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Light-stable Rhodopsin

II. AN OPSIN MUTANT (TRP-265 → PHE) AND A RETINAL ANALOG WITH A NONISOMERIZABLE 11-*cis* CONFIGURATION FORM A PHOTOSTABLE CHROMOPHORE*

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In order to prepare a completely light-stable rhodopsin, we have synthesized an analog, II, of 11-*cis* retinal in which isomerization at the C_{11}–C_{12} *cis*-double bond is blocked by formation of a cyclohexene ring from the C_{10} to C_{13}-methyl. We used this analog to generate a rhodopsin-like pigment from opsin expressed in COS-1 cells and opsin from rod outer segments (Bhattacharya, S., Ridge, K. D., Knox, B. E., and Khorana, H. G. (1992) *J. Biol. Chem.* 267, 6763–6769). The pigment (λ_{max}, 512 nm) formed from opsin and analog II (rhodopsin-II) showed ground state properties very similar to those of rhodopsin, but was not entirely stable to light. In the present work, 12 opsin mutants (Ala-117 → Phe, Glu-122 → Gln(Ala, Asp), Trp-126 → Phe(Leu, Ala), Trp-265 → Ala(Tyr, Phe), Tyr-268 → Phe, and Ala-292 → Asp), where the mutations were presumed to be in the retinal binding pocket, were reconstituted with analog II. While all mutants formed rhodopsin-like pigments with II, blue-shifted (12–30 nm) chromophores were obtained with Ala-117 → Phe, Glu-122 → Gln(Ala), Trp-126 → Leu(Ala), and Trp-265 → Ala(Tyr, Phe) opsins. The extent of chromophore formation was markedly reduced in the mutants Ala-117 → Phe and Trp-126 → Ala. Upon illumination, the reconstituted pigments showed varying degrees of light sensitivity; the mutants Trp-126 → Phe(Leu) showed light sensitivity similar to wild-type. Continuous illumination of the mutants Glu-122 → Asp, Trp-265 → Ala, Tyr-268 → Phe, and Ala-292 → Asp resulted in hydrolysis of the retinyl Schiff base. Markedly reduced light sensitivity was observed with the mutant Trp-265 → Tyr, while the mutant Trp-265 → Phe was light-insensitive. Consistent with this result, the mutant Trp-265 → Phe showed no detectable light-dependent activation of transducin or phosphorylation by rhodopsin kinase.

In attempts to prepare light-stable analogs of rhodopsin for use in structural studies, we described the preparation of retinal analog II (Fig. 1) in which the 11-*cis* configuration is rendered nonisomerizable by inclusion in a cyclohexene ring (1). Analog II allowed effective reconstitution of a rhodopsin-like pigment (rho-II)[1] from bovine opsin expressed in COS-1 cells as well as opsin from bovine rod outer segments (ROS). Further, rho-II and rhodopsin (rho-I) showed comparable ground state properties. Thus, 1) the visible absorbance maximum (~512 nm) was only 12 nm red-shifted from that of rho-I; 2) the molar extinction coefficient (41,200 M^{-1} cm^{-1}) and opsin shift (2600 cm^{-1}) of rho-II were virtually identical with that of rho-I (ϵ, 40,600 M^{-1} cm^{-1} and 2650 cm^{-1} opsin shift); and 3) rho-II was completely stable, like rho-I, in the presence of hydroxylamine and detergents such as dodecyl maltoside. However, rho-II was not entirely light-stable. Lack of complete photochemical stability has also been reported previously for rhodopsin analogs prepared by reconstituting ROS opsin with II and a retinal analog where the 11-*cis* configuration is maintained by inclusion in a cyclopentene ring (2, 3). These findings suggest that the structural constraints on the photosensitive C_{11}–C_{12} *cis*-double bond in II are not sufficient to render rhodopsin completely light-insensitive.

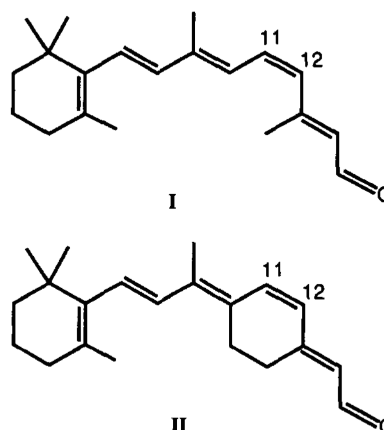

FIG. 1. **Structures of 11-*cis* retinal, I, and the cyclohexatrienylidene retinal analog, II.** Retinal analog II was prepared as described under "Experimental Procedures" in Ref. 1.

In further efforts to obtain a light-stable rhodopsin-like pigment, we have now investigated reconstitution of a set of opsin mutants with retinal analog II. The amino acid residues substituted in these mutants (Fig. 2) are presumed to line the retinal binding pocket based on chemical cross-linking and

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[1] The abbreviations used are: rho-I, wild-type bovine opsin reconstituted with 11-*cis* retinal, I; rho-II, wild-type bovine opsin reconstituted with retinal analog II; ROS, rod outer segments; bR, bacteriorhodopsin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The standard three and one letter abbreviations are used for amino acids.

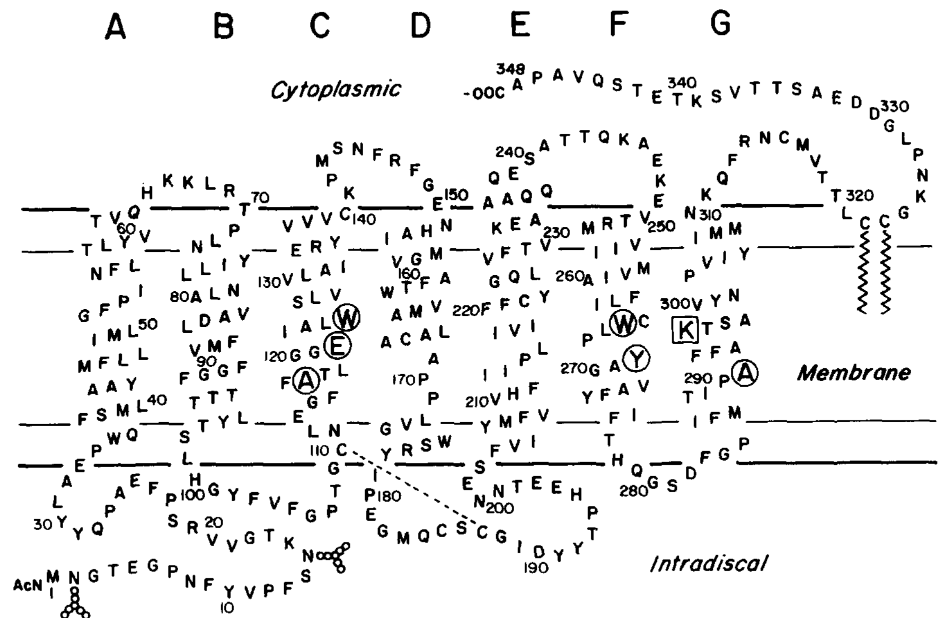

FIG. 2. **Proposed secondary structure model of bovine rhodopsin.** The amino acid substitutions investigated in the present work are *circled*, and Lys-296, the attachment site of retinal (8), is *boxed*. The amino terminus of rhodopsin is acetylated (9), and Asn residues 2 and 15 are glycosylated (10, 11). Cys residues 110 and 187 participate in a disulfide bond (12), while those at 322 and 323 are palmitoylated (13). The borders of the helices (indicated by the letters *A* to *G*) and connecting loops have been arbitrarily drawn.

mutagenesis studies (4–6). Upon reconstitution with 11-*cis* retinal, **I** (Fig. 1), these mutants displayed altered ground and/or excited state properties. We show that of the 12 mutants with single amino acid substitutions (A117F,[2] E122Q(D, A), W126F(L, A), W265Y(F, A), Y268F, and A292D), all form rhodopsin-like chromophores with **II**. The reconstituted mutants showed significant differences in their light sensitivity, indicating that the specific amino acid alterations can selectively influence the photochemistry of the chromophore. The goal of obtaining a light-stable rhodopsin-like pigment was accomplished upon reconstitution of the opsin mutant W265F with **II**. As expected, this pigment did not show any light-dependent signal transduction. Thus, by combining two independent approaches, namely synthetic retinal chemistry and site-specific mutagenesis, a light-stable rhodopsin analog has been prepared.

EXPERIMENTAL PROCEDURES

Materials—Dodecyl maltoside was from Anatrace. Hydroxylamine hydrochloride was from Aldrich Chemical Co. Sequenase (version 2.0) was purchased from U. S. Biochemicals. 11-*cis* Retinal was a gift of Dr. P. Sorter (Hoffman-La Roche). Adenosine 5′-[γ-^{32}P]triphosphate, deoxyadenosine 5′-[α-^{35}S]thiotriphosphate, and guanosine 5′-[γ-^{32}P]triphosphate were from Du Pont-New England Nuclear. The retinal analog, **II**, was prepared as described in Ref. 1.

*Expression and Purification of Rhodopsin-**II** Mutants*—The construction of mutant opsin genes used in this investigation have been described previously (6). The wild-type and mutant opsin genes were transiently expressed in COS-1 cells essentially as described (7). Pigments were generated with **I** or **II** and purified by immunoaffinity adsorption (1).

*Spectral Characterization of Rhodopsin-**II** Mutants*—Spectroscopic measurements were performed with a Perkin-Elmer λ7 UV/visible spectrophotometer. All spectra were recorded in 10 mM Tris-HCl, pH 7.0, containing 0.1% (w/v) dodecyl maltoside and 150 mM NaCl at 20 °C. Pigments were illuminated for the indicated time periods with a 300-watt light source through a 495 or 475 nm long pass filter. Pigments were acid-denatured by adjusting the pH of the sample to 1.9 with 2 N H_2SO_4. The reactivity of hydroxylamine was examined by adding a solution of hydroxylamine hydrochloride (adjusted to pH 7.0 with NaOH) to a final concentration of 100 mM. All visible absorbance maxima are ±3 nm.

Functional Assays—Light-dependent activation of transducin and phosphorylation by rhodopsin kinase was done essentially as described (1).

RESULTS

*Chromophore Formation with Opsin Mutants and Retinal Analog **II***

Twelve opsin mutants, in which selected amino acid residues presumed to be in the retinal binding pocket were substituted (6), have presently been studied by reconstitution with retinal analog **II**. The mutants include A117F, E122Q(A, D), and W126F(L, A) in helix C; W265A(Y, F) and Y268F in helix F; and A292D in helix G. A summary of their ground state spectral properties is presented in Table I. All of the opsin mutants generated rhodopsin-like chromophores with **II**. Chromophores, blue-shifted by 12–30 nm, relative to rho-**II**, were obtained with A117F, E122Q(A), W126L(A), and W265A(Y, F) mutants (Table I and Fig. 3). Similar results were obtained previously on reconstitution of these mutants with **I** (6). Opsin mutants E122D and A292D showed wild-type chromophores, λ_{max} 508 and 509 nm, respectively, upon reconstitution with **II**, while blue-shifted chromophores were obtained upon reconstitution of these mutants with **I** (6, 14, 16). The molar extinction coefficient values for E122Q(A)-**II**, W126F-**II**, and W265Y-**II** were increased from 41,200 M^{-1} cm^{-1} for rho-**II**, while those for W126L-**II** and W265A-**II** were slightly lower (Table I). The extent of chromophore formation in these mutants varied considerably. The mutants E122Q(A) and W265F generated chromophores with **II** to a level equivalent to or greater than wild-type and to a significantly higher extent than with **I** (Table I). In contrast, the mutants A117F and W126F(L, A) formed chromophores to a markedly lower extent relative to wild-type and when compared with **I**. The low level of chromophore generation in these mutants is presumably due to steric interference because of the cyclohexene ring in **II**.

*Photochemical Behavior of Rhodopsin-**II** Mutants*

*Mutants with Light Sensitivity Similar to Rhodopsin-**II***

*Rho-**II***—On illumination at >495 nm for 10 s, rho-**II** showed a 3 nm blue-shift in λ_{max} and ~3% loss of visible

[2] Amino acid substitutions are designated by the wild-type amino acid residue, its position in the sequence, and the replacing amino acid residue. Thus, "A117F" signifies the mutant in which the alanine at position 117 has been replaced by phenylalanine.

TABLE I
Absorption maxima, molar extinction coefficients, and extent of chromophore formation of rhodopsin-II mutants

The spectral properties were measured as described under "Experimental Procedures."

Mutation	Absorption maxima[a]	Extinction coefficient[b]	Chromophore formation[a]
	nm	M^{-1} cm^{-1}	%
Rho	512 (498)	41,200	86 (84)
A117F	~481 (490)	ND[c]	~9[d] (44)
E122Q	501 (482)	43,400	94 (78)
E122A	500 (476)	44,400	99 (83)
E122D	508 (476)	40,200	60 (94)
W126F	512 (498)	45,100	38 (89)
W126L	494 (490)	38,500	59 (94)
W126A	~482 (486)	ND	~12[d] (84)
W265A	493 (470)	39,200	29 (22)
W265Y	492 (483)	45,200	54 (68)
W265F	493 (480)	40,900	78 (58)
Y268F	512 (493)	42,200	59 (84)
A292D	509 (488)	40,500	54 (52)

[a] The values in parentheses are for opsin mutants reconstituted with 11-*cis* retinal, **I** (6).

[b] Molar extinction coefficients were determined as described (14). The extinction coefficient of rhodopsin was assumed to be 40,600 M^{-1} cm^{-1} (15). All values have been rounded to the nearest 100 M^{-1} cm^{-1}.

[c] ND, the value could not be accurately determined because of the low level of chromophore formation.

[d] Calculated with ϵ, 41,200 M^{-1} cm^{-1}.

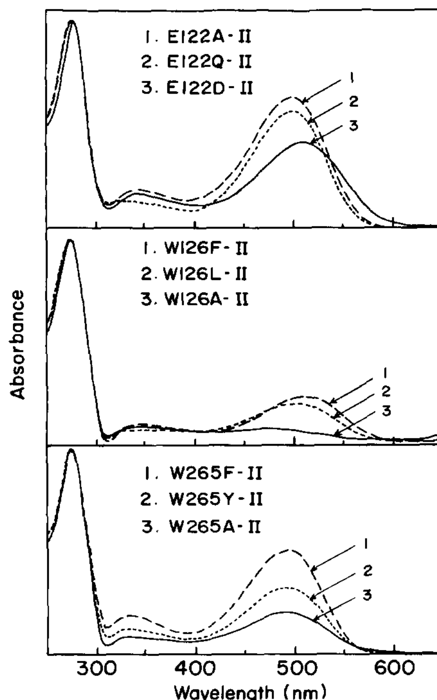

FIG. 3. **UV/visible absorption spectra of rhodopsin-II mutants E122Q(A, D), W126F(L, A), and W265A(Y, F).** Opsin mutants were reconstituted with retinal analog **II**, solubilized in dodecyl maltoside, and purified by immunoaffinity adsorption as described under "Experimental Procedures" in Ref. 1. Spectra recorded in the dark are shown. The corresponding absorption maxima are listed in Table I.

absorbance (Fig. 4). Further illumination resulted in a progressive blue-shift in λ_{max} (up to 20 nm) with a concomitant lowering of visible absorbance (maximally up to ~40%) and the appearance of a broad absorbtion band between 475 and 310 nm. No clear isosbestic point was evident, suggesting that more than one species was being formed. After 12 min of

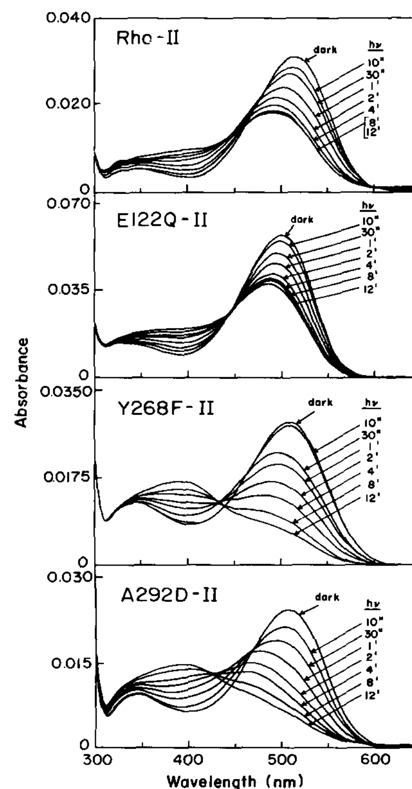

FIG. 4. **UV/visible absorption spectra of rhodopsin-II and rhodopsin-II mutants E122Q, Y268F, and A292D upon continuous illumination.** The purified pigments were illuminated at >495 nm for the indicated time periods at 20 °C.

continuous illumination, an apparent photochemical steady state resulted, and no further changes were subsequently detected in the spectrum. We have defined this as "saturating illumination" (1), and all opsin mutants reconstituted with analog **II** were compared under these conditions. The presence of a Schiff base in the 12-min illuminated pigment was tested by acid denaturation. A species with a λ_{max} at 450 nm, which is characteristic of the protonated retinyl Schiff base (17), was observed. Thus, the covalent linkage between opsin and **II** was preserved under these conditions.

The Mutants W126F-II and W126L-II—Similar to rho-**II**, W126F-**II** showed a 20 nm blue-shift in λ_{max} and a 50% loss of visible absorbance under the above conditions of illumination. An 18 nm blue-shift in λ_{max} and a 73% loss of visible absorbance was observed with W126L-**II** under identical illumination conditions. No isosbestic point was apparent with either mutant, and subsequent acid denaturation indicated that the covalent linkage between the protein and **II** remained intact.

Mutants with Light Sensitivity Different from Rhodopsin-II

The Mutants E122Q-II, E122A-II, and E122D-II—Continuous illumination (>495 nm) of E122Q-**II** resulted in a 15 nm blue-shift in λ_{max} and a 34% decrease in visible absorbance (Fig. 4). Unlike rho-**II**, a single isosbestic point at 445 nm was observed. This finding suggests that a single species is being produced in E122Q-**II** during 12 min of continuous illumination. Similar to E122Q-**II**, a 10 nm blue-shift in λ_{max}, a 37% loss of visible absorbance, and a broad isosbestic point at ~445 nm were observed with E122A-**II** under identical illumination conditions. A species with a λ_{max} at 450 nm was apparent after acid denaturation of the 12-min illuminated E122Q-**II** and E122A-**II** pigments. E122D-**II** showed an 18

nm blue-shift in λ_{max} and a 52% loss of visible absorbance upon 12 min of continuous illumination. Unlike E122Q-**II** and E122A-**II**, no single isosbestic point was observed. The appearance of a 390 nm species was evident after 8 min of illumination; an increase in this peak was observed upon further illumination. As free **II** and the unprotonated Schiff base of **II** both have a λ_{max} at ~390 nm (1), the 12-min illuminated E122D-**II** was further characterized by acid denaturation. A mixture of species with a λ_{max} at 390 and 450 nm was evident upon acidification, indicating the Schiff base had partly hydrolyzed.

The Mutants Y268F-II and A292D-II—Illumination of Y268F-**II** at >495 nm for 10 s caused only a minor decrease (~3%) in λ_{max} (Fig. 4). Further illumination (30 s–12 min) resulted in a progressive blue-shift in λ_{max}, an incremental loss of visible absorbance, and the appearance of a 390 nm species. A single isosbestic point (435 nm) became apparent for this conversion after 30 s of continuous illumination. Acid denaturation indicated that the 390 nm species did not contain a Schiff base linkage.

Continuous illumination of A292D-**II** at >495 nm caused a gradual blue-shift in λ_{max}, a pronounced loss of visible absorbance, and the generation of a 390 nm species. Two distinct isosbestic points at 465 nm and 425 nm were observed (Fig. 4). As with E122D-**II** and Y268F-**II**, acid denaturation of the illuminated A292D-**II** indicated that the Schiff base linkage had undergone partial hydrolysis.

The Mutants W265A-II, W265Y-II, and W265F-II—The three Trp-265 mutants showed striking differences in light sensitivity. W265A-**II** showed only a marginal blue-shift (~3 nm) in λ_{max} after 12 min of continuous illumination (>475 nm), but a 47% decrease in visible absorbance. In addition, a species with a λ_{max} at 390 nm appeared (Fig. 5). A broad isosbestic point at ~430 nm was observed for this conversion. Subsequent acid denaturation indicated that the 390 nm species represented the analog **II** with no Schiff base linkage.

W265Y-**II** displayed markedly reduced light sensitivity when compared with W265A-**II** or rho-**II**. After 4 min of continuous illumination (>475 nm), only a 3 nm blue-shift in λ_{max} and a 16% loss of visible absorbance was evident (Fig. 5). No significant change in λ_{max} or visible absorbance was evident after an additional 8 min of illumination, suggesting that a photochemical steady state was achieved.

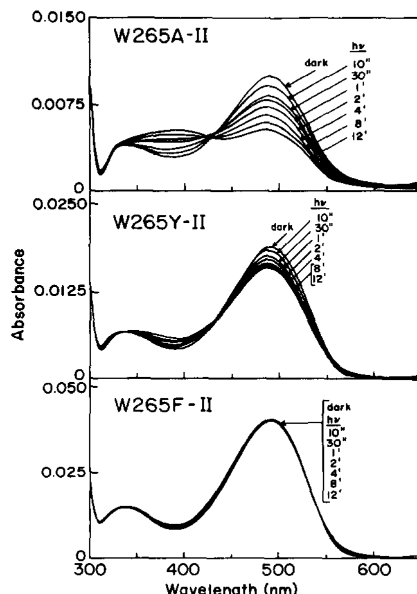

FIG. 5. **UV/visible absorption spectra of rhodopsin-II mutants W265A, W265Y, and W265F upon continuous illumination.** The purified pigments were illuminated at >475 nm for the indicated time periods at 20 °C.

In contrast to the above mutants, the mutant W265F-**II** showed complete insensitivity upon illumination. Thus, no detectable change in λ_{max} or visible absorbance was evident even after 12 min of continuous illumination at >475 nm (Fig. 5). The light minus dark difference spectra comparing the 12-min illuminated rho-**II** and W265F-**II** highlights the remarkable photostability of W265F-**II** (Fig. 6). In rho-**II**, a major depletion appeared at a wavelength corresponding to 530 nm. No such changes occurred with W265F-**II**.

W265F-II Does Not Activate Transducin or Undergo Phosphorylation

Transducin Activation—Activation of transducin was examined by measuring the light-dependent hydrolysis of GTP. Determination of the specific activities revealed that the level of GTP hydrolysis by rho-**II** was ~12% of that of ROS rhodopsin (Table II). Consistent with light insensitivity, W265F-**II** exhibited no detectable capacity to activate transducin. The level of GTP hydrolysis was indistinguishable from that obtained with opsin.

Phosphorylation by Rhodopsin Kinase—Rho-**II** was maximally phosphorylated by rhodopsin kinase to ~10% the level of that observed with ROS rhodopsin (Fig. 7*A*). Phosphorylation by rhodopsin kinase showed that the stoichiometry of ^{32}P incorporation into W265F-**II** was similar to that of opsin

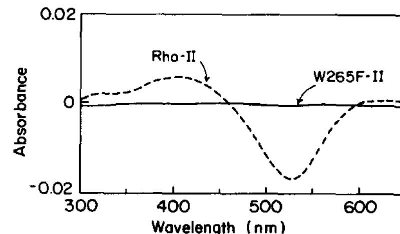

FIG. 6. **Light minus dark difference spectra of rhodopsin-II and W265F-II.** The spectra recorded before and after 12 min of continuous illumination of rho-**II** (*broken line*) and W265F-**II** (*continuous line*) were subtracted. The pigments were of identical chromophore absorbance prior to illumination.

TABLE II

Transducin activation as measured by GTP hydrolysis

GTP hydrolysis was measured as described under "Experimental Procedures" in Ref. 1.

Sample[a]	Specific activity[b]		Activation[c]
	Light	Dark	
	mol $^{32}P_i$ released/mol rho/min		%
ROS rhodopsin	34 ± 5.0	0.9 ± 0.5	100
Rho-I	31 ± 1.5	0.8 ± 0.4	90
Rho-II	5 ± 0.6	0.8 ± 0.5	12
W265F-II	1.3 ± 0.6	0.9 ± 0.5	1
Opsin	1.2 ± 0.6	0.8 ± 0.4	1

[a] The amount of pigment assayed was based on the molar extinction coefficient. The extinction of opsin was assumed to be 65,000 M^{-1} cm^{-1}.

[b] Each value is the average of four independent determinations ± S.D. The reaction rate for the hydrolysis of GTP in the light and the dark was plotted as a function of the amount of pigment used in the assay. The specific activities were calculated from the slope of the lines.

[c] The values were normalized to the activity of purified ROS rhodopsin.

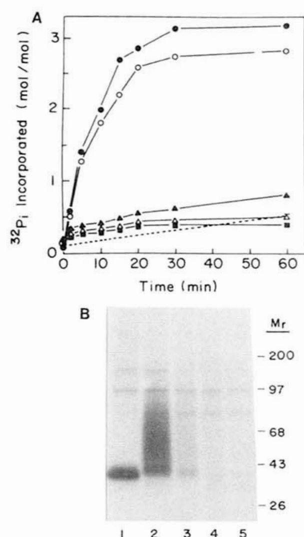

FIG. 7. **Rhodopsin kinase-catalyzed phosphorylation.** *A*, time course of rhodopsin kinase-catalyzed phosphorylation. Light-dependent phosphorylation by rhodopsin kinase was performed as described under "Experimental Procedures" in Ref. 1. Aliquots from the phosphorylation reactions were removed at the indicated time periods, and the stoichiometry of ^{32}P incorporation was determined. ●, ROS rhodopsin; ○, rho-**I**; ▲, rho-**II**; △, W265F-**II**; ■, opsin; - - -, dark controls. *B*, autoradiograph of the phosphorylated pigments. Aliquots from the phosphorylation reactions were removed after 60 min and analyzed by 10% reducing SDS-PAGE and autoradiography. *Lane 1*, ROS rhodopsin; *lane 2*, rho-**I**; *lane 3*, rho-**II**; *lane 4*, W265F-**II**; *lane 5*, opsin. The glycosylation pattern of COS-1 cell opsin contributes to the apparent molecular weight heterogeneity observed on SDS-PAGE (18). The higher molecular weight phosphorylated species present in all the lanes correspond to proteins which co-purify with rhodopsin kinase. Molecular weight markers are shown on the *right*.

and to the dark controls. An autoradiograph revealed that no phosphorylated species was present at a molecular weight corresponding to W265F-**II** (Fig. 7*B*, *lane 4*).

DISCUSSION

As described in the preceding paper (1), we attempted to prepare a light-stable analog of rhodopsin by the use of a retinal analog, **II** (Fig. 1), in which the 11-*cis* configuration is rendered nonisomerizable by formation of a cyclohexene ring with the C_{10} and C_{13}-methyl of the polyene (1). Reconstitution of the bovine opsin expressed in COS-1 cells or opsin from bovine ROS with **II** yielded the analog, rho-**II**, which displayed ground state properties very similar to those of rho-**I**. As expected, rho-**II** was substantially photoresistant, but not completely light-stable. Similar findings with a number of retinal analogs have been reported previously (2, 3). In bacteriorhodopsin (bR), a light-insensitive pigment is formed with a retinal analog in which the C_{13}–C_{14} *trans*-double bond is included in a cyclopentene ring (19). Clearly, the retinal binding pocket of bR can accommodate this analog such that the above structural constraint is sufficient to obtain a photoresistant pigment. While it is possible that a different retinal analog would yield a light-stable rhodopsin, we have now investigated a complementary approach in which opsin mutants carrying amino acid replacements are reconstituted with **II** to form rhodopsin-like pigments. Twelve opsin mutants, which showed altered interactions with **I** in the ground and/or excited state (6), were reconstituted with **II** and their properties were examined.

All the opsin mutants tested formed rhodopsin-like chromophores with retinal analog **II** (Table I). The mutants A117F, E122Q(A), W126L(A), and W265A(Y, F) showed blue-shifted chromophores, as was previously observed upon reconstitution of these opsin mutants with 11-*cis* retinal, **I** (6). These findings suggest that the interactions of **II** in the retinal binding pocket with these opsins closely resemble those of **I**. Two notable exceptions were E122D and A292D, which showed wild-type chromophores with **II** and blue-shifted chromophores with **I**.

All the Trp-126 mutants, and the mutant A117F, showed relatively low levels of chromophore formation with **II** (Table I and Fig. 3). A possible reason may be steric because of the introduced cyclohexene ring in **II**. Both W126F-**II** and W126L-**II** showed light sensitivity similar to that of rho-**II**, suggesting that these mutations have little or no effect on chromophore structure during illumination. Of the other helix C mutants investigated, E122Q and E122A formed chromophores with **II** almost quantitatively (94–99%), while the opsin produced from E122D generated 60% of the expected pigment (Table I and Fig. 3). Unlike rho-**II**, both E122Q-**II** and E122A-**II** presented a single isosbestic point during illumination suggesting that a single species was being produced (Fig. 4). Thus, these amino acid substitutions at Glu-122 appear to influence the photochemistry of the chromophore during illumination. Continuous illumination caused hydrolysis of the retinyl Schiff base in E122D-**II**, as well as in W265A-**II**, Y268F-**II**, and A292D-**II** (Figs. 4 and 5). Presumably, these amino acid substitutions cause significant perturbations in the retinal binding pocket such that photochemical changes in **II** may allow the Schiff base to become accessible to water.

Of the 12 mutants examined, only one, W265F, yielded a rhodopsin-like pigment with **II** that was light-stable and therefore devoid of signal transduction. Several ground state properties of W265F-**II** also suggest that it closely resembles rho-**I**. First, the visible absorbance maximum (493 nm) is only 7 nm blue-shifted relative to rho-**I** (Table I and Fig. 3). As W265F shows a 20 nm blue-shift upon reconstitution with **I** (6), the observed λ_{max} represents an intermediate between the red-shift due to **II** and the blue-shift arising from the opsin mutation. Second, the molar extinction coefficient (40,900 M^{-1} cm^{-1}) and opsin shift (1940 cm^{-1}) are similar to that of rho-**I**. As the opsin shift of W265F-**I** is 1900 cm^{-1}, the difference from rho-**I** is solely due to the opsin mutation. Third, W265F-**II**, like rho-**I**, is stable in the presence of hydroxylamine, indicating that the Schiff base is inaccessible to the aqueous environment.

Tryptophan 265 is a highly conserved amino acid among the visual opsins as well as in many other guanine nucleotide binding protein-coupled receptors with the seven helix structural motif. Previously, using a photoactivatable analog of 11-*cis* retinal, Trp-265 was found to be proximal to the β-ionone ring of the retinal molecule (4, 5). Additionally, the amino acid substitutions made at this position had profound effects on chromophore λ_{max}, extinction, bleaching behavior, and the kinetics of transducin activation when these opsin mutants are reconstituted with **I** (6). The present findings further support the importance of this tryptophan residue in rhodopsin function. Reconstitution of the opsin mutants W265Y and W265F with **II** had a remarkable effect on the ability of the chromophore to undergo photochemical changes. W265Y-**II** showed markedly reduced light sensitivity compared to that of rho-**II**. An apparent photochemical steady state was reached after 4 min of illumination (Fig. 5). Replacement of Trp-265 with Phe yielded a pigment which displayed no detectable change in λ_{max} or visible absorbance upon illumination (Figs. 5 and 6). Consistent with complete resistance to

light, W265F-**II** displayed no signal transducing capacity when assayed for light-dependent activation of transducin or phosphorylation by rhodopsin kinase (Table II and Fig. 7).

Whether a direct or indirect effect of replacing Trp-265 by Phe, and to some extent by Tyr, accounts for the increased light stability is not fully understood. From the cross-linking results (5), it is conceivable that these mutations introduce a steric constraint on **II** in the vicinity of the β-ionone ring. Consequently, any repositioning of the β-ionone end of **II** within the protein during illumination is restricted. In the case of W265F, such "fine tuning" would effectively "lock" the orientation of **II** in the retinal binding pocket. This may prevent accompanying or subsequent rotation around single bonds and/or isomerization around the unblocked double bonds of **II**, as is thought to occur in rho-**II** (1, 2). As a result, conformational changes in the protein cannot be elicited. The extent of such an interaction would depend on the amino acid residue at position 265. In Trp and Tyr, -NH or -OH, respectively, can act as putative electron donors to a neighboring electron deficient acceptor (*e.g.* protonated Schiff base of a polyene network), particularly in the excited state. Such an interaction is less favorable with Phe because of the lack of any electron donating functional group. Further, both Trp and Tyr can hydrogen bond, which is not possible with Phe. Clearly, a three-dimensional structure of rhodopsin would be very useful in determining how the interactions between the chromophore and the amino acid side chains in these mutants have been affected.

A combination of synthetic retinal chemistry and site-specific mutagenesis has allowed us to prepare a light-stable rhodopsin-like pigment. When coupled with a large scale overexpression system and an efficient method for purifying quantitatively reconstituted pigment, the profound photostability of W265F-**II** provides us with an attractive candidate for further structural analyses by Fourier transform infrared spectroscopy, resonance Raman, and three-dimensional crystallography.

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