Unusual Thermal and Conformational Properties of the Rhodopsin Congenital Night Blindness Mutant Thr-94 → Ile*

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Naturally occurring point mutations in the opsin gene cause the retinal diseases retinitis pigmentosa and congenital night blindness. Although these diseases involve similar mutations in very close locations in rhodopsin, their progression is very different, with retinitis pigmentosa being severe and causing retinal degeneration. We report on the expression and characterization of the recently found T94I mutation associated with congenital night blindness, in the second transmembrane helix or rhodopsin, and mutations at the same site. T94I mutant rhodopsin folded properly and was able to bind 11-cis-retinal to form chromophore, but it showed a blue-shifted visible band at 478 nm and reduced molar extinction coefficient. Furthermore, T94I showed dramatically reduced thermal stability, extremely long lived meta-rodopsin II intermediate, and highly increased reactivity toward hydroxylamine in the dark, when compared with wild type rhodopsin. The results are consistent with the location of Thr-94 in close proximity to Glu-113 counterion in the vicinity of the Schiff base linkage and suggest a role for this residue in maintaining the correct dark inactive conformation of the receptor. The reported results, together with previously published data on the other two known congenital night blindness mutants, suggest that the molecular mechanism underlying this disease may not be structural misfolding, as proposed for retinitis pigmentosa mutants, but abnormal functioning of the receptor by decreased thermal stability and/or constitutive activity.

Naturally occurring mutations in rhodopsin (most of them single amino acid replacements) are associated with retinal disease. Most of these are the cause of retinitis pigmentosa (RP), a group of inherited retinal degenerative diseases (1–3) that leads to blindness by causing photoreceptor cell death (4). Over 100 mutations have been found to date in the opsin gene associated with RP, most of them being inherited as an autosomal dominant trait (1). These are located in all the three domains of rhodopsin, namely the intradiscal, the transmembrane, and the cytoplasmic domains of the protein (5). Mutations in the transmembrane and intradiscal domains of rhodopsin that cause RP have been shown to cause misfolding of the mutant proteins (6–8). Only a very small number of mutations have been associated with the retinal disease characterized by a congenital night blindness (CNB) phenotype. CNB appears to be a stable condition that does not seem to cause photoreceptor degeneration resulting mainly in night vision impairment. Two of these mutations were previously studied, namely G90D (9) and A292E (10) in transmembrane helices II and VII of rhodopsin, respectively. The mechanism of action of the G90D and A292E mutations was proposed to be persistent activation of the phototransduction pathway by constitutive activity of the mutant proteins (9–11). Another possible explanation for the observed G90D mutant phenotype has been proposed, i.e. enhanced rate of thermal isomerization due to lowered activation energy caused by the mutation (12, 13). More recently, a third mutation associated with CNB, T94I, in the second transmembrane helix of rhodopsin, has been described (14). The study of mutations associated with CNB can provide important information about the differences between RP and CNB at the molecular level. A region of rhodopsin in the transmembrane helix II, toward the intradiscal site of the protein, is particularly interesting to investigate these differences. In this region, similar mutations in close proximity in rhodopsin cause RP and CNB: G87D (RP), G89D (RP), G90D (CNB), and T94I (CNB) (Fig. 1). Thus, although these diseases involve similar mutations in very close locations in rhodopsin, it is still a puzzle that their progression is very different, with RP being responsible for a severe phenotype causing retinal degeneration.

We report here on the expression and characterization of the T94I CNB mutant and other mutations at the 94 site, T94D, T94S, and T94K. Our results are consistent with the location of Thr-94 close to the retinal Schiff base linkage and suggest a role for this residue in keeping the correct structure of the retinal binding pocket of rhodopsin. A very unusual property is the extremely slow Meta II decay process of the mutant T94I. Although no misfolding is detected for this mutant, the high hydroxylamine reactivity indicates that its dark structure is significantly altered. Also, the low thermal stability in the dark may be relevant to the molecular mechanism of CNB (12, 13). The results obtained for the Thr-94 mutants highlight the tight coupling between the structural domains of rhodopsin in in vivo folding of this receptor. They also provide evidence that the molecular mechanism of CNB does not involve misfolding of the mutated proteins, as has been proposed (or shown) for many RP mutations, but it may be related to conformational stability changes of the mutant receptors.

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§ The abbreviations used are: RP, retinitis pigmentosa; CNB, congenital night blindness; DM, n-dodecyl β-D-maltoside; Metal, metarhodopsin I; Meta II, metarhodopsin II; PSB, protonated Schiff base; WT, wild type.
DnAs were analyzed first by restriction analysis (using NcoI transfected monkey kidney cells (COS-1) as described (17). Transfected the absence of 5 mM ATP and 5 mM MgCl2 (2 ml/plate) for 1 h at 4°C. (w/v) DM; buffer C, buffer A plus 0.05% DM; buffer D, 2 mM NaH2PO4, 321 in the boxed in a secondary structure model of rhodopsin. The lengths of the trans-

yellow green blue bolder black circles

321 in the boxed in a secondary structure model of rhodopsin. The lengths of the trans-

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Conformation of Rhodopsin Thr-94 → Ile Mutant

EXPERIMENTAL PROCEDURES

Materials—11-cis-Retinal was purified from illuminated all-trans-retinal using a modified version of a protocol described previously (15). Dodecyl maltoside (DM) was from Antrace (Maumee, OH). Antirhodopsin monoclonal antibody rho-1D4 was obtained from the National Cell Culture Center (Minneapolis, MN) and was coupled to cyanogen bromide-activated Sepharose 4B (Sigma). The binding capacity of the resulting antibody Sepharose was 0.6 µg/µl of the beads.

The buffers used are as follows: buffer A, 1.8 mM KH2PO4, 10 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl, pH 7.2; buffer B, buffer A plus 5 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride, and 1% (v/v) DM; buffer C, buffer A plus 0.05% DM; buffer D, 2 mM NaH2PO4, pH 6.0, 0.05% DM; buffer E, buffer D plus 150 mM NaCl.

Cloning, Expression, and Purification of Thr-94 Mutants—Construction of the mutant opsin genes at position 94 was carried out by replacement of the BlaII-NcoI restriction fragment in the synthetic opsin gene in the pMT4 vector (16). The cloning strategy involved the ligation of a large fragment from EcorI/VnoI digestion (5128 bp), a small fragment from EcoRI/BglII digestion (261 bp), and a small fragment from NcoI/VnoIXn1 digestion (752 bp) with the appropriate annealed synthetic oligonucleotide duplexes. The synthetic oligonucleotides used were 51-mer (both strands), with the following sequence for the wild type for the top strand 5′-gAT TCT TCC ATg gTC TTT gGC TCC ACC ACC CCT TAC ACC TCT CTC-3′, and 5′-CTAg gAg AgA ggT gTA gAg gAG gGT gAA gCC ACC gAA gAC CAT gAA gA-3′ for the bottom strand. The mutants contained the following codon changes at the threonine codon (ACC) underlined above: T94I, ACC → ATC; T94D, ACC → TCC; and T94S, ACC → GAC. Plasmid DNAs were analyzed first by restriction analysis (using NcoI enzyme) followed by DNA sequencing by the dyeoxy chain-terminated method.

The wild type and the mutant genes were expressed in transiently transfected monkey kidney cells (COS-1) as described (17). Transfected cells were harvested 50 h from the time of DNA incubation, washed twice with buffer A, and treated with 50 µl 11-cis-retinal for 12 h at 4°C for reconstitution. The cells were then solubilized in buffer B but in the absence of 5 mM ATP and 5 mM MgCl2 (2 ml/plaque) for 1 h at 4°C. Regeneration with all-trans-retinal was carried out by addition of 50 µm purified all-trans-retinal to the COS-cell suspension for 12 h at 4°C. Purification was carried out using rho-1D4 Sepharose in a newly de-

FIG. 1. Secondary structure model of rhodopsin. Two sites of RP (yellow) and CNB (green) rhodopsin mutants in transmembrane helix II in a secondary structure model of rhodopsin. The lengths of the trans-

membrane helices (boxed) are shown as in the crystal structure of rhodopsin (31). The helical region sequence amino acid 311–321 in the cytoplasmic region is also boxed as in the crystal structure (31). The secondary structure shows, in addition, the two palmitoyl groups (wig-

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 yellow green blue bolder black circles

Regeneration with all-

 C for reconstitution. The cells were then solubilized in buffer B but in

high salt conditions with very little protein being eluted at high salt conditions was very high for the wild type protein, the slightly higher

 wild type helix II restriction fragment in the synthetic

PEL digestion (5128 bp), a

Eco

I digestion (251 bp), and a small frag-

mental amount of protein eluted with 150 mM salt (Fig. 2C). In contrast, the protein from T94K mutant eluted predominantly at high salt conditions with very little protein being eluted at low salt condition indicating that most of the protein is misfolded.

UV-visible absorption spectroscopy—Absorption spectra were recorded with a PerkinElmer Life Sciences Lambda spectrophotometer equipped with water-jacketed spectroscopic sample holders containing a circulating water bath. All spectra were recorded with a bandwidth of 2 nm, response time of 1 s, and scan speed of 480 nm/min at 20°C. For photobleaching experiments, the samples were illuminated with a 150-watt fiber optic light equipped with a 495-nm long pass filter for 10 s, and spectra were immediately recorded. In some cases the bleaching was carried out for further periods of time up to 2 min.

Hydroxylation treatment was performed by adding an aliquot of a 1 M NH2OH, pH 7.0, stock solution to the sample, to a final concentration of 30 mM. Thermal bleaching experiments involved measuring the decrease of absorption at Amax in the visible with time at 37°C.

Rate of Metarhodopsin II (MetaII) Decay as Measured by Retinal Release—The rate of MetaII decay was measured, after illumination of the samples for 30 s, by means of fluorescence spectroscopy (18). Briefly, 2 µg of protein in 200 µl of buffer D were allowed to equilibrate at 20°C for 30 min, then bleached for 30 s, and the fluorescence increase measured. The excitation and emission wavelengths were 295 (slit = 0.25 nm) and 330 nm (slit = 12 nm), respectively. Spectra were normalized and fitted to single exponential functions using SigmaPlot to derive the t1/2 values.

RESULTS

Purification and Spectral Properties of Thr-94 Rhodopsin Mutants in the Dark—The expressed opsin proteins from T94I, T94D, T94S, and T94K were purified (Fig. 2) using separation on a rho-1D4 Sepharose column as under “Experimental Procedures.” A selective elution chromatographic method (19) was used to separate the folded (retinal-binding species; eluted at pH 6, no salt) and misfolded fractions (non-retinal binding species; eluted at pH 6, 150 mM NaCl) of the different recombinant rhodopsin studied. The method was previously used in the study of mutants associated with RP (6–8). Similar elution profiles were obtained for the wild type and the T94I and T94D mutants (Fig. 2A–C) with most of the protein eluting with pH 6 buffer, with no salt, in the third fraction, and only a very small amount of non-retinal binding fraction eluting with added salt. In the case of the T94S mutant the elution behavior was similar to that for the wild type, but, in addition, a significant amount of protein eluted with 150 mM salt (Fig. 2D). In contrast, the protein from T94K mutant eluted predominantly at high salt conditions with very little protein being eluted at low salt condition indicating that most of the protein is misfolded.
cially the T94I mutant with an absorption maximum in the visible region at 478 nm (Table I). The shift for the other mutants is less pronounced, with \( \lambda_{\text{max}} \) of 494 nm for T94S and 496 nm for T94D, respectively.

**Photobleaching and MetaII Decay Rates**—The photobleaching of the T94I and T94S mutants showed wild type-like behavior on illumination (Fig. 4). Thus, after 10 s of illumination with light \( \lambda = 495 \text{ nm} \) formed the characteristic 380 nm-absorbing species with concomitant disappearance of the visible band (Fig. 4, A–C). In contrast, T94D showed a very abnormal behavior (Fig. 4D). After 10 s of illumination, the product showed \( \lambda_{\text{max}} \) at 480 nm, presumably a MetaI-like product that changed little upon further illumination for up to 2 min and then being kept in the dark for 1 h. Altered photobleaching has also been reported for the G90D mutant (9), but in this case the MetaI-like species formed upon illumination decayed in the dark in a 1-h period even at the lower temperature of 15 °C (9). Altered photobleaching has been also reported for other mutants involving the introduction of Asp, like the RP G89D mutant (8). In this case, the species formed upon illumination decayed in the dark to free opsin plus all-trans-retinal which is different from the behavior observed for the T94D mutant we describe here which does not decay in the dark in the same period of time.

An aspect relevant to the functionality of the mutant proteins is the stability of their photoactive conformations, i.e., their MetaII species. The decay of the photointermediate MetaII formed upon rhodopsin illumination was examined for the wild type and the Thr-94 mutants by fluorescence spectroscopy. The fluorescence curves were fitted to a single exponential function, and the \( t_{1/2} \) values for the MetaII decay for the wild type rhodopsin is typically about 15 min under the conditions of the assay. All the mutants studied showed very abnormal values compared with the wild type. T94D and T94I showed very slow decay processes, being about 2- and 6.5-fold slower than the wild type, respect-

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**TABLE I**

| Recombinant rhodopsin | \( \lambda_{\text{max}}^a \) | Retinal release, \( t_{1/2}^b \) |
|-----------------------|----------------|-----------------|
| Wild type             | 500            | 15.8            |
| T94I                  | 478            | 96.9            |
| T94D                  | 496            | 30.1            |
| T94S                  | 494            | 7.2             |
| T94K                  | ND             | ND              |

\( ^a \) \( \lambda_{\text{max}} \), wavelength of the absorption band in the visible region for wild type and the mutants.

\( ^b \) By the fluorescence increase assay as described under “Experimental Procedures.”

\( ^c \) ND, not determined due to the extremely low amount of chromophore formed by this mutant.
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FIG. 4. UV-visible photobleaching spectra for the WT rhodopsin and the 94 mutants. Spectra labeled dark are the corresponding spectra taken in the dark, and spectra labeled light are those taken after 10 s of illumination with light ~495 nm. All photobleaching spectra are normal except for T94D. This mutant shows a very abnormal bleaching behavior. Shown are the spectra, for T94D, after 10 s and after 2 min of illumination which were essentially identical. After 2 min of illumination, the sample was kept in the dark for 60 min, and the spectrum recorded (labeled 60') showed essentially no change.

Thermal Stability and Reactivity to Hydroxylamine in the Dark—Although the purified mutants showed good \( A_{280}/A_{500} \) ratio indicating the presence of correctly folded proteins, it was important to investigate the stability of these proteins in the dark state and their Schiff base accessibility. To this goal, assays were carried out to measure the chromophore stability in the dark (thermal bleaching) and the accessibility of the protonated Schiff base (PSB) in the dark (hydroxylamine reactivity).

Thermal bleaching was followed by monitoring the decay of the visible absorption band with time at 37 °C in the dark (Fig. 5A). The results obtained show that wild type rhodopsin and the mutant T94S had the same behavior in their stability in the period studied. The mutant T94D was less stable (20% decrease in 5 h), and T94I, in particular, showed reduced thermal stability (loss of 70% absorption in 5 h, Fig. 5A). The accessibility of the PSB toward hydroxylamine was also measured in the dark (Fig. 5B). Again, wild type rhodopsin and the mutant T94S were completely stable to NH2OH reactivity in the dark for 2 h, and the susceptibility of the mutants T94D and T94I to hydroxylamine again followed the order they showed for thermal instability. After 2 h about 45% of the mutant T94D had survived, although the mutant T94I had reacted with hydroxylamine more than 80%.

All-trans-retinal Binding by Mutant T94I—As seen in Fig. 6, the T94I mutant also bound all-trans-retinal forming a chromophore with its visible absorption band located at 466 nm, close to the 478 nm band observed for the same mutant regenerated with 11-cis-retinal (Fig. 6). Because the \( \lambda_{\text{max}} \) for the PSB under denaturing conditions is 440 nm, the chromophore for all-trans-retinal-constituted pigment reflects most likely the presence of a PSB linkage. The presence of the PSB was confirmed by acification of the sample that resulted in the formation of the 440-nm characteristic absorption (data not shown).

DISCUSSION

RP mutations in rhodopsin have been shown to affect correct folding of the receptor resulting in impaired 11-cis-retinal binding (6–8). This misfolding would be caused by structural uncoupling between the packing of the helices and the formation of the tertiary structure in the intradiscal domain of the protein (8). Rhodopsin mutations associated with CNB have been proposed to act by a constitutive activity mechanism involving disruption of the electrostatic salt bridge between Glu-113 and the PSB (9, 11). In contrast, it has been shown that constitutive activity is not the cause of photoreceptor degeneration in transgenic mice carrying RP mutations (20). An alternative mechanism, involving thermal instability of the mutant rhodopsins, has also been proposed for CNB mutations, like G90D (12, 13). Thus, different molecular mechanisms underlying RP and CNB have been outlined previously (34). We wanted to provide new insights into the differences between the molecular mechanisms of RP and CNB by studying the phenotypic features of the recently found T94I mutant associated with CNB, and comparing them with those observed for other CNB and RP mutants reported previously (6–13, 36).

The T94I mutant protein could be eluted at low salt condi-
tions indicating that the protein is folded and forms the retinal binding pocket. However, in the case of T94S, a small amount of misfolded protein could be eluted at high salt conditions indicating the presence of some misfolded opsin. T94K was mostly misfolded because most of the protein was eluted at high salt conditions, and only a very small amount could be eluted at low salt conditions. The mutants that formed chromophore showed blue-shifted $\lambda_{\text{max}}$ in the visible region. The observed blue shifts in the $\lambda_{\text{max}}$ in the visible absorption bands, particularly for T94I, involving a change from a polar to a hydrophobic side chain, indicate that Thr-94 in rhodopsin is located in proximity to the retinal binding pocket. The results obtained indicate that the dark state conformation of the T94I mutant must be significantly different from that of wild type rhodopsin. This conclusion can be reached from the following results. (i) This purified mutant protein showed reduced thermal stability in the dark, and even at the moderate temperature of 37 °C the decay of the chromophoric band was greatly accelerated compared with that of the wild type rhodopsin (mutant T94I samples stored at 4 °C also decayed in several weeks). In contrast, T94S mutant shows the same stability as wild type rhodopsin. (ii) T94I mutant protein shows reactivity toward hydroxylamine in the dark. This may be interpreted as an increase in the accessibility of the PSB by the reagent, reflecting structural alterations in the PSB environment and in the intradiscal domain, in the dark state. (iii) T94I mutant is able to regenerate chromophore with all-trans-retinal to give a PSB.

A different behavior can be observed for the mutants upon illumination. T94I and T94S showed the same photobleaching behavior than wild type, whereas T94D showed a very abnormal photobleaching pattern. Both T94I and T94S showed the formation of the 380-nm Meta II conformation. T94D showed the formation of a Meta-like photoproduct at 480 nm that is stable at 20 °C for at least 1 h. This result is in contrast with those found for other mutants, like the RP G89D mutant (8) and the CNB G90D mutant, which showed also an altered photobleaching (with formation of the of the Meta-like species), but the resulting species did decay in the dark in 1h in these latter cases (even at 15 °C in the case of G90D (9)). Thus, mutations involving the introduction of an Asp residue in the transmembrane domain show an altered photobleaching pattern, but the T94D mutation here described is different in the very slow decay of the species formed after bleaching.

The T94I mutant can regenerate chromophore with all-trans-retinal to form a PSB linkage in the dark. Several mutant rhodopsins have been described previously that can bind all-trans-retinal to form stable complexes. Among them, E113Q, involving mutation at the site of the counterion to the PSB which alters the $pK_a$ of the PSB, was found to be able to bind all-trans-retinal to form a 380 nm absorbing species (21). More recently, mutations at position 257, M257A for example, have been shown to bind all-trans-retinal to form a covalent complex that could be purified in detergent solution (22). The ability to bind all-trans-retinal has been correlated to the constitutive activity of the Met-257 mutants in the opsin state (23). However, in all these previously reported cases, all-trans-retinal-bound forms of the mutants had absorbance maxima at 380 nm reflecting the presence of an unprotonated Schiff base linkage.

In our case, the all-trans-retinal-bound mutant conformation resulting from photobleaching of the T94I (originally regenerated with 11-cis-retinal) mutant must be different from the all-trans-retinal form obtained from regenerating the mutant T94I opsin with all-trans-retinal, because both conformations show very different spectra, with the former showing an absorption band at 380 nm and the latter at 466 nm. It is inter-
phobic Ile would plausibly explain the marked blue shift in the $\lambda_{\text{max}}$ of T94I relative to wild type rhodopsin, as well as the stability and conformational specific properties of this mutant when compared with wild type rhodopsin. The T94K mutant shows the e-amino group of Lys-94 extending to Lys-296 (site of the PSB) with steric and electrostatic effects (Fig. 7C) that would explain the extensive misfolding observed for the mutant protein. The model for the mutant T94D shows close proximity of a carboxyl oxygen of Asp with the oxygen of the Glu-113 counterion (Fig. 7D). This proximity would cause a change in the electrostatic interactions network around the PSB and would account for the observed effects on the spectral and bleaching properties of this mutant rhodopsin. It is also interesting to note that a similar mutation to the T94I studied, T90A, has been recently shown to affect the properties of bacteriorhodopsin by affecting the Schiff base environment of this bacterial rhodopsin (35).

From the reported results, taken together with those reported previously for G90D (9) and A292E (10), it was foreseen that T94I mutant could be also constitutively active. In fact, constitutive activity of the T94I mutant has been recently reported previously for G90D (9) and A292E (10), it was foreseen that T94I mutant could be also constitutively active. In fact, constitutive activity of the T94I mutant has been recently reported previously. From the reported results, taken together with those reported previously for G90D (9) and A292E (10), it was foreseen that T94I mutant could be also constitutively active. In fact, constitutive activity of the T94I mutant has been recently reported previously. From the reported results, taken together with those reported previously for G90D (9) and A292E (10), it was foreseen that T94I mutant could be also constitutively active. In fact, constitutive activity of the T94I mutant has been recently reported previously. From the reported results, taken together with those reported previously for G90D (9) and A292E (10), it was foreseen that T94I mutant could be also constitutively active. In fact, constitutive activity of the T94I mutant has been recently reported previously.
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