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Structure and function in rhodopsin: The role of asparagine-linked glycosylation

(membrane protein/signal transduction/carbohydrate/structure–function relationship)

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ABSTRACT **Rhodopsin, the dim light photoreceptor of the rod cell, is an integral membrane protein that is glycosylated at Asn-2 and Asn-15. Here we report experiments on the role of the glycosylation in rhodopsin folding and function. Nonglycosylated opsin was prepared by expression of a wild-type bovine opsin gene in COS-1 cells in the presence of tunicamycin, an inhibitor of asparagine-linked glycosylation. The nonglycosylated opsin folded correctly as shown by its normal palmitoylation, transport to the cell surface, and the formation of the characteristic rhodopsin chromophore (λ_{max}, 500 nm) with 11-*cis*-retinal. However, the nonglycosylated rhodopsin showed strikingly low light-dependent activation of G_T at concentration levels comparable with those of glycosylated rhodopsin. Amino acid replacements at positions 2 and 15 and the cognate tripeptide consensus sequence [Asn-2 → Gln, Gly-3 → Cys (Pro), Thr-4 → Lys, Asn-15 → Ala (Cys, Glu, Lys, Gln, Arg), Lys-16 → Cys (Arg), Thr-17 → Met (Val)] showed that the substitutions at Asn-2, Gly-3, and Thr-4 had no significant effect on the folding, cellular transport, and/or function of rhodopsin, whereas those at Asn-15 and Lys-16 caused poor folding and were defective in transport to the cell surface. Further, mutant pigments with amino acid replacements at Asn-15 and Thr-17 activated G_T very poorly. We conclude that Asn-15 glycosylation is important in signal transduction.**

Asparagine-linked (N-linked) glycosylation of membrane and secreted proteins is observed frequently, although the role that glycosylation may serve is not always evident (1–3). Bovine rhodopsin is glycosylated at Asn residues 2 and 15 (Fig. 1) by the hexasaccharide sequence $Man_3GlcNac_3$ (4, 5). We have now examined the role of N-linked glycosylation in rhodopsin folding and function. We expressed the wild-type bovine opsin gene (6) in the presence of the glycosylation inhibitor tunicamycin (TM). The resulting nonglycosylated opsin was normally palmitoylated, was transported to the cell surface, and formed the characteristic rhodopsin chromophore with 11-*cis*-retinal. However, the nonglycosylated rhodopsin showed strikingly diminished light-dependent activation of transducin (G_T) when compared with glycosylated rhodopsin. We next studied opsin mutants that contained amino acid replacements in the regions of the two glycosylation sites (Fig. 1). Mutations at or near Asn-2 had little effect on cell-surface expression, chromophore formation, and/or G_T activation. In contrast, mutations at Asn-15 and Lys-16 resulted in opsins that were defective in cellular transport and formed little or no chromophore with 11-*cis*-retinal. The mutations at Asn-15 and Thr-17 resulted in pigments that were defective in signal transduction. These results show that while glycosylation of rhodopsin is not required for its folding to an apparently correct ground-state structure, it is necessary for full activity in signal transduction.

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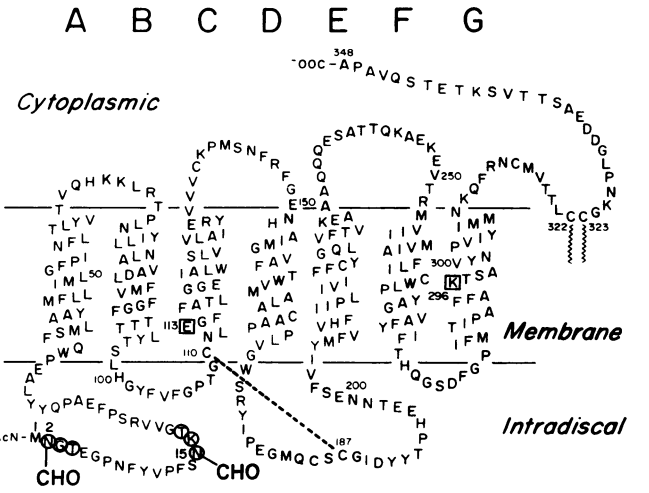

FIG. 1. A secondary structure model of bovine rhodopsin. The amino acid substitutions investigated here are circled, and the attachment sites for the carbohydrate are designated CHO. Cys-110 and Cys-187 form a disulfide bond (indicated by the boldface dashed line), whereas Cys-322 and Cys-323 are palmitoylated. The attachment site of retinal, Lys-296, and the counterion for the protonated Schiff base, Glu-113, are boxed. The seven membrane-embedded helical segments (indicated by letters *A–G*) are bordered approximately by the interrupted horizontal lines.

MATERIALS AND METHODS

Materials. The sources of most materials have been reported (7). N-Glycosidase F (PNGase F), TM, and rhodamine-conjugated goat anti-mouse IgG were from Boehringer Mannheim. D-[2-^{3}H]Mannose (20 Ci/mmol; 1 Ci = 37 GBq) was from DuPont/NEN, and DNA purification kits were from Qiagen (Chatsworth, CA).

Construction of Opsin Mutants. All mutants were prepared by cassette replacements of the synthetic bovine opsin gene in the pMT3 expression vector (6, 8). The N2Q, G3C/P, and T4K mutants were prepared by replacement of the *Eco*RI–*Fsp* I restriction fragment in the opsin gene. The N15A,-C, -E,-K,-Q,-R,K16C/R, and T17M/V mutants were prepared by replacement of the *Kpn* I–*Fsp* I restriction fragment in the opsin gene. The sequences of the mutants were confirmed by the dideoxynucleotide chain-termination method (9).

Expression and Purification of Wild-Type and Mutant Rhodopsins. The transient transfection of COS-1 cells with the opsin genes by a DEAE-dextran procedure has been described (7, 10). After chloroquine treatment (8 hr after

Abbreviations: PNGase F, N-glycosidase F; N-linked, asparagine-linked; ROS, rod outer segment; TM, tunicamycin; DM, 1-dodecyl β-D-maltoside; MII, metarhodopsin II; GTP[γS], guanosine 5′-[γ-thio]triphosphate; vis, visible; G_T, transducin. Mutants are named in single letter code with position number between the original and the substituted amino acid.

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addition of DNA), TM was added to the transfected cells at a concentration of 0.8 μg/ml. The cells were harvested 40–60 hr later, washed with 10 mM NaH_2PO_4 (pH 7.0) containing 150 mM NaCl (phosphate-buffered saline), and incubated with 5 μM 11-*cis*-retinal for 3 hr at 4°C in the dark. After solubilization in 1% 1-dodecyl β-D-maltoside (DM), the opsin was purified by immunoaffinity chromatography on 1D4-Sepharose (10) in (*i*) 10 mM Tris·HCl, pH 7.0/150 mM NaCl/0.1% DM or (*ii*) 2 mM NaH_2PO_4, pH 6.0/0.1% DM (7).

Cleavage of Oligosaccharide Chains with PNGase F. Purified rod outer segment (ROS) and COS-1 cell rhodopsins were deglycosylated with PNGase F (0.5 units per μg of opsin) in 20 mM Tris·HCl, pH 8.0/0.05% DM/0.5% SDS/5 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride for 1 hr at 20°C. The pigments were analyzed by SDS/PAGE (11) with a 5% stacking and a 10% or 12% resolving gel and immunoblotting (12) using the rho 1D4 antibody.

Labeling of Opsin in COS-1 Cells with [^{3}H]Mannose and [^{3}H]Palmitic Acid. Forty-eight hours after transfection, the COS-1 cells were incubated in glucose-free medium/10% fetal calf serum for 1 hr at 37°C. [^{3}H]Mannose (100 μCi/ml) was then added to the medium for 2 hr at 37°C. The cells were washed twice with cold phosphate-buffered saline and harvested; the opsin was then solubilized and immunoaffinity purified. Opsin was labeled with [^{3}H]palmitic acid as described (7).

Immunofluorescent Staining of Transfected COS-1 Cells. The procedures used were similar to those described by Doi *et al.* (13), except that the fixed and permeabilized cells were incubated with the rho 4D2 antibody (5 μg/ml) before staining with rhodamine-conjugated goat anti-mouse IgG (1:200).

Spectral Characterization of Wild-Type and Mutant Rhodopsins. Spectroscopic measurements were done with a λ7 UV/visible (vis) spectrophotometer. Molar extinction coefficients were determined as described (14, 15). Chromophore stability toward hydroxylamine was measured in a 100 mM (pH 7.0) solution. Pigments were illuminated for 10 s at 20°C with a 150-W light source through a >495-nm long-pass filter. Samples were acid denatured by adjusting the pH to 1.9 with 2 M H_2SO_4.

G_T Activation Assays. G_T was prepared from bovine ROS by the method of Fung *et al.* (16). The ability of wild-type and mutant rhodopsins to catalyze GTP–GDP exchange by G_T (17, 18) was analyzed as a function of rhodopsin concentration. The reaction mixtures (250 μl) contained 0.04–40 nM, pH 6.0, purified wild-type or mutant rhodopsin, 0.3 μM G_T, and 0.6 μM guanosine 5′-[γ-[^{35}S]thio]triphosphate (GTP[γS]) in 10 mM Tris·HCl, pH 7.5/0.012% DM/100 mM NaCl/5 mM $MgCl_2$/2 mM dithiothreitol. The samples were illuminated (>495 nm) for 2 min at 20°C and kept in the dark for 2 hr. The extent of GTP[γS] bound was determined by using a nitrocellulose filter-binding assay (7).

RESULTS

Expression of the Bovine Opsin Gene in COS-1 Cells in the Presence of TM. Bovine opsin was expressed in transiently transfected COS-1 cells in the presence of TM (0.8 μg/ml). The SDS/PAGE pattern of the wild-type opsin formed in the absence and presence of TM is shown in Fig. 2. While ROS opsin migrated as a species of 38 kDa, the expressed wild-type COS-1 cell opsin migrated as an elongated band with a sharp leading edge at 41 kDa and a trailing smear (Fig. 2*A*). This difference is apparently due to heterogeneity in the oligosaccharide chains because deglycosylation of this and ROS opsin with PNGase F both gave a species of 33 kDa. A small amount of the opsin dimer (60 kDa) was also present. Expression of the wild-type opsin gene in COS-1 cells in the presence of TM also yielded a 33-kDa opsin (Fig. 2*A*). An opsin of similar mobility has previously been observed upon expression of the bovine opsin gene in *Xenopus* oocytes and Sf9 insect cells in the presence of TM (19, 20). The mobility

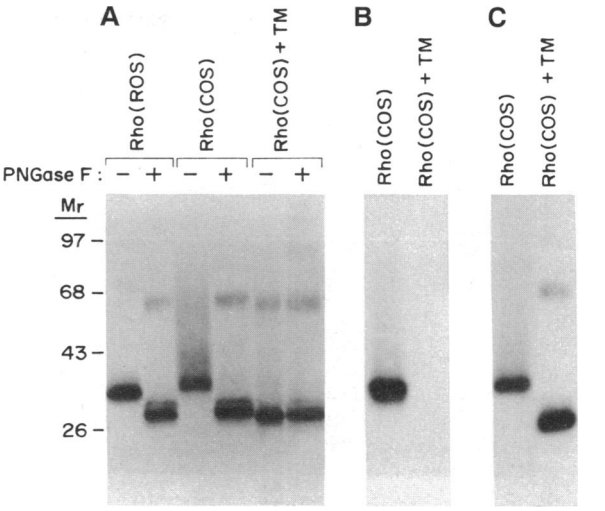

FIG. 2. Characterization of wild-type opsin expressed in the absence and presence of TM. (*A*) Sensitivity of ROS rhodopsin [Rho (ROS)], wild-type COS-1 cell opsin [Rho (COS)], and wild-type COS-1 cell opsin expressed in the presence of TM [Rho (COS) + TM] to PNGase F. Equivalent amounts of protein were analyzed by SDS/PAGE and immunoblotting before and after treatment with PNGase F. Fluorograph of [^{3}H]mannose (*B*) and [^{3}H]palmitic acid-labeled wild-type COS-1 cell opsin [Rho (COS)] expressed with and without TM (*C*). Equivalent amounts of protein were analyzed by SDS/PAGE and fluorography.

of this opsin, the absence of any change upon treatment with PNGase F (Fig. 2*A*), and the lack of [^{3}H]mannose incorporation (Fig. 2*B*) suggested that the above concentration of TM effectively prevented N-linked glycosylation.

Wild-type opsin undergoes palmitoylation (7) and is transported to the cell surface in COS-1 cells (13). Wild-type opsin expressed in the presence of [^{3}H]palmitic acid and TM showed that this opsin was also palmitoylated (Fig. 2*C*). Indirect immunofluorescence stains of COS-1 cells expressing the nonglycosylated wild-type opsin also demonstrated that it is transported to the cell surface (Fig. 3 *A* and *B*).

Spectral Properties of the Nonglycosylated Wild-Type Rhodopsin. The UV/vis absorption spectra of purified glycosylated and nonglycosylated wild-type rhodopsins are shown in Fig. 4 *A* and *B*, respectively. The nonglycosylated wild-type opsin formed the characteristic rhodopsin chromophore (λ_{max}, 500 nm) with 11-*cis*-retinal. The absorbance ratio (A_{280}/A_{500}, 1.8) and the molar extinction coefficient (ε, 39,800 $M^{-1}\cdot cm^{-1}$) for the nonglycosylated rhodopsin were similar to those of the glycosylated rhodopsin (A_{280}/A_{500}, 1.8; ε, 40,600 $M^{-1}\cdot cm^{-1}$). The sensitivity of the retinyl-Schiff base linkage in glycosylated and nonglycosylated wild-type rhodopsin to hydroxylamine was compared. Both rhodopsins were completely stable in the dark but were quantitatively converted to the 360-nm-absorbing retinaloxime upon illumination (>495 nm) for 10 s (data not shown).

The bleaching behavior of glycosylated and nonglycosylated wild-type rhodopsin was compared. Upon illumination (>495 nm) for 10 s (Fig. 5), both pigments yielded a 380-nm species characteristic of metarhodopsin II (MII). The decay of this species to opsin and free retinal was followed by monitoring the loss of the 440-nm-absorbing protonated retinyl-Schiff base (21) formed in acid (Fig. 5). Acidification at different time intervals (1–30 min) showed a progressive blue-shift from 440 nm to 390 nm, presumably due to the release of all-*trans*-retinal. Because the rates of decay of the 380-nm MII species formed from the two pigments were similar, the absence of glycosylation did not affect the stability of retinyl–opsin linkage after photobleaching.

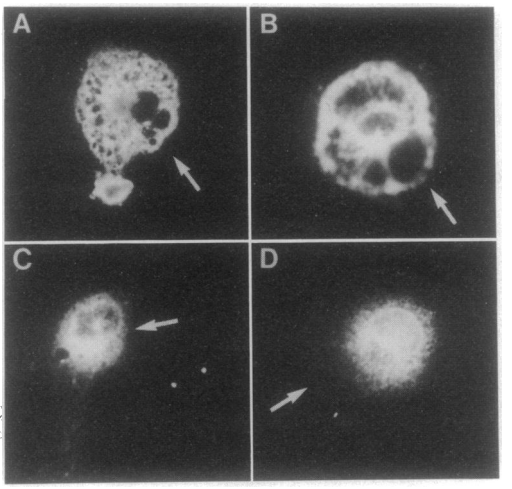

FIG. 3. Cellular localization of glycosylated and nonglycosylated opsins by immunofluorescence. (*A*) Wild-type COS-1 cell opsin. (*B*) Wild-type COS-1 cell opsin expressed in the presence of TM. (*C*) Mutant opsin N15Q. (*D*) Mutant opsin N2, 15Q. Rhodamine-conjugated goat anti-mouse antibody was used to probe for the mouse rho 4D2 rhodopsin monoclonal antibody. Arrows indicate surface of the COS-1 cells.

Rhodopsin Mutants with Asn → Gln Replacements at the Glycosylation Sites. As Asn → Gln substitution represents the most conservative amino acid change, the glycosylation-site mutants N2Q, N15Q, and N2, 15Q were studied. Fig. 6*A* shows immunoblots, after SDS/PAGE, of the expressed mutants and their sensitivity to PNGase F. The opsin from the N2Q mutant (36 kDa) migrated slightly faster than the wild-type protein and showed the trailing smear. The N2Q opsin was also sensitive to treatment with PNGase F, indicating glycosylation at N-15. Both the N15Q and N2, 15Q mutants formed distinct opsin species with mobilities between the mature form (41 kDa) and the nonglycosylated form (33 kDa). However, only the N15Q opsin was sensitive to PNGase F, consistent with it being glycosylated at Asn-2. The multiple opsin species formed in the N2, 15Q mutant may represent partially folded forms of the protein. That the opsins formed from the mutants carrying single Asn → Gln replacements were, indeed, glycosylated, whereas that from the double mutant was not glycosylated was further demonstrated by [^{3}H]mannose labeling (Fig. 6*B*).

Indirect immunofluorescence stains of COS-1 cells expressing the above Asn → Gln mutants indicated that the opsin from the N2Q mutant was at the cell surface (data not shown), whereas the N15Q and N2, 15Q opsins (Fig. 3 *C* and *D*) were located in the perinuclear region, presumably the endoplasmic reticulum.

Spectral Properties of Asn → Gln Glycosylation-Site Mutants. The UV/vis absorption spectra of the pigments from the single Asn → Gln mutants are shown in Fig. 4 *C* and *D*. The N2Q mutant opsin formed the rhodopsin chromophore in amounts comparable to that of the wild-type protein and showed a similar absorbance ratio (A_{280}/A_{500}, 1.8) and molar extinction coefficient (ε, 41,700 $M^{-1}\cdot cm^{-1}$). However, the N15Q opsin poorly regenerated the chromophore with 11-*cis*-retinal (A_{280}/A_{500}, 6.2; ε, 38,300 $M^{-1}\cdot cm^{-1}$). The same was true for the nonglycosylated N2, 15Q mutant opsin. Presumably, the purified N15Q and N2, 15Q proteins contain mixtures of correctly folded opsins that bind retinal to form the chromophore and misfolded opsin(s) that do not bind retinal. This result was confirmed by immunopurification in low salt buffer at pH 6.0 (7), which allowed separation of the correctly folded and reconstituted N15Q rhodopsin (A_{280}/A_{500}, 1.8) from the misfolded opsin(s) (Fig. 4*D*). As described above for the glycosylated and nonglycosylated wild-type pigments, the rhodopsins reconstituted from the N2Q, N15Q, and N2, 15Q mutants were also stable toward hydroxylamine in the dark and formed the 360-nm retinaloxime species only upon illumination (data not shown).

The bleaching properties of the above mutant rhodopsins and their subsequent decay to opsin and free retinal were also studied. Upon illumination (>495 nm) for 10 s, all three

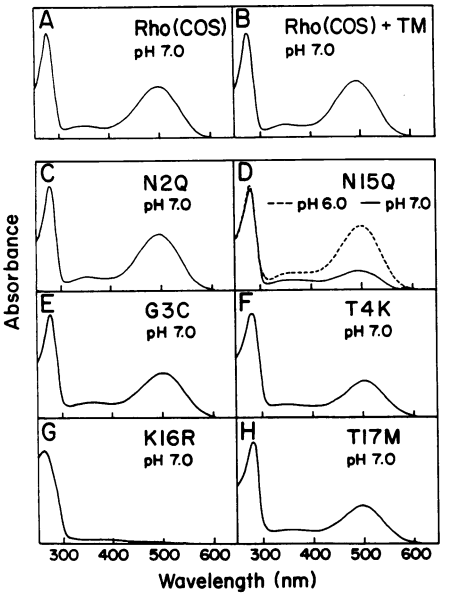

FIG. 4. UV/vis absorption spectra of nonglycosylated wild-type rhodopsin and glycosylation-site mutants. Opsin and opsin mutants expressed in transiently transfected COS-1 cells were reconstituted with 11-*cis*-retinal, solubilized in DM, and immunopurified at pH 7.0 or pH 6.0 as indicated. Spectra recorded at 20°C in the dark are shown. The absolute A_{280} values varied from 0.1 for glycosylated wild-type to 0.04 for K16R mutant.

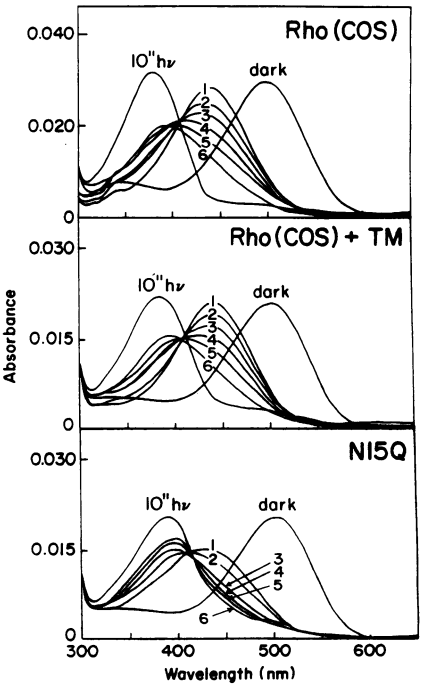

FIG. 5. UV/vis absorption spectra of glycosylated and nonglycosylated wild-type and N15Q rhodopsins upon illumination. The pH 6.0-purified pigments were illuminated (>495 nm) for 10 s at 20°C and subsequently denatured at the indicated time periods by the addition of 2 M H_2SO_4 to a final pH of 1.9.

pigments yielded the 380-nm absorbing species. However, the bandwidth of this species was broader in the N15Q (Fig. 5) and N2, 15Q mutant rhodopsins. Acid denaturation of the illuminated N2Q pigment after 1 min showed the appearance of the 440-nm protonated retinyl-Schiff base. This species was converted to the 390-nm species at a rate similar to that of the glycosylated wild-type pigment. In contrast, acid denaturation of the illuminated N15Q mutant rhodopsin after 1 min resulted in the appearance of a 420-nm species, indicating a mixture of covalently bound (440 nm) and free (380–390 nm) retinal (Fig. 5). The finding that the 420-nm species was completely converted to the 390-nm-absorbing species within 20 min indicated that the 380-nm MII or MII-like species formed upon illumination was less stable than that of glycosylated wild-type rhodopsin.

Mutations in the Glycosylation Tripeptide Sequences. In further work, we constructed a series of mutants in which single amino acids in the tripeptide consensus sequence (Asn-Xaa-Ser/Thr) were substituted. The replacements Gly-3 → Cys/Pro and Thr-4 → Lys were introduced at the Asn-2 glycosylation site. The latter is a naturally occurring point mutation (22) found in autosomal dominant retinitis pigmentosa (23). Both the G3P and T4K mutant opsins showed higher mobilities on SDS/PAGE (36 kDa) than the glycosylated wild-type and the mutant G3C opsins. All mutants closely resembled the glycosylated wild-type protein in the spectral characteristics of their chromophores (Fig. 4 *E* and *F*).

Additional mutations were also constructed at amino acids in the second glycosylation-site tripeptide sequence. These mutants included N15A,-C,-E,-K, and -R; K16C and -R; and T17M and -V. The T17M mutation has also been shown to be associated with autosomal dominant retinitis pigmentosa (24, 25). All of the N15 mutants showed SDS/PAGE band patterns and spectral characteristics similar to the N15Q mutant. The mutants K16C and K16R also showed a band pattern similar to the N15Q mutant but failed to form the rhodopsin chromophore with 11-*cis*-retinal (Fig. 4*G*). In contrast, the opsins from the T17M (Fig. 4*H*) and T17V mutants resembled the N2Q opsin by immunoblot analysis and in the extent of chromophore formation with 11-*cis*-retinal (A_{280}/A_{500}, 2.5). Although the molar extinction coefficients of the chromophores in these mutants (ε, 35,800 and 37,200 $M^{-1}\cdot cm^{-1}$, respectively) were lower than that of glycosylated wild-type rhodopsin, they were stable toward hydroxylamine in the dark (data not shown).

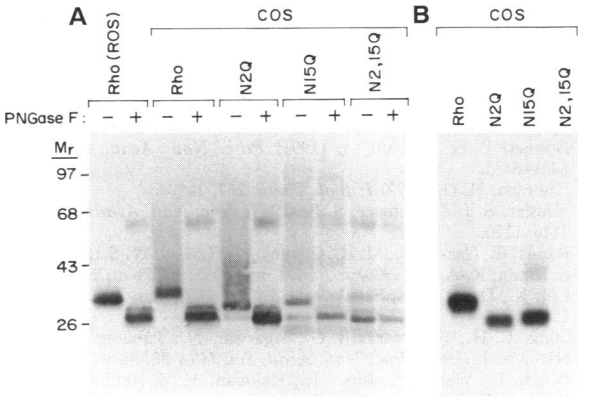

FIG. 6. Characterization of glycosylation-site mutants of opsin. (*A*) Sensitivity of ROS rhodopsin [Rho (ROS)], wild-type COS-1 cell opsin [Rho (COS)], and glycosylation-site mutant opsins N2Q, N15Q, and N2, 15Q to PNGase F. Equivalent amounts of protein were analyzed by SDS/PAGE and immunoblotting before and after treatment with PNGase F. (*B*) Fluorograph of [^{3}H]mannose labeling of wild-type COS-1 cell opsin [Rho (COS)] and glycosylation-site mutant opsins N2Q, N15Q, and N2, 15Q. Equivalent amounts of protein were analyzed by SDS/PAGE and fluorography.

G_T Activation by Nonglycosylated Wild-Type Rhodopsin and Glycosylation-Site Mutants. G_T activation was measured in the presence of limiting G_T (Fig. 7). The nonglycosylated wild-type rhodopsin was only 10% as efficient as the glycosylated pigment in achieving half-maximal turnover of G_T (Fig. 7). To determine whether the lack of glycosylation at Asn-2 or Asn-15 or both affected signal transduction, the glycosylation-site mutants were also tested. Although the N2Q mutant was virtually identical to the glycosylated wild-type protein, the N15Q and N2, 15Q mutants were only 1% as efficient (Fig. 7). G_T activation by the T17V mutant was similar to that of nonglycosylated wild-type rhodopsin, whereas the T17M mutant resembled the N15Q pigment (Fig. 7). Thus, in the absence of glycosylation at Asn-15, the light-activated rhodopsin couples poorly to G_T, and the N15Q and T17M mutations further destabilize the light-activated conformation of rhodopsin.

DISCUSSION

To obtain insights into the specific structural role of rhodopsin (Fig. 1), we have previously reported studies on the effects of amino acid replacements and/or deletions therein (26). In particular, we found that a variety of short deletions in any one of the intradiscal polypeptide segments resulted in the formation of defective opsin molecules (13). We proposed that the formation of a specific tertiary structure comprising all of the intradiscal polypeptide segments was required for the assembly of rhodopsin.

In this paper, we have further studied the formation and function of the putative intradiscal structure. We have examined the role of N-linked glycosylation as well as the effects of amino acid substitutions in the NH_2-terminal region. The remarkable result is that while Asn-15 glycosylation is not required for the *in vivo* folding, assembly, or transport of the opsin in COS-1 cells, it is important for activity in signal transduction. Thus, wild-type opsin expressed in the presence of TM undergoes palmitoylation (Fig. 2*C*), is transported to the cell surface (Fig. 3*B*), and forms the characteristic chromophore with 11-*cis*-retinal (Fig. 4*B*). Similarities in chromophore extinction and inertness toward hydroxylamine in the dark further show that the ground-state structure of the nonglycosylated wild-type pigment is similar to its glycosylated counterpart. Previously, Fliesler and Basinger (27), reported that TM treatment of frog retina virtually abolished opsin transport from the rod inner segment to the ROS. Therefore, glycosylation of opsin might be

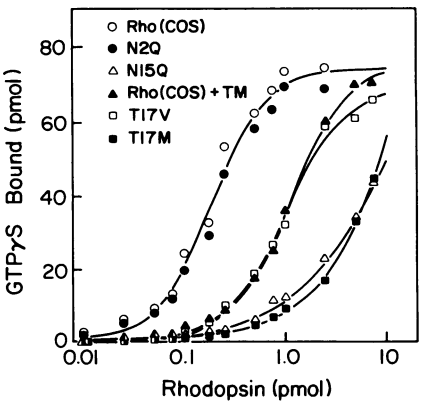

FIG. 7. Transducin activation by nonglycosylated wild-type rhodopsin and glycosylation-site mutants. The activation of G_T as a function of rhodopsin concentration was measured by using a GTP[γS] filter-binding assay. The amount of pigment used in the assays was based on the molar extinction coefficient. Wild-type COS-1 cell rhodopsin [Rho (COS)] expressed in the absence (○) and presence of TM (▲); N2Q (●); N15Q (△); T17M (■); and T17V (□).

important for proper folding, assembly, and/or transport in the rod cell.

To determine whether glycosylation affected signal transduction, mutants lacking glycosylation sites were prepared (Fig. 1). The properties of the opsin from the N2Q mutant indicated that glycosylation at Asn-2 is not required for the folding, cellular transport, or light-dependent activation of G_T (Figs. 4*C*, 6, and 7). In contrast, N15Q substitution formed an opsin that poorly generated the rhodopsin chromophore (Fig. 4*D*). The same phenotype was observed for additional amino acid replacements (N15A,-C,-E,-K, and -R). Further, the opsin from the N15Q mutant as well as the completely nonglycosylated opsin from the N2, 15Q mutant were not transported to the cell surface (Fig. 3 *C* and *D*). Similar effects on cell-surface expression have been reported for other G-protein coupled receptors containing equivalent Asn → Gln substitutions (28, 29). The additional substitutions at the Asn-15 glycosylation site allowed us to further test some of the structural requirements in this region. The K16C and K16R mutants (Fig. 4*G*) formed opsins that failed to bind 11-*cis*-retinal. However, the T17M (Fig. 4*H*) and T17V mutants expressed opsins that formed the rhodopsin chromophore in good yield. However, a different result, namely the inability to bind retinal, has previously been reported for the T17M mutant expressed in 293S cells (30). Taken together, the above effects observed for the Asn-15 glycosylation-site substitutions support the proposal that the NH_2-terminal region is involved in the formation of a specific intradiscal structure.

The G_T activation results show that both the nonglycosylated wild-type rhodopsin and the T17V mutant are only 10% as efficient as glycosylated wild-type rhodopsin (Fig. 7). Therefore, the lack of Asn-15 glycosylation somehow affects the light-activated form required for signal transduction. Since, in the above described receptors, the formation of the ground-state molecules is evidently normal, it appears that only the light-dependent conformational changes are affected and result in poor coupling between photoactivated rhodopsin and G_T. Similarly, expression of the β_2-adrenergic receptor in A431 cells in the presence of TM impaired agonist-dependent signal transduction (31). In a previous study (32), nonglycosylated bovine rhodopsin produced by *in vitro* translation was stated to be 90% as active as ROS rhodopsin in stimulating G_T GTPase activity. Possible reasons for the difference between these results and ours could be the conditions used to prepare the G_T assays.

The following possibilities arise for the differences in G_T activation between glycosylated and nonglycosylated rhodopsin. Illumination of both the glycosylated and nonglycosylated wild-type rhodopsins results in a 380-nm-absorbing species with the characteristics of MII (Fig. 5). Further, the rate of decay of this species to opsin and free retinal is virtually identical for the two rhodopsins in the time range studied. Thus, the lack of Asn-15 glycosylation does not affect either the formation of MII or the stability of retinyl–opsin linkage. Therefore, the possibility must be considered that the glycosylated and nonglycosylated rhodopsins differ in the stabilities of their "active" MII or MII-like conformation. For the N15Q mutant, the difference between its bleaching behavior and that of glycosylated wild-type rhodopsin is quite apparent (Fig. 5). The 380-nm MII or MII-like intermediate is less stable as shown by the formation and rapid decay of a 420-nm-absorbing species. This may account for the low efficiency of G_T activation by this mutant as well as by the T17M mutant.

In the accompanying paper (35), the conserved intradiscal Cys-110 and -187 residues have been substituted by Ala. Interestingly, these mutants also fold *in vivo* to an apparently correct ground-state structure but show altered signal transduction. The results presented here and those cited above strongly suggest that the intradiscal region is also involved in the process of light-dependent activation of rhodopsin.

This is paper no. 5 in the Structure and Function in Rhodopsin series. Papers 1–4 are ref. 13, 33, 7, and 34, respectively. The contributions of Sadashiva S. Karnik and Daniel D. Oprian in early work are gratefully acknowledged. We thank Drs. Phillips W. Robbins, Peter S. Kim, Florence F. Davidson, Zhijian Lu, Cheng Zhang, and Xun Liu for critical reading of the manuscript and Drs. U. L. RajBhandary and Barry E. Knox for helpful discussions, and Judith Carlin for assistance during the manuscript preparation. This work was supported by Grant GM 28289 from the National Institutes of Health (NIH). S.K. was supported by NIH Cancer Training Grant 5 T32-CA09112, and K.D.R. was supported by NIH Research Service Award 5 F32-EY06269.

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