Mutagenesis Studies of Human Red Opsin: Trp-281 Is Essential for Proper Folding and Protein−Retinal Interactions†

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Received August 26, 1998; Revised Manuscript Received October 12, 1998

ABSTRACT: Human red and green opsins contain a strikingly large number of tryptophan residues. These tryptophans are highly conserved among all red and green opsins. To investigate possible roles of these tryptophans in folding and structure, we have systematically replaced each tryptophan of human red opsin. When expressed in COS cells, wild-type red opsin undergoes N-linked glycosylation, forms a light-sensitive pigment with absorption maximum at 560 nm upon reconstitution with 11-*cis*-retinal, and is transported to the plasma membrane. We used the extent of glycosylation, pigment generation, and intracellular localization of mutant red opsins as our criteria for assessing the effect of substitution. Replacement of eight tryptophans, Trp-59, Trp-90, Trp-149, Trp-152, Trp-183, Trp-191, Trp-195, and Trp-243, with Phe or Ala did not affect the wild-type phenotype significantly. However, replacement of Trp-5 and Trp-51 in the putative N-terminal domain and Trp-142, Trp-177, Trp-179, and Trp-281 in the transmembrane domain with Phe had profound effects, indicating that these substitutions affected red opsin folding. Judged by the severity of the effects, we propose that Trp-5, Trp-51, Trp-177, and Trp-281 are important for red opsin folding. Although substitution of Trp-281 with Phe and Cys did not permit normal glycosylation and transport, substitution with Tyr and His permitted these processes but resulted in blue-shifted pigment. Thus, polar aromatics appear to substitute for Trp-281 to allow red opsin folding. The large spectral shift indicates that Trp-281 is essential for the proper interaction of the protein with 11-*cis*-retinal.

Red, green, and blue cone visual pigments are light-sensing proteins in cone photoreceptor cells that play a central role in mediating color vision and spatial resolution in humans. These three cone pigments and rhodopsin, the visual pigment that mediates dim light vision in rod photoreceptor cells, are G-protein coupled receptors and share a common structural and functional motif. Each of the four visual pigments consists of a seven membrane spanning apoprotein, opsin (*1*, *2*), and the covalently bound 11-cis retinal chromophore. The differences in the amino acid sequence of each opsin give rise to different spectral and functional properties unique to each visual pigment.

Red, green, blue, and rod opsins absorb light maximally at 560, 530, 430, and 500 nm, respectively. Spectral tuning of red and green pigments have been extensively investigated. By genetic analysis and spectral sensitivity studies of primate visual pigments (*3*), mutagenesis studies of human red and green visual pigments (*4*−*6*), and mutagenesis studies of bovine rhodopsin (*7*), the major spectral shifts from red to green pigments were shown to originate from amino acid changes in only three positions in the transmembrane domain. The spectral tuning is also modulated by the Schiff base environment of the visual pigments. Resonance Raman spectroscopy studies of recombinant human visual pigments and chicken red sensitive iodopsin indicate that a part of the opsin shift of long-wave sensitive pigments results from the weakening of the protonated Schiff base-counterion interaction (*8*, *9*).

We recently reported that coexpression of red opsin with a C-terminal fragment of bovine retinal Ran binding protein 2 (RanBP2)[1] increases the production of folded red opsin in COS-1 cells and proposed that RanBP2 functions as a chaperone for long-wave-sensitive opsins (*10*, *11*). Early autoradiographic studies of frog cone photoreceptors show that newly synthesized cone pigments in the inner segment are transported to the outer segment and then become diffusely distributed throughout the lamellae in the outer segment (*12*). However, the synthesis and transport of cone opsins remain largely unknown. In contrast, the biosynthesis process of rhodopsin is better understood. Rod opsin is synthesized in the rod inner segment and co- and posttranslationally modified (amino terminal acetylation, N-glycosylation, disulfide bond formation, and palmitoylation; see

† This research was supported by the Fight for Sight research division of Prevent Blindness America, American Cancer Society Grant IN152L-144, University of Connecticut Health Center Faculty Research Grant, and Connecticut Lions Eye Research Foundation.

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[1] Abbreviations: PBS, phosphate-buffered saline; ER, endoplasmic reticulum; GFP, green fluorescent protein; Endo F, *N*-glucosidase F.; Endo H, *N*-glucosidase H.; RanBP2, Ran binding protein 2; RBD4-CY, a C-terminal fragment of RanBP2 consisting of Ran binding domain 4 and cyclophilin domain. Mutants are named in single letter code with the position number between the original and the substituted amino acid.

10.1021/bi982077u CCC: $15.00
Published on Web 11/20/1998

reviews in refs *13*−*15*). Studies of many rhodopsin mutants, including those associated with retinitis pigmentosa, in cell culture systems have shown that rhodopsin folding is highly sensitive to mutations in the intradiscal (extracellular) and transmembrane domains of rhodopsin (*16*−*20*). Many of these mutants accumulate in the ER and do not bind 11-*cis*-retinal. In photoreceptor cells, normal rhodopsin is transported to the plasma membrane at the outer segment base (*12*), where individually separated disks containing newly synthesized rhodopsin are formed. Newly assembled disks are then gradually displaced from the base to the tip of the outer segment (*12*, *21*). Recent studies indicate that the C-terminus of rhodopsin is required for the transport of rhodopsin to the outer segment (*22*−*24*).

One feature of human red and green opsins is that they contain a strikingly large number of tryptophan residues. While rod and blue opsin contain only five and seven tryptophans, respectively, red and green opsins contain fourteen tryptophans (Figure 1) that are highly conserved among all vertebrate red and green opsins (Table 1). Tryptophan has the largest side chain among the twenty common amino acids and is predominantly found inside proteins (*25*). To investigate whether these tryptophans play a role in folding, structure, and/or the retinal binding site of red and green opsins, we systematically replaced each tryptophan of human red opsin. We found that replacement of six tryptophans (Trp-5, Trp-51, Trp-142, Trp-177, Trp-179, and Trp-281) had profound effects on red opsin folding. In particular, Trp-5 and Trp-51 at the N-terminus and Trp-177 and Trp-281 in the transmembrane domain appear to be important for red opsin folding in an early stage of biosynthesis. Moreover, Trp-281 exhibited extreme sensitivity to the amino acid used for replacement. Spectral analysis indicated that Trp-281 is essential for proper interaction of the protein moiety with the 11-*cis*-retinal chromophore.

EXPERIMENTAL PROCEDURES

Materials. Complementary DNA encoding human red opsin was a gift from Dr. Jeremy Nathans (Johns Hopkins University). 11-*cis*-Retinal was kindly provided by Dr. Rosalie Crouch (Medical University of South Carolina and the National Eye Institute), complementary DNA encoding green fluorescent protein-KDEL by Dr. Mark Terasaki (University of Connecticut Health Center), and monoclonal anti-rhodopsin antibody 1D4 by Dr. Robert Molday (University of British Columbia). *N*-Glucosidases F and H were purchased from Boehringer Mannheim. *n*-dodecyl β-D-maltoside was from Anatrace. Dideoxyadenosine 5′-[α-[^{35}*S*]-thio-triphosphate (500 Ci/mmol) was from Dupont-New England Nuclear. Phagemids pSL1180 and 1190 were from Pharmacia. Sculptor in vitro mutagenesis kit was from Amersham. Restriction enzymes were from New England Biolab and GIBCO. Fluorescein-conjugated goat anti-mouse IgG was from Organon Teknika Corp. Cy-5 conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs were from Boehringer Mannheim. The enhanced chemiluminescence detection system was from Amersham.

Construction of Red Opsin Mutants. For mutagenesis of Trp-5, Trp-51, and Trp-59, the *Eco*R1-*Bam*H1 (270 bp) fragment of wild-type red opsin cDNA was cloned into phagemid pSL1190. For mutagenesis of Trp-142, Trp-149, Trp-152, Trp-177, Trp-179, and Trp-183, the *Bam*H1-*Sph*1 fragment (370 bp) of wild-type red opsin cDNA was cloned into phagemid pSL1180. For mutagenesis of Trp-243 and Trp-281 mutant red opsins, the *Sma*1-*Sph*1 (220 bp) fragment of wild-type red opsin cDNA was cloned into pSL1190. Mutagenesis was carried out by the oligo nucleotide mediated mutagenesis method (*26*) using the Sculptor in vitro mutagenesis kit. The nucleotide sequence of DNA fragments containing a desired mutation was confirmed by the dideoxy method (*27*). The mutant red opsin cDNAs were constructed using a mutant fragment and fragments of a modified red opsin cDNA and cloned into the pMT3 expression vector as previously described (*10*). Thus, the wild-type and all the mutants described here carry the rhodopsin C-terminal 14 amino acids containing the monoclonal antibody 1D4 epitope at their C-termini (amino acid 362). Complementary DNA fragments used to construct mutant red opsin cDNA are as follows: the *Eco*R1-*Bam*H1 fragment containing a mutation and the *Bam*H1-*Not*1 fragment of wild-type red opsin cDNA for the Trp-5, Trp-51, and Trp-59 mutants, the *BamH*1-*Sph*1 fragment containing the mutation and the *EcoR*1-*BamH*1 and *Sph*1-*Not*1 fragments of wild-type red opsin cDNA for the Trp-142, Trp-149, Trp-177, Trp-179, and Trp-183 mutants, and the mutant *Sma*1-*Sph*1 fragment and the *Eco*R1-*Sma*1 and *Sph*1-*Not*1 fragments of wild-type red opsin cDNA for the Trp-243 and Trp-281 mutants.

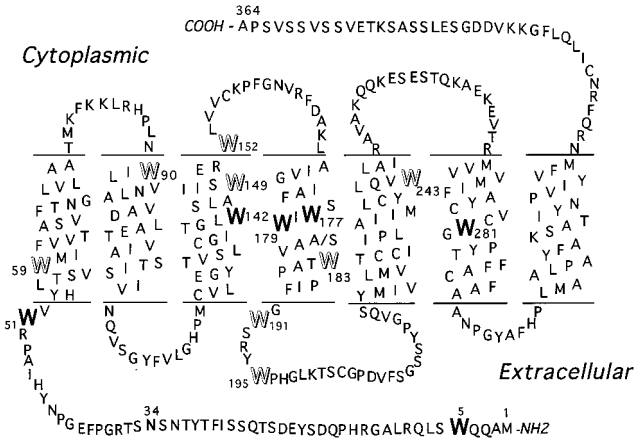

FIGURE 1: Secondary structure model of human red opsin showing tryptophans studied in the present work. Tryptophan mutants that resemble wild-type red opsin are shown in large white bold letters, and tryptophan mutants with significantly altered properties are shown in large dark bold letters. The membrane−aqueous boundaries are arbitrarily drawn. The potential glycosylation site, asparagine-34 in the N-terminal tail, is shown in a small bold letter. The helices are referred to as 1−7 from left to right in the text.

Expression of Wild-Type and Mutant Red Pigments in COS-1 Cells. The wild-type and mutant red opsin were transiently expressed with and without a C-terminal fragment of Ran binding protein 2 consisting of Ran binding domain 4 and cyclophilin homologous domain (RBD4-CY) in COS-1 cells as previously described (*10*). Pigment generation by addition of 11-*cis*-retinal (20 μM) and the purification of wild-type and mutant red pigments with monoclonal antibody 1D4-Sepharose were done as previously described (*10*). Typically, COS cells cultured in four to six 15 cm plates were transfected for the expression of each mutant.

Table 1: Amino Acids in Blue and Rod Opsins Located in Positions Corresponding to Tryptophans in Human Red and Green Opsins

tryptophans in human red/green	amino acids in human blue	amino acids in human rhodopsin[a]	conservation among vertebrate opsins	
			red/green	blue/rhodopsin
W5	none	none	W(50%)/A(19%)/L(19%)	none
W51	W32	W35	W(100%)	W(83%)
W59	F40	Y43	W(94%)	Y(71%)/F(29%)
W90	Y71	Y74	W(100%)	Y(92%)
W142	W123	W126	W(100%)	W(100%)
W149	F130	I133	W(100%)	I(52%)/F(33%)/V(15%)
W152	Y133	Y136	W(100%)	Y(73%)/W(23%)
W177	W158	W161	W(100%)	W(100%)
W179	I160	M163	W(100%)	M(71%)/I(19%)
W183	V164	C167	W(100%)	C(73%)/V(17%)/A(10%)
W191	W172	W175	W(100%)	W(100%)
W195	I176	I179	W(100%)	I(92%)
W243	L224	V227	W(100%)	V(50%)/L(42%)
W281	Y262	W265	W(100%)	W(81%)/Y(19%)[b]

[a] Human and bovine rhodopsins contain identical residues at these positions. [b] UV- and near-UV-sensitive blue and violet opsins contain Y.

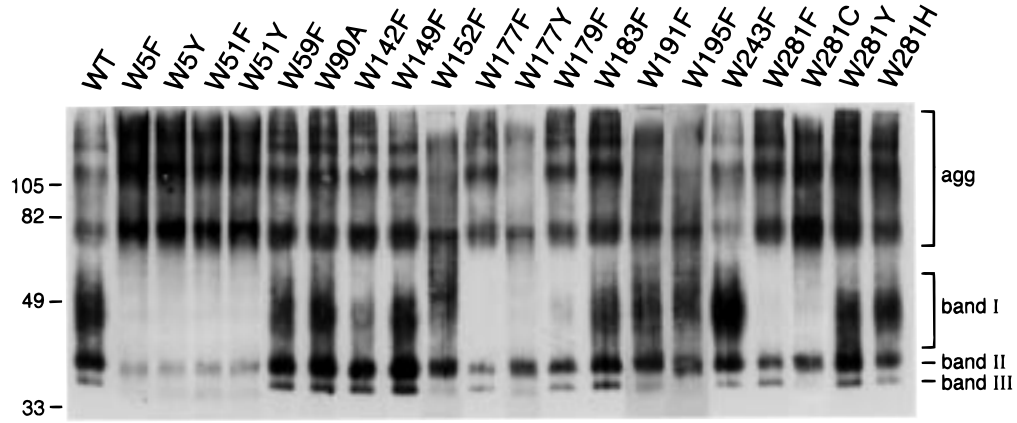

FIGURE 2: Western blot analysis of wild-type and mutant red opsins expressed in COS-1 cells. Aliquots of the purified proteins were subjected to 10% SDS polyacrylamide gel electrophoresis. The proteins were visualized by immunoblotting with monoclonal antibody 1D4 and using chemiluminescence. Bands of higher molecular mass (>80 kDa) indicated as agg are aggregated red opsin.

Characterization of Purified Wild-Type and Mutant Red Pigments. UV−vis absorption spectra of mutant red pigments were recorded on a Perkin-Elmer lambda 14 UV−visible spectrophotometer. Bleaching spectra were recorded after illuminating samples through a 550 or 495 nm long-pass filter. For Western blot analysis, aliquots (1−3 μL) of purified wild-type and mutant proteins were subjected to 10% SDS/polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. The protein bands were visualized by using monoclonal antibody 1D4 and the chemiluminescence method. For deglycosylation of wild-type and mutant red opsins, aliquots (0.5 μL) of purified protein were incubated with Endo F in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 0.1% SDS or with Endo H in phosphate buffered saline (PBS), pH 6.0, containing 0.02% SDS for 1 h at 37 °C. Deglycosylated proteins were subjected to Western blot analysis as described above.

Immunofluorescence Labeling of COS-1 Cells. Cells were grown on glass cover slips and transfected. Following 48 h after transfection, cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized in ice-cold methanol for 3 min, and labeled with monoclonal antibody 1D4 (10 μg/mL), followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (10 μg/mL) essentially as previously described (*16*, *17*). To visualize the ER and to confirm that some mutants are retained in the ER, the W5F mutant was expressed with green fluorescent protein (GFP) attached to the ER signal sequence and the KDEL ER retention sequence (*28*). For colocalization of GFP and W5F, CY5-conjugated goat anti-mouse IgG was used as a secondary antibody to visualize W5F. Cells were examined with a Zeiss LSM410 confocal microscope.

RESULTS

Wild-Type Red Opsin Expressed in COS-1 Cells. The wild-type and mutant red opsins were purified as described in Experimental Procedures, and the purified proteins were characterized. Western blot analysis of wild-type red pigment showed multiple protein bands plus high molecular weight aggregates and suggested that human red opsin expressed in COS cells is glycosylated (Figure 2). Glycosidases were used to determine the type of carbohydrate added to red opsin. Endo F cleaves all N-linked oligosaccharides, whereas Endo H cleaves high mannose oligosaccharides attached in the ER but not the complex oligosaccharides generated in the Golgi apparatus. Treatment of purified red opsin with Endo F converted the broad band of molecular mass centered around 50 kDa (band I) and a 40 kDa band (band II) to a 38 kDa band (band III). Upon treatment with Endo H, band II converted to band III, while the mobility of band I did not change (pattern 1 in Figure 3). The results indicate that band I is a mature form of red opsin with complex carbohydrates, band II is a mannose-rich form, and band III is unglycosylated red opsin. Immunofluorescence studies showed that

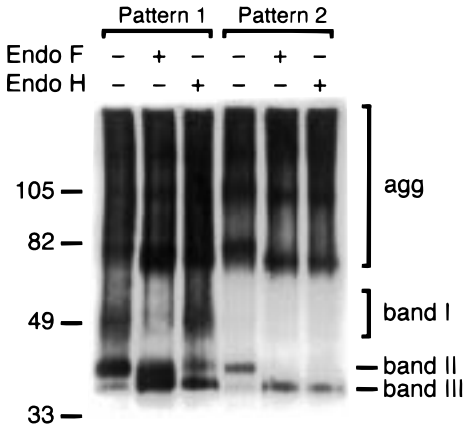

FIGURE 3: Characterization of two glycosylation patterns observed in the present study. Wild-type red opsin (pattern 1) and W177F (pattern 2), representing two different glycosylation patterns, were analyzed by treatment with Endo F and Endo H. Deglycosylated samples were subjected to 10% SDS polyacrylamide gel electrophoresis. The proteins were visualized by immunoblotting using monoclonal antibody 1D4 and chemiluminescence. agg indicates aggregated red opsin.

wild-type red opsin was efficiently transported to the plasma membrane (Figure 4). These results show that human red opsin undergoes *N*-glycosylation and is transported efficiently to the plasma membrane when expressed in COS-1 cells, similar to rhodopsin expressed in cultured cells. We estimated that approximately 4−6 μg of wild-type red opsin was purified from a 15 cm culture plate. This value was calculated from absorbance at 280 nm based on the molar extinction coefficient (95 000 cm^{-1} M^{-1}) calculated by the method described by Edelhock (*29*). A similar expression level of color opsins has been previously reported (*30*). We estimated that approximately 50% of purified red opsin bound 11-*cis*-retinal and generated pigment as previously described (*10*).

Mutants That Resemble Wild-Type Red Opsin. Out of twenty mutants characterized, eight (W59F, W90F, W149F, W152F, W183F, W191F, W195F, and W243F) showed the wild-type glycosylation and pigment formation. The W59F, W90F, W149F, W152F, W183F, W191F, and W195F mutants produced the fully glycosylated protein (band I, Figure 2), and the proteins were transported to the plasma membrane as wild-type red opsin (data not shown). These mutants bound 11-*cis*-retinal and generated wild-type pigment; however, the extent of pigment formation ranged from 30 to 70% of that of wild-type red opsin (Figure 5A, Table 2). In contrast, the W243F mutant gave a similar or occasionally slightly higher yield of fully glycosylated protein and pigment generation (Figures 2 and 5A). The level of pigment recovered correlates with the glycosylation pattern, in which the fully glycosylated red opsin, band I, is present in high yields when pigment levels are high. In addition, the W243F mutation reduced protein aggregation (Figure 2). These results suggest that the substitution of W243 slightly increased the folding efficiency and thus the stability of the protein, while other mutations in this group slightly decreased the folding efficiency and destabilized the protein.

Previously, we have observed that coexpression of wild-type red opsin with the RBD4-CY fragment of bovine retinal RanBP2 nearly doubled the amount of folded red opsin (*10*, *11*). To examine whether RBD4-CY has effects on the folding of mutant red opsins, we also coexpressed the mutant

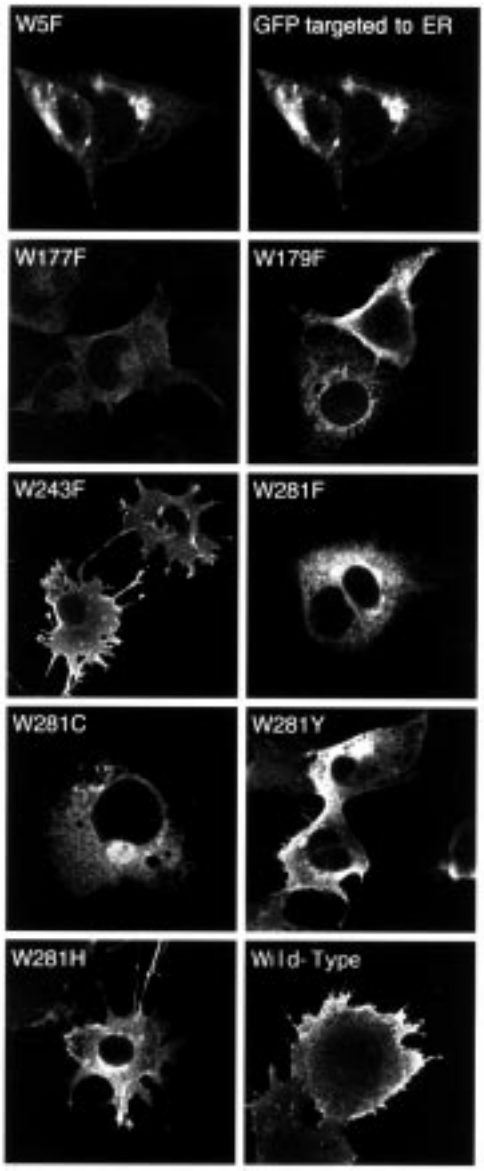

FIGURE 4: Immunofluorescence staining of COS-1 cells expressing wild-type and mutant red opsins. Mutants representing different phenotypes are shown. Cells expressing wild-type, W177F, W179F, W243F, and W281F/C/Y/H red opsins were fixed and labeled with monoclonal antibody 1D4 followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The W5F mutant was coexpressed with GFP targeted to the ER to demonstrate the localization of W5F in the ER. W5F was visualized with monoclonal antibody 1D4 as a primary antibody and Cy-5 conjugated goat anti-mouse IgG as a secondary antibody.

red opsins and RBD4-CY. Coexpression of RBD4-CY resulted in only a minimal increase in the extent of pigment formation (~20%), indicating that RBD4-CY was unable to fully rescue folding defects in these mutants.

Mutants That Profoundly Affected Glycosylation, Transport, and Pigment Generation. Substitution of five tryptophans (Trp-5, Trp-51, Trp-142, Trp-177, and Trp-179) produced little or no fully glycosylated protein and pigment. The W142F and W179F mutants produced a small amount of fully glycosylated protein (Figure 2) and formed pigment to about 10% of the wild-type level (Figure 5B, Table 2). The bleaching difference spectra show that the W142F and W179F generated the wild-type pigment, suggesting that

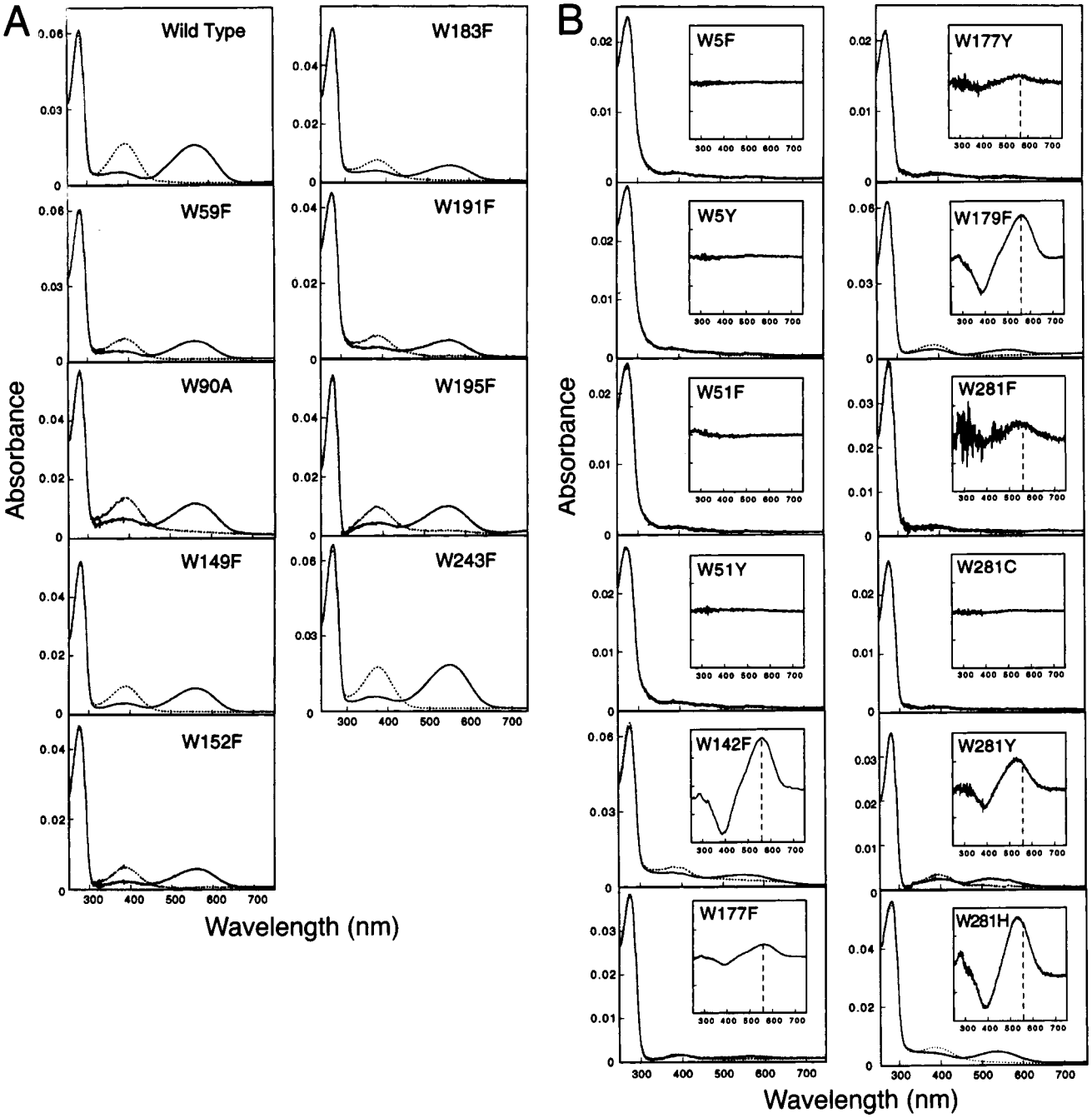

FIGURE 5: UV−vis absorption spectra of wild-type and mutant red pigments. Spectra were recorded before (solid line) and after (dotted line) bleaching. (A) Eight mutants generated wild-type pigment with absorption maximum at 560 nm. The extent of pigment generation varied from 30 to 110% of the wild-type level. (B) Mutants with little or no pigment generation. Bleaching difference specta are shown in inset. Absorbance scale in bleaching spectra were from +0.006 to −0.006 for W5F/Y, W51F/Y, W142F, and W179F and from +0.003 to −0.003 for W177F/Y and W281F/C/Y/H. Vertical lines in the insets indicate 560 nm. The W281F/Y/H pigments were blue-shifted by 20−30 nm.

these mutations did not interfere with the protein−retinal chromophore interaction. Immunofluorescence microscopy showed that W142F and W179F were retained in the ER in most cells, although they were observed in the plasma membrane in a small portion of cells. The immunolocalization of W179F is shown in Figure 4.

The mutants W5F and W51F, located in the putative N-terminal domain, and W177F, located in the fourth helix, did not produce the fully glycosylated protein (band I, Figure 2) and failed to generate pigment with 11-*cis*-retinal. In some experiments, W177F generated a very small amount (~5% of the wild-type level) of pigment with the wild-type absorption maximum at 560 nm (Figure 5B). Treatment of these mutant red opsins with endoglucosidases F and H confirmed that W5F, W51F, and W177F mutant red opsins were glycosylated only in the ER. The deglycosylation of W177F is shown in Figure 3 (pattern 2) as a representative of this group of mutants. Consistent with the glycosylation pattern, these proteins were accumulated in the nuclear periphery and cytoplasm, the region corresponding to the ER. To confirm that these mutant proteins are in the ER, W5F was coexpressed with a GFP construct targeted to the ER as described in Experimental Procedures. W5F fluorescence colocalizaed with GFP, confirming its localization with

Table 2: Characteristics of Tryptophan Mutants of Human Red Opsin[a]

mutant	abs max (nm)	pigment generation[b] (% of the wild type)	rel amt of red opsin with complex oligosaccharide
Wild type	560	100	+++
W5F		0	
W5Y		0	
W51F		0	
W51Y		0	
W59F	560	50	++
W90A	560	60−70	++
W142F	560	5−10	+
W149F	560	50−60	++
W152F	560	50−60	++
W177F	560	0−10	
W177Y		0−5	
W179F	560	5−10	+
W183F	560	30−40	++
W191F	560	30	++
W195F	560	60	++
W243F	560	100−110	++++
W281F	∼540	0−5	
W281C		0	
W281Y	530	20	++
W281H	530	25	++

[a] Purified mutant proteins and their spectra were analyzed from at least three different expressions. [b] % pigment generation was a relative amount of absorbance at their λmax with respect to that of wild-type red pigment.

the ER (Figure 4). We further substituted these three tryptophans with tyrosine to examine if it improved the severe effects observed with the Phe substitution since tryptophan is most frequently substituted with Phe and Tyr in proteins of high homology (*25*). However, there was no difference in the observed phenotype between the substitutions with phenylalanine or tyrosine (Figures 2 and 5B). The amount of purified W5F/Y, W51F/Y, and W177F/Y mutant proteins varied from 60 to 80% of that of the wild-type, suggesting reduced expression of these proteins, cellular degradation of these proteins, or both.

Since it is possible that 11-*cis*-retinal is inaccessible to W5F/Y, W51F/Y, W142F, W177F/Y, and W179F because they were not transported to the plasma membrane, we prepared membranes of COS cells and added 11-*cis*-retinal. Alternatively, these mutant proteins may be unstable and may require 11-*cis*-retinal during biosynthesis. Therefore, we incubated COS cells in the presence of 11-*cis*-retinal during the expression period. However, these attempts did not increase pigment generation. These results suggest that the substitution of Trp-5, Trp-51, Trp-142, Trp-177, and Trp-179 caused a defect in protein structure, resulting in misfolded proteins in the ER. Coexpression of these mutant red opsins with RBD4-CY had little effect on their glycosylation and pigment generation.

Replacement of Trp-281 with Tyr and His, but Not with Phe or Cys, Permitted Glycosylation, Transport, and Pigment Generation. The W281F mutant failed to produce fully glycosylated protein and to generate pigment and accumulated in the ER, essentially exhibiting the same phenotype as W5F/Y, W51F/Y, and W177F/Y described in the previous section. However, substitution of Trp-281 with Tyr produced fully glycosylated protein (Figure 2), and the protein was transported to the plasma membrane, although some are also present in the ER (Figure 4). Consistently, W281Y generated pigment to a small extent (∼20% of the wild-type level), but its absorption maximum was significantly blue-shifted (∼30 nm). To examine what amino acid side chain is tolerated at this position, we further constructed W281H and W281C. We chose histidine and cysteine because histidine preserves a half of the heterocyclic indole ring and cysteine offers a hydrophilic nonaromatic side chain. The W281H mutant was fully glycosylated (Figure 2), was transported to the plasma membrane (Figure 4), and generated blue-shifted pigment (∼30 nm, Figure 5B), resembling the W281Y phenotype closely. On the other hand, the W281C mutant was not fully glycosylated (Figure 2), accumulated in the ER (Figure 4), and failed to generate pigment (Figure 5B), resembling the W281F phenotype. Coexpression of the mutant proteins with RBD4-CY did not alter the W281F, W281Y, W281H, and W281C phenotypes. With respect to the relative amount of fully glycosylated protein present in purified W281Y and W281H, the extents of their pigment generation were low as compared to the other mutants. This observation suggests that the mutation destabilized the protein−retinal interactions. Further, large spectral shifts suggest that the substitution of Trp-281 perturbed the proper protein−retinal interactions.

DISCUSSION

Previously we have shown the expression of wild-type red opsin in COS cells and described an increase in the extent of red opsin folding when red opsin was coexpressed with the RBD4-CY fragment of bovine RanBP2. Using this expression system, we have now investigated the role of tryptophans in human red opsin folding. In an attempt to evaluate the tryptophan specific effects, we used conservative substitutions (mostly Phe and Tyr) to minimize changes in side chain properties. Fourteen tryptophans found in human red and green opsins are highly conserved throughout vertebrate red and green opsins (Table 1). Human red opsin contains one cognate glycosylation site (Asn-34) (*1*), and wild-type red opsin was *N*-glycosylated and transported to the plasma membrane when expressed in COS cells. Similar observations have been made in Sf-9 cells (*31*) and 293-EBNA cells (*32*). Therefore, we evaluated mutant red opsin folding using the criteria of the extent of glycosylation, the ability of mutant red opsins to generate pigment, and their intracellular localization. The phenotypes of the tryptophan mutants are largely divided into two groups: one relatively insensitive to the amino acid substitution and the other with profound mutational effects.

Out of fourteen tryptophans, eight tryptophans comprise the first group. They are Trp-59, Trp-90, Trp-49, Trp-152, Trp-183, and Trp-243, likely located in the helices 1−5 toward either the cytoplasmic or extracellular aqueous phase or in their membrane border, and Trp-191 and 195, likely located in the second extracellular loop or in the membrane border (Figure 1). The relatively small effects of Trp-191 and Trp-195 substitutions were somewhat surprising. This loop contains a cysteine residue (Cys-203) presumed to be involved in a conserved disulfide bond important in the structure and function of visual pigments (*33*, *34*). In human and bovine rhodopsins, many amino acid substitutions and deletions in this loop cause misfolding and/or destabilization of the protein (*16*−*18*, *20*). Either these two tryptophans have no dominant role in the structure of this extracellular loop,

or red opsin may not have a structural requirement similar to that of rhodopsin, or both. In red and green opsins, His-197 and Lys-200 in this loop are involved in chloride ion binding, which causes a spectral shift (*35*). The wild-type pigments generated from W191F and W195F indicate that a conservative substitution of Trp-191 and Trp-195 did not perturb the chloride ion binding.

Six tryptophans, Trp-5, Trp-51, Trp-142, Trp-177, Trp-179, and Trp-281, comprise the second group. Our results suggest that these mutations had impacts on folding in an earlier biosynthesis step. Trp-5 is located in the N-terminus, and Trp-51 is likely located in the membrane border as shown in Figure 1. The N-terminal amino acid sequences of red and green opsins are highly divergent from rhodopsins and blue opsins (*1*). All vertebrate red and green opsins contain a stretch of approximately 15−20 extra amino acids at their N-termini, and a tryptophan residue corresponding to Trp-5 in human red opsin is present in approximately 50% of them (Table 1). The significance of these long N-termini is not known. Some single amino acid substitutions or short deletions in the N-terminus of rhodopsin cause misfolding as do mutations in intradiscal loops (*16*−*20*). In contrast to W191F and W195F described above, the effects of the replacement of Trp-5 and Trp-51 were parallel to or more drastic than those of mutations in the rhodopsin N-terminus. The results may suggest a specific structural requirement of the red opsin N-terminus for correct folding of red opsin.

Substitution of Trp-142, Trp-177, and Trp-179, most likely located in the middle of the lipid bilayer (Figure 1), apparently perturbed the transmembrane domain structure, and therefore folding. While Trp-179 is conserved only among red and green opsins, Trp-142 and Trp-177 are conserved throughout rod and cone opsins (Table 1). Trp-142 and Trp-177 are analogous to Trp-126 and Trp-161 in bovine rhodopsin, and we have previously replaced them with various amino acids (*37*). Although substitution of Trp-126 reduced the ability to activate transducin, substitution of either Trp-126 or Trp-161 had no effect on folding. The bulky indole rings at positions 142, 177, and 179 could be important in a folding intermediate structure of red opsin, whereas they apparently do not play such a role in rhodopsin.

The highly conserved Trp-281 (Table 1) exhibited unusual sensitivity to amino acid substitution in red opsin. The Trp-281 mutants were glycosylated and transported to different extents depending on the amino acid used for the substitution. Substitutions with Tyr and His permitted red opsin folding and transport to the plasma membrane, but substitutions with Phe and Cys did not permit protein folding and, thus, transport. We hypothesize that the NH or OH group in the aromatic side chain could form an intra- or possibly intermolecular interaction which allows proper protein folding. In this regard, the role of Trp-281 in red opsin may resemble that of the conserved cysteines in the extracellular domain in rhodopsin. Trp-281 is conserved in all opsins except for UV-sensitive blue and violet opsins which contain tyrosine at this position (Table 1, ref *36*). It is equivalent to Trp-265 in bovine rhodopsin. Previously, we and others have shown that Trp-265 is located near the β-ionone ring of 11-*cis*-retinal (*38*, *39*), and W265F combined with an analogue of 11-*cis*-retinal generated an unbleachable pigment, whereas W265Y generated a bleachable pigment (*40*). On the basis of this observation, we postulated that the NH or OH group in Trp or Tyr, respectively, plays a role in light-activated conformational changes of the protein. However, replacement of Trp-265 had almost no effect on rhodopsin folding. While the role of Trp-281 in the bleaching of red pigment remains to be investigated, our present results on Trp-281 as well as Trp-142, Trp-177, and Trp-179 described above suggest a structural role for these residues, which has not been observed for the equivalent residues in rhodopsin.

In addition to folding, Trp-281 is clearly important in the protein−retinal chromophore interaction. Large blue shifts (~30 nm) caused by the substitution of Trp-281 in red opsin, like Trp-265 in rhodopsin (*37*) and Trp-182 and Trp-189 in bacteriorhodospin (*41*), indicate that their substitution perturbed the proper interaction of the protein moiety with 11-*cis*-retinal. In fact, Thr-285, one of the three amino acids found to cause the major spectral shift (20 nm) from green to red pigments (*4*−*6*), is four amino acid residues, i.e., one helical turn, away from Trp-281 in the sixth helix. Moreover, the other two amino acids responsible for the major shift, Ala/Ser-180, and Tyr-277 (*4*−*6*), are also suggested to be close to the ionone ring of 11-*cis*-retinal (*8*). Therefore, the replacement of Trp-281 is likely to cause the perturbation of the interactions of Thr-285, Ala/Ser-180, and Tyr-277 with 11-*cis*-retinal and remove the spectral shift caused by them. A 10 nm additional shift is possibly caused by structural perturbation of the overall retinal binding site. Compared to other mutants, a relatively small recovery of the W281Y/H pigments with respect to the amount of fully glycosylated protein suggests that the substitution indeed perturbed the retinal binding site and reduced the stability of the mutant pigments.

Last, coexpression of the RBD4-CY fragment of RanBP2 had little or no effect on folding of the tryptophan mutated red opsins. Possible explanations for this observation include the following. First, the introduced mutations caused fundamental defects in structure and folding such that RBD4-CY could not increase folding of the mutants. Second, RBD4-CY may exert effects of red opsin folding/maturation in the biosynthetic pathway downstream of the ER; therefore, RBD4-CY had no effects on misfolded protein that accumulated in the ER. Third, mutations may have prevented or interfered with the proper interaction of red opsin mutant with RBD4-CY. The effects of RBD4-CY on the stability of the mutant proteins and the effects of other domains of RanBP2 on the wild-type and mutant red opsins remain to be addressed.

ACKNOWLEDGMENT

We thank Dr. Jeremy Nathans for providing us red opsin cDNA, Dr. Rosalie Crouch for 11-*cis*-retinal, Dr. Robert Molday for monoclonal antibody 1D4, Dr. Mark Terasaki for the GFP-KDEL cDNA, Dr. Shozo Yokoyama for unpublished aligned amino acid sequences of rod and cone opsins and helpful discussion, Dr. Laurinda Jaffe for critical reading of the manuscript, and Ms. Susan Krueger for her excellent assistance in the immunofluorescence studies. Fluorescence microscopy was carried out at the Center for Biomedical Imaging Technology at the University of Connecticut Health Center.

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BI982077U