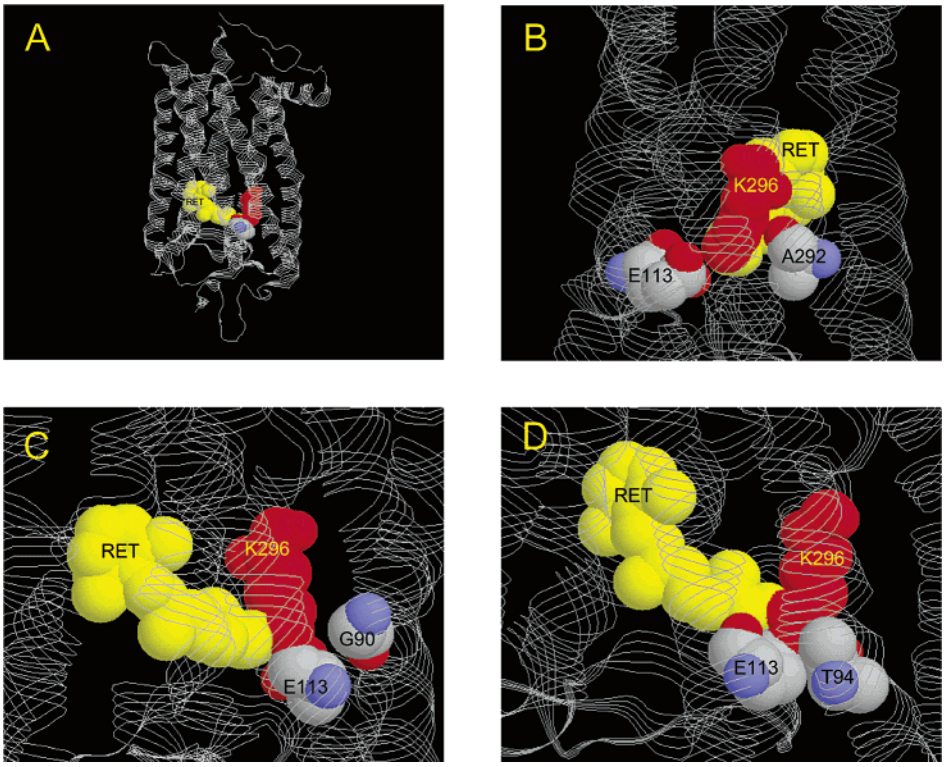

FIGURE 1: Structure of bovine rhodopsin showing an expanded view of the active site. 11-*cis*-Retinal (RET), Lys296 (K296), the Schiff base counterion Glu113 (E113), and positions harboring congenital night blindness mutations are shown in a space-filling diagram. (A) Overall structure of rhodopsin. (B−D) Close-up of the active site showing locations of the three night blindness mutations: (B) Ala292 (A292), (C) Gly90 (G90), and (D) Thr94 (T94). The rhodopsin coordinates are from PDB entry 1L9H (*18*).

1) (*18*, *19*), but the nature of the change is different. In G90D, a neutral amino acid residue is changed for one that is negatively charged (similar to the change found for the A292E mutant). In the T94I mutant, a neutral (polar) residue is replaced with another neutral residue. Therefore, it was important to determine whether the effects of the Thr94 → Ile mutation on the biochemical properties of rhodopsin were similar to or different from those found in the other previously characterized night blindness mutants.

EXPERIMENTAL PROCEDURES

Materials. 11-*cis*-Retinal was synthesized according to published procedures (*20*). Frozen bovine retinae were from Schenk Packing Co., Inc. (Stanwood, WA). Transducin was purified from bovine retinae as described previously (*21*) and was monitored for purity by gel electrophoresis and for contamination with rhodopsin by Western blot analysis with the anti-rhodopsin monoclonal antibody 1D4 (*22*, *23*). [^{35}S]-GTPγS (1156 Ci/mmol) was from Perkin-Elmer (Boston, MA), and nonradiolabeled GTPγS (tetralithium salt) was from Amersham Biosciences (Piscataway, NJ). Sodium acetate, 2-(*N*-morpholino)ethanesulfonic acid (MES), tris-(hydroxymethyl)aminomethane (Tris), 10 mM 1,3-bis[tris-(hydroxymethyl)methylamino]propane (BTP), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and glycine were from Sigma. Oligonucleotides for cassette mutagenesis were from QIAGEN Operon (Alameda, CA). The anti-rhodopsin monoclonal antibody 1D4 (*22*, *23*) was purified from hybridoma culture medium (National Cell Culture Center, Minneapolis, MN) by ammonium sulfate precipitation and ion exchange chromatography on DE-52 (Sigma, St. Louis, MO). The 1D4-Sepharose 4B immunoaffinity matrix used for purification of rhodopsin from transfected COS cells was prepared as previously described (*24*). Peptide I (DEASTTVSKTETSQVAPA), corresponding to the 18 carboxy-terminal amino acids of rhodopsin, was purchased from American Peptide Co., Inc. (Santa Clara, CA). Peptide I contains the sequence for the 1D4 epitope and was used for elution of rhodopsin from the 1D4-Sepharose 4B matrix.

Mutagenesis of the Opsin Gene and Expression of Mutants. The opsin mutants were constructed from a synthetic opsin gene (*25*) in a pMT3-based vector (*26*), and cassette mutagenesis was used to create mutations in the gene as previously described (*3*, *27*). The rhodopsin gene and mutants were transiently transfected into COS-1 cells by the DEAE-dextran method (*28*) using either 2 μg of DNA/100 mm plate or 10 μg of DNA/150 mm plate.

Preparation of COS Membranes. Membranes were prepared from COS cells harvested 72 h after transfection as previously described (*9*), except that the final membrane pellet was resuspended in 500 μL of 10 mM Tris buffer (pH 7.4), containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1 mM EDTA.

Reconstitution and Purification of Pigments. Protein from transfected COS cells was reconstituted with a 11-*cis*-retinal chromophore and purified at 4 °C as previously described (*29*, *30*). Briefly, 72 h after transfection, cells were harvested and washed with 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl (PBS). Cells were then solubilized in PBS containing 1% (w/v) DDM and 1 mM phenylmethanesulfonyl fluoride, and the postnuclear supernatant fraction was allowed to bind to the 1D4-Sepharose 4B

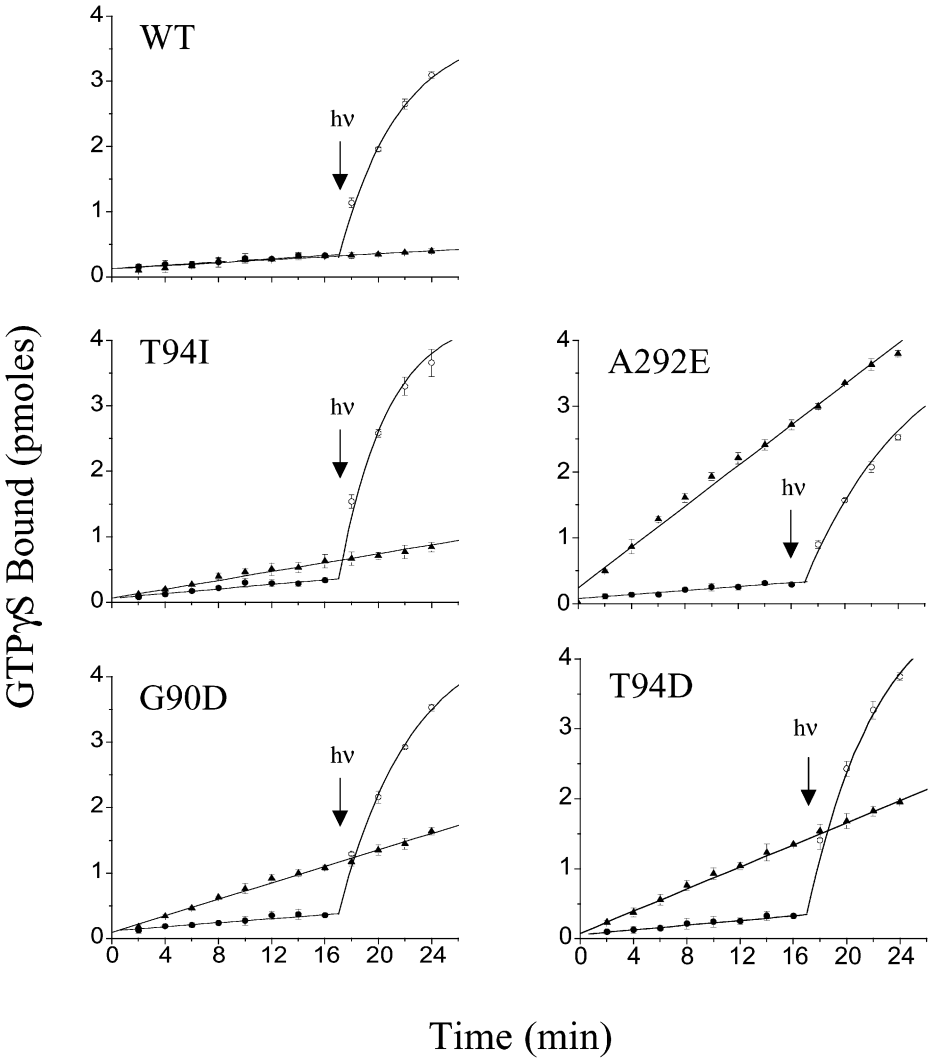

FIGURE 2: Transducin activation by wild-type and mutant opsins in the presence and absence of the chromophore. Transducin activity was assayed using membranes from transfected COS cells as described in Experimental Procedures: (▲) in the absence of 11-*cis*-retinal, (●) in the presence of 11-*cis*-retinal, and (○) time course for the reaction in the presence of retinal after exposure to light (*hν*). Error bars are standard deviations ($n = 3$).

column. Bound protein was washed with PBS containing 0.1% (w/v) DDM and eluted at room temperature with 0.18 mg/mL Peptide I in PBS containing 0.1% (w/v) DDM. For experiments with Glu113 counterion mutants, the samples were washed and eluted using 10 mM HEPES buffer (pH 8.0) supplemented with 150 mM NaCl and 0.1% (w/v) DDM.

Transducin Activation Assays. The ability of receptors to catalyze the exchange of radiolabeled [^{35}S]GTPγS for bound GDP in transducin was monitored using a filter binding assay as described previously (*21*, *31*), except that time points were taken every 2 min, and light was introduced at 17 min. The assays were performed with rhodopsin in COS cell membranes at a final concentration of 1 nM. Reaction mixtures contained 10 mM BTP buffer (pH 6.7) containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 2 μM transducin, and 3 μM [^{35}S]GTPγS (5 Ci/mmol). Reactions were initiated by addition of [^{35}S]-GTPγS. For light-dependent reactions, membranes were incubated with 100 μM 11-*cis*-retinal for at least 1 h at 4 °C prior to use. The final reaction volume was 150 μL, and 10 μL aliquots were withdrawn and assayed at the indicated times. The temperature was 23−25 °C. All assays were performed at pH 6.7. Under these conditions, the reaction rate was directly proportional to the rhodopsin concentration.

Absorption Spectroscopy. UV−visible absorption spectra of immunopurified proteins were recorded using 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., Salem, NH). All spectra were recorded from samples with a path length of 1.0 cm at 25 °C.

Determination of Schiff Base pK_a Values in E113Q and T94I,E113Q Mutants. Purification of the E113Q and T94I,E113Q mutants for determination of the Schiff base pK_a values was as described above except that each pigment was prepared in a series of buffers at different pHs. This was necessary because the large number of manipulations required for a direct pH titration of a single protein sample resulted in denaturation of the protein and optical deterioration of the samples. The samples were prepared as follows. The 1D4-Sepharose 4B matrix was divided into six equal portions. Each portion was eluted with Peptide I in one of the following buffers (10 mM) containing 150 mM NaCl and 0.1% (w/v) DDM: sodium acetate from pH 4.0 to 5.0,

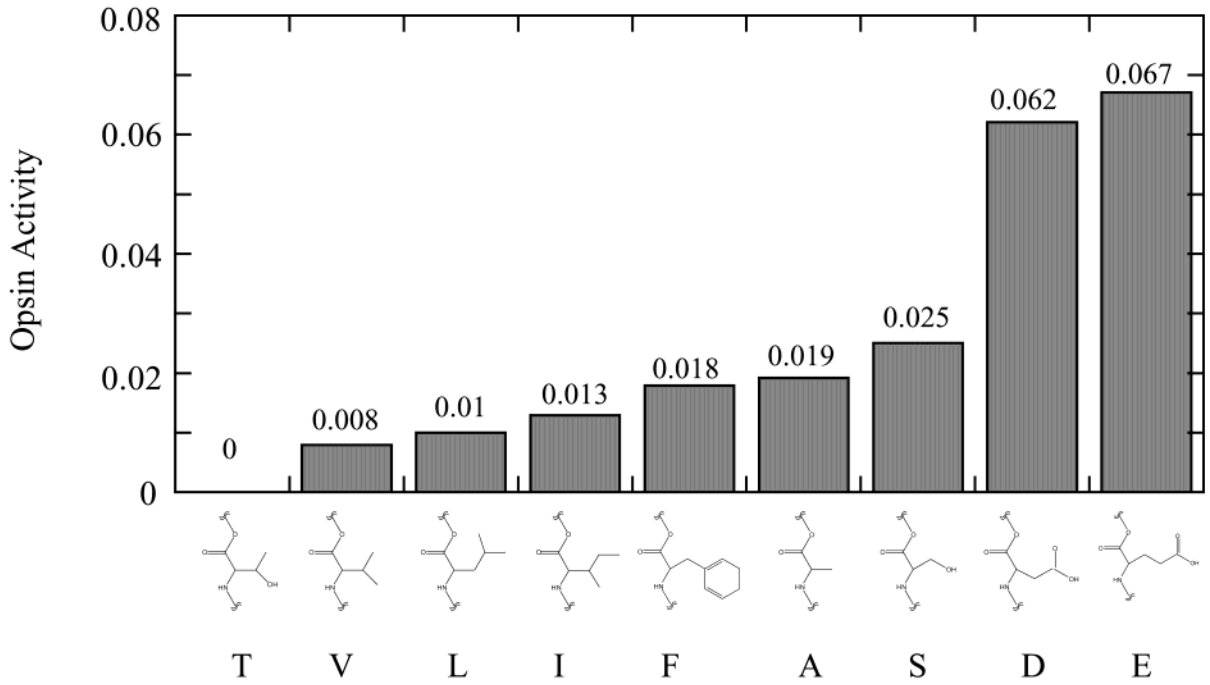

FIGURE 3: Levels of constitutive activity for opsins with different amino acids at position 94. Transducin activation assays were performed as described in the legend of Figure 2 and in Experimental Procedures. The level of constitutive activity was determined relative to background activity observed for the reaction in the presence of 11-*cis*-retinal in the dark and is in units of picomoles of GTPγS bound per minute. The identity of the amino acid at position 94 is indicated for each mutant that was assayed.

MES from pH 5.5 to 6.0, Tris from pH 7.0 to 8.5, and glycine from pH 9.0 to 10.0. Spectra were recorded on each sample and overlaid on the same plot to create a set of spectra used to determine the pK_a. The pH titration curve for the reaction followed at 500 nm was fit to the equation

$$\Delta A = [H^+]/(K_a + [H^+])$$

where $\Delta A = A/(A_m - A)$, A is the absorbance at 500 nm, A_m is the maximum absorbance at 500 nm (limiting value at low pH), $[H^+]$ is the proton concentration, and K_a is the equilibrium constant for the acid dissociation reaction. The pH titration curve for the reaction followed at 380 nm was fit to the equation

$$\Delta A = K_a/(K_a + [H^+])$$

where $\Delta A = (A - A_l)/(A_h - A_l)$, A is the absorbance at 380 nm, A_l is the limiting 380 nm absorbance found at low pH, and A_h is the limiting 380 nm absorbance at high pH. The pH titration curves were fit using pK_a values of 6.4 for E113Q and 5.6 for T94I,E113Q.

Time Course of MII Decay. The rate of MII decay was determined by two different methods. For samples that generate a long-wavelength intermediate (protonated MII) upon exposure to light, as with A292E and G90D, the rate of MII decay was determined as follows. The spectrum of the purified pigment in PBS containing 0.1% (w/v) DDM was recorded in the dark. The sample was then exposed to light from a 300 W tungsten bulb that was passed through a 480 nm cut-on filter for 5 s, and the spectrum of the resulting protonated MII was recorded. Successive spectra were recorded in the dark until no further absorbance decrease was observed at 480 nm.

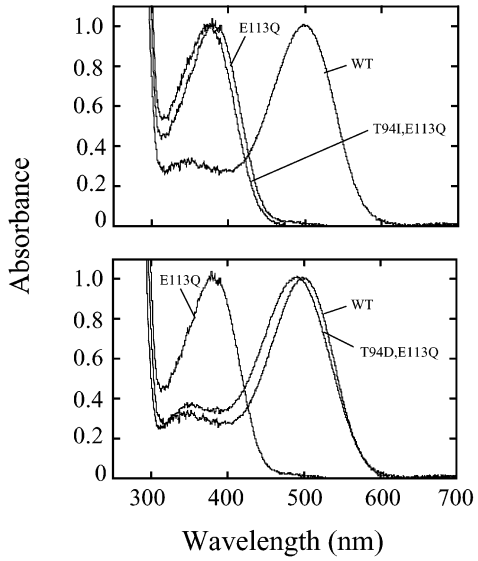

FIGURE 4: Counterion rescue of the E113Q mutant with Asp but not Ile at position 94. In each panel, absorption spectra are shown for the wild type (WT), the E113Q counterion mutant, and either T94I,E113Q (top panel) or T94D,E113Q (bottom panel). Spectra have been normalized to an absorbance of 1.0, and all samples were at pH 8.0.

For samples that bleach immediately to a 380 nm maximum after exposure to light, as with wild-type rhodopsin and the T94I mutant, the rate of MII decay was determined by taking advantage of the fact that 11-*cis*-retinal binds to opsin much faster than MII decays (*32*, *33*). First, the spectrum of the purified pigment in PBS containing 0.1% (w/v) DDM was recorded in the dark. The sample was then exposed to light from a 300 W tungsten bulb that was passed through a 480 nm cut-on filter for 5 s, and the spectrum of the resulting MII was recorded. Approximately 1.5 equiv of 11-*cis*-retinal was added to the sample, and successive spectra

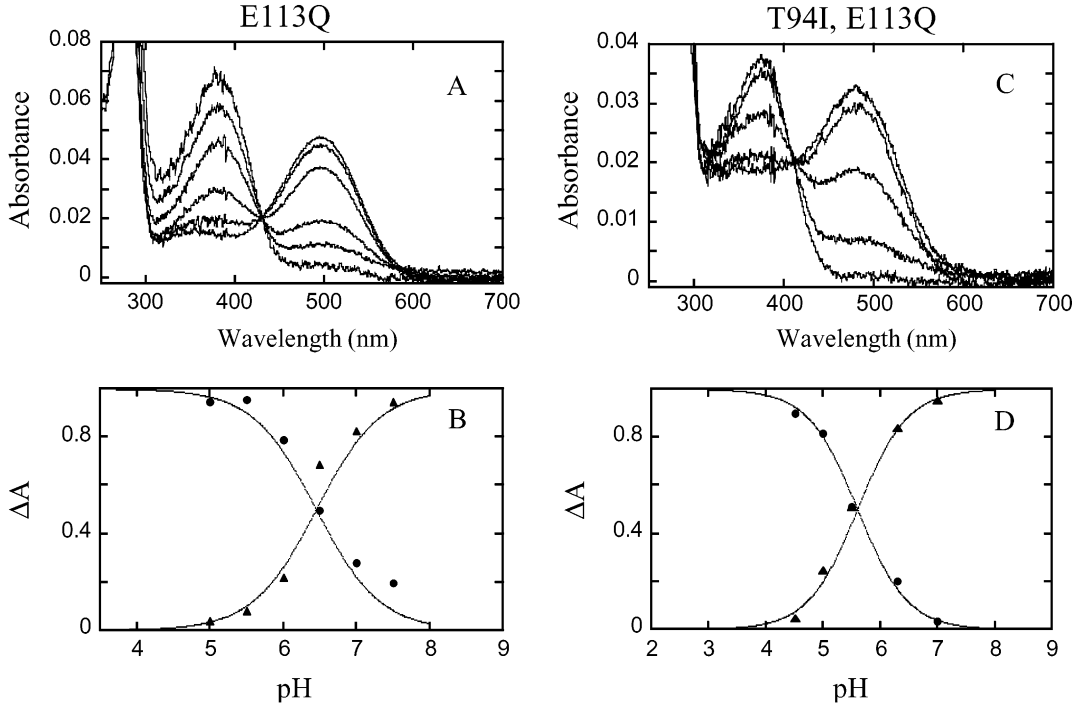

FIGURE 5: Determination of the pK_a for the Schiff base nitrogen in E113Q and T94I,E113Q. (A) Absorption spectra for E113Q at pH 7.5, 7.0, 6.5, 6.0, 5.5, and 5.0 (in order of increasing absorbance at 500 nm). (B) Plot of the normalized absorbance change (ΔA) for E113Q vs pH at 500 (●) and 380 nm (▲). (C) Absorption spectra for T94I,E113Q at pH 7.0, 6.3, 5.5, 5.0, and 4.5 (in order of increasing absorbance at 500 nm). (D) Plot of the normalized absorbance change (ΔA) for T94I,E113Q vs pH at 500 (●) and 380 nm (▲). Solid lines were simulated using pK_a values of 6.4 for E113Q and 5.6 for T94I,E113Q. See Experimental Procedures for details.

were recorded until no further absorbance increase was observed at 500 nm.

Rate constants for the reactions were determined from semilogarithmic plots of ΔA versus time (as presented in the insets of Figure 7), where $\Delta A = (A - A_\infty)/(A_0 - A_\infty)$, A is the absorbance recorded at 500 nm for each time point, A_∞ is the absorbance at time ∞, and A_0 is the absorbance at time zero. The temperature in these experiments was 25 °C.

RESULTS

Activity of the T94I Mutant. When assayed in isolated COS cell membranes, the T94I mutant rhodopsin displays activity for the light-dependent activation of transducin that is comparable to that of wild-type rhodopsin under the same conditions (Figure 2). The opsin form of the T94I mutant, however, is different from the wild type in that it displays a small but significant level of constitutive ability to activate transducin. The activity seen with T94I is lower than that observed for the two other night blindness mutants but clearly greater than the background activity measured in the presence of 11-*cis*-retinal, and clearly above the activity observed for wild-type opsin (Figure 2). Thus, the T94I mutant is similar to the other two rhodopsin night blindness mutants, G90D and A292E, in that T94I opsin is constitutively active.

In an effort to determine if different amino acid residues at position 94 result in different levels of constitutive activity, we examined a total of eight different mutant opsins (including T94I, Figure 3). Interestingly, all eight mutants display some degree of elevated constitutive activity over the wild type; while the activity varies with amino acid side chain, no clearly discernible trend is apparent. Steric bulk does not appear to play a major role (the Ala, Ile, Val, and Leu mutants all have similar activities). Loss of a hydroxyl

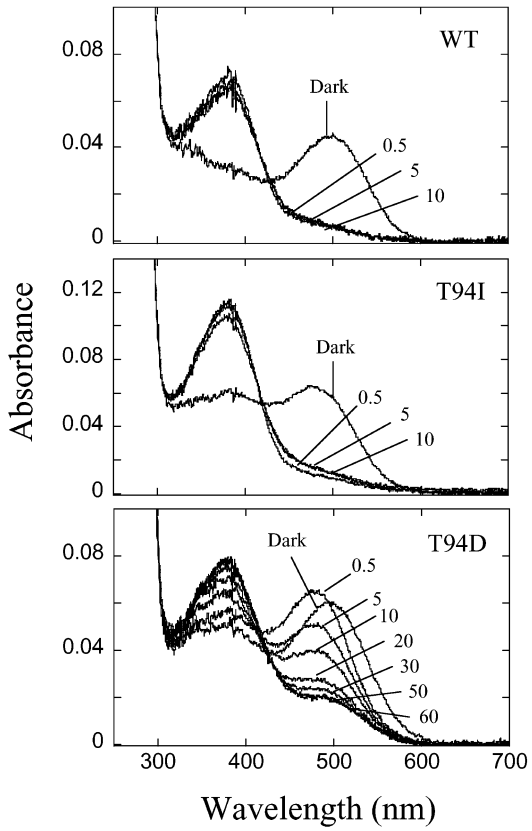

FIGURE 6: Bleaching behavior of wild-type (WT), T94I, and T94D rhodopsin. An absorption spectrum for each pigment was first recorded in the dark. The samples were then exposed to light for 5 s using a 300 W tungsten bulb filtered through a 480 nm cut-on filter, and successive spectra were recorded in the dark at the indicated postflash times (in minutes). The scan rate was 300 nm/min.

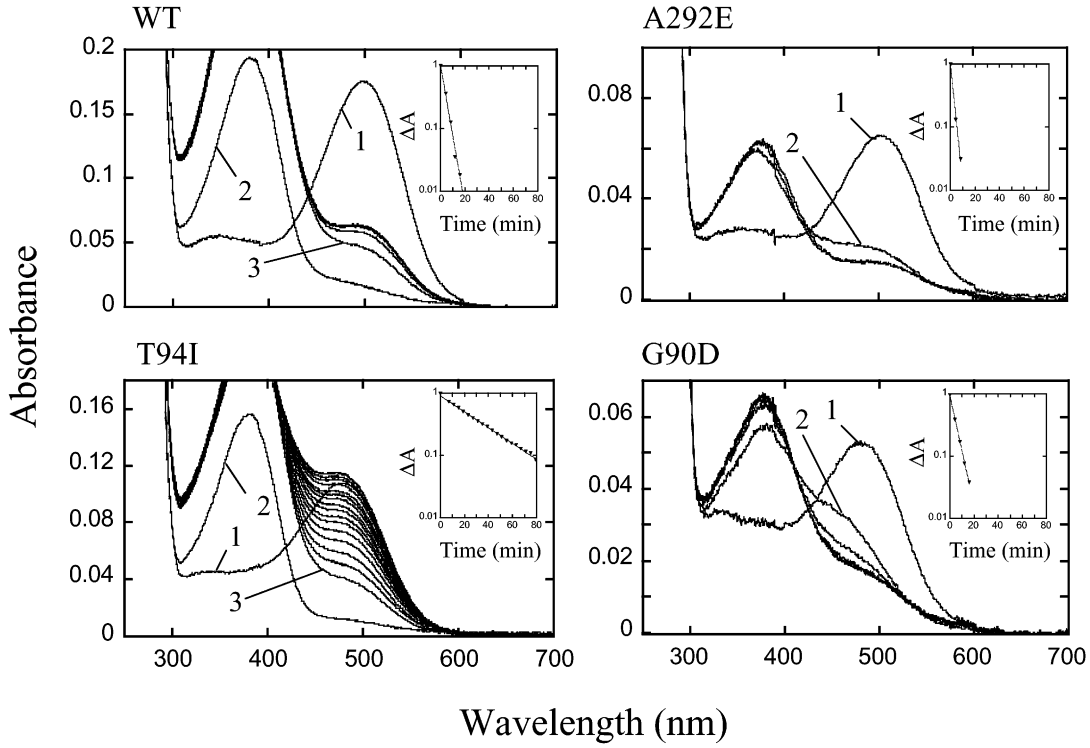

FIGURE 7: Time course of MII decay in the wild type (WT), T94I, A292E, and G90D. For the wild type and T94I, the rate of MII decay was measured by exposing the sample to light, adding 11-*cis*-retinal, and following reconstitution of the pigment in the dark. For A292E and G90D, the loss of long-wavelength absorbance of the protonated MII species at 480 nm could be followed directly. In each panel, spectrum 1 is the spectrum of the purified pigment in the dark, spectrum 2 is a set of spectra recorded after a 5 s exposure to light from a 300 W tungsten bulb passed through a 480 nm cut-on filter, and spectrum 3 is a set of spectra recorded at various times after the addition of ~1.5 equiv of 11-*cis*-retinal. The insets show semilogarithmic plots of the normalized absorbance change ΔA vs time after retinal added for WT and T94I and ΔA vs time after exposure to light for A292E and G90D. Retinal was added 3 min after light for the wild type and T94I. Half-lives determined from the semilogarithmic plots are 2.6, 1.4, 2.9, and 20 min for the wild type, A292E, G90D, and T94I, respectively. Because the half-life of the wild type is 2.6 min and opsin is unstable and irreversibly denatures under these conditions, less than half of the pigment (representing the amount of MII remaining at the time retinal was added to the sample) was recovered in this experiment. We note that the kinetics of MII decay appear to be highly dependent on the experimental conditions as the half-times reported here are significantly shorter than those previously observed under different conditions (*20*, *33*).

per se does not appear to be responsible (the Ser mutant is more active than the Ala mutant), and the presence of an acidic side chain is not determinant (although the Asp and Glu mutants display higher activity than other mutants in the group and, therefore, may have an electrostatic component).

Counterion Rescue. Changing the Schiff base counterion Glu113 to Gln results in a dramatic reduction of the pK_a of the Schiff base nitrogen such that the mutant pigment when purified at pH 8 contains an unprotonated Schiff base and displays an absorption maximum at 380 nm, shifted 120 nm to the blue relative to that of the wild-type pigment (*1*−*3*). Under the same conditions, the double mutant G90D,E113Q displays a long-wavelength maximum of 472 nm, showing that the newly introduced Asp90 is capable of substituting for the Glu113 counterion and rescuing the wild-type phenotype with a higher pK_a and long-wavelength absorption maximum (*10*). While we did not expect the Ile substitution at position 94 to rescue the counterion mutant in a similar manner (Figure 4, top panel), we did think that an Asp residue might. As expected, the T94D,E113Q double mutant when purified at pH 8 displays a single long-wavelength absorption maximum at 490 nm (Figure 4, bottom panel). This experiment demonstrates that position 94 is close enough to the Schiff base nitrogen in rhodopsin that an Asp residue can functionally substitute for the Glu113 counterion. Interestingly, an Ile at position 94 in the double mutant T94I,E113Q has an effect opposite to that of Asp at this position; a positive charge on the Schiff base nitrogen is less stable in the T94I,E113Q double mutant, and the pK_a is shifted ~1 unit lower than in the single mutant E113Q (Figure 5). Therefore, we conclude that Thr94 aids in stabilization of a positive charge on the Schiff base nitrogen in the E113Q mutant.

Bleaching Kinetics and MII Lifetime. When rhodopsin in DDM solution is exposed to light, the absorption spectrum converts within milliseconds to that of the active MII intermediate with an absorption maximum at 380 nm, characteristic of an unprotonated Schiff base. Under identical conditions, the G90D mutant accumulates an intermediate with a long-wavelength absorption maximum (*10*). This intermediate has wild-type activity; it decays to opsin and free retinal, and it is thought to be a protonated MII (i.e., it has the active MII conformation but contains a protonated Schiff base as a consequence of Asp90 being located close to the Schiff base nitrogen in the activated state). Similar slow-bleaching kinetics are observed with the A292E mutant (*34*).

We did not expect the T94I mutant to exhibit slow-bleaching kinetics (Figure 6), but we thought that the T94D mutant might. In accord with this expectation, the T94D mutant does indeed accumulate a long-wavelength intermediate upon exposure to light (Figure 6). This intermediate is active (Figure 2), and it has an absorption maximum at 480 nm which we presume to be a protonated MII (*10*, *35*),

similar to the G90D and A292E intermediates. Decay of the MII intermediate is, however, much slower than for the wild-type, G90D, and A292E intermediates (see below).

While the T94I mutant does not exhibit slow-bleaching kinetics, it does display slow decay of MII. As can be seen from the MII lifetime determinations in Figure 7, wild-type MII decays with a half-life of ~2.6 min at 25 °C. The protonated MII intermediates of A292E and G90D decay with comparable half-lives of ~1.4 and ~2.9 min, respectively. In stark contrast, the T94I MII intermediate decays with a half-life of ~20 min, 8-fold slower than the decay of wild-type MII.

DISCUSSION

The biochemical characterization described here has shown that the T94I mutant differs from wild-type rhodopsin in two important ways: T94I has a long-lived MII intermediate (*36*), and the opsin form of T94I is constitutively active. It seems unlikely that the long-lived MII would be responsible for night blindness symptoms in individuals with the T94I mutation since the intrinsic decay rate of wild-type MII is not rate-limiting in turn-off of the photoresponse in rod photoreceptor cells (*37*). In addition, the long-lived MII phenotype is unique to T94I among night blindness mutants; the MII intermediates of G90D and A292E decay with kinetics similar to those of the wild-type intermediate. While there is no a priori requirement that the different night blindness mutants cause the disease by a common mechanism, the fact that the three mutations are found close together within the three-dimensional structure of rhodopsin (Figure 1) leads one inevitably to this expectation.

The phenotype of constitutive activity could account for the symptoms of night blindness. According to this model, thermal dissociation of 11-*cis*-retinal from the mutant rhodopsin gives rise to constitutively active opsin which activates the rod photoreceptor cell and results in inappropriate desensitization of the photoresponse (*8*, *10*, *11*). Constitutive activity also accounts for the dominant inheritance pattern observed for the disease**.** While an alternative model has been proposed for the G90D mutant in which activation results from an enhanced rate of thermal isomerization of the retinal chromophore in the mutant pigment (*14*, *38*), it should be noted that this is an activity that has never been experimentally demonstrated for G90D or any of the other night blindness mutants. In contrast, the model based on constitutively active opsin invokes a phenotype that has been documented in all three known rhodopsin night blindness mutants.

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