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Rhodopsin Phosphorylation Suggests Biochemical Heterogeneities of Retinal Rod Disks

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Frogs (Rana pipiens) were injected subcutaneously with (^{3}H)-leucine and allowed to incorporate the radioactive amino acid into newly assembled disks in the retinal rod outer segment. The labeled disks served as a temporal marker for following the turnover of rod outer segments. Animals were killed at different times after injection and outer segments were isolated and phosphorylated with ATP in the light. The visual pigment (as isorhodopsin) was regenerated with 9-cis retinal, extracted, and chromatographed on epichlorohydrin triethanolamine cellulose so that phosphorylated pigment could be separated from unphosphorylated pigment. The ratio of (^{3}H)-radioactivity of phosphorylated pigment to that of unphosphorylated pigment was then plotted against the time after injection. The ratio was high when (^{3}H)-labeled disks were largely associated with the basal region of the rod and decreased as the labeled disks moved toward the rod apical region. The results were interpreted as suggesting that newer disks are phosphorylated preferentially to older disks. Papain digestion of (^{3}H)-labeled disks indicated that rhodopsin in newer disks is more susceptible to proteolysis than that in older disks.

Key words: frog retina, rod membranes, rhodopsin, phosphorylation, disk heterogeneity, papainolysis

The rod visual pigment rhodopsin is phosphorylated by adenosine triphosphate (ATP) in the light [1–4]. Rhodopsin kinase, the enzyme that catalyzes transfer of the γ-phosphate from ATP to bleaching pigment, has been purified to homogeneity and characterized [4]. We have shown that only about 20% of the total rhodopsin is phosphorylated and the phosphorylated rhodopsin has multiple phosphorylation sites [4, 5]. Similar results have recently been reported by Akhtar's group [6]. We thought that this might suggest that highly phosphorylated rhodopsin is localized within the rod outer segment. In attempts to determine the location of the phosphorylation reaction, we have taken advantage of the continuous turnover of the rod membrane system and scanned the whole length of the outer segment with respect to rhodopsin phosphorylating activity. The rod disks are known to be continuously assembled at the basal region (proximal to the inner segment) and shed at the distal tip of the outer segment [7]. Displacement of radio-labeled disks from the base because of continuous assembly of unlabeled newer disks was used as a temporal marker for scanning the rod segment over a period of 5 weeks. We present evidence that newer disks are preferentially phosphorylated and are therefore different biochemically from older disks.

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METHODS

Preparation of (^{3}H)-Leucine-Labeled Frog Rod Outer Segments

Into each of 91 frogs (Rana pipiens; Lemberger) was injected subcutaneously 75 μCi of 4,5-(^{3}H)-leucine (105 Ci/mmole; Amersham) in 150 μl Ringer's solution. At appropriate times after injection, a group of 18 frogs each was dark-adapted for 12 h and killed by decapitation. The retinas were dissected under dim red light and rod outer segments were prepared by flotation in 50% sucrose/67 mM potassium phosphate, pH 7.4. The outer segments from 12 frogs were used immediately for phosphorylation studies and the rod outer segments from six frogs were stored as a pellet at −70°C until ready for proteolytic digestion.

Phosphorylation of Rhodopsin Associated With Rod Membranes

Rod outer segments were homogenized in 5.0 ml of 0.2 M potassium phosphate, pH 7.4 (15 nmoles rhodopsin per milliliter) and incubated for 10 min with 0.5 ml of 20 mM ATP/40 mM $MgCl_2$ at 25°C in the dark. The suspension was then mixed with an additional 0.5 ml of 20 mM ATP/40 mM $MgCl_2$ in the light and incubated for 15 min. The mixture was centrifuged at 48,000g for 10 min and washed three times with 67 mM potassium phosphate, pH 7.4.

Regeneration and Purification of Rhodopsin

A sufficient volume of 9-cis retinal (31 nmoles/μl ethanol) was added to a phosphorylated rod suspension in a 4:1 ratio of retinal to opsin. After incubation in the dark at 23°C for 2.5 h, the regenerated sample was diluted to 10 ml with 1 mM potassium phosphate buffer, extracted with 1.5 ml of 3% Emulphogene BC720 (a generous gift from General Anilin and Film Corp.) in 1mM potassium phosphate by homogenization and freeze-thawing, and centrifuged at 183,000g for 60 min. The clear extract was applied to a column of epichlorhydrin triethanolamine cellulose (30.0 × 0.9 cm, Bio-Rad) preequilibrated with 1% Emulphogene/1 mM potassium phosphate, pH 7.2 [4]. The column was eluted with the same buffer containing 1% Emulphogene, and 2.0-ml fractions were collected (flow rate = 1 ml/5.5 min). The optical density of fractions at 280 nm was measured with a Beckman Model 25 spectrophotometer, and pigment concentrations were determined from the absorption at 485 nm recorded in the presence of hydroxylamine on a Cary 14 recording spectrometer and a molar extinction coefficient of 43,000 [8]. The radioactivity (as DPM) of tritiated fractions was determined by mixing 10-μl aliquots of each fraction with 10 ml Aquasol (NEN) and by counting the samples in a Beckman LS9000 scintillation counter.

Proteolytic Digestion of (^{3}H)-Leucine-Labeled Frog Rod Outer Segments

(^{3}H)-labeled outer segments (from six frogs) were suspended in 2.0 ml of 6.7 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 5 mM cysteine. Papain (10–25 units/mg protein, Worthington, New Jersey) was diluted 10 times with the same buffer. Both the rod suspension and papain were preincubated separately at 30°C in the dark for 10 min. Digestion was initiated by the addition of 40 μl of the diluted enzyme to the rod suspension and continued for a maximum of 160 min. An aliquot (250 μl) was withdrawn at various time intervals and emptied directly into 2.0 ml of 10 mM iodoacetamide at 0°C. Centrifugation of the digested pellet at 183,000g for 30 min yielded a pellet that was subsequently solubilized with 250 μl of an 8% sodium dodecyl sulfate gel containing 100 mM Tris-HCl, 25% glycerol, and bromophenol blue (0.02 mg/ml). The solubilized pellet was centrifuged and an aliquot (50 μl) of the clear supernatant was applied to 8% poly-

acrylamide gel preequilibrated by electrophoresis with a running buffer consisting of 0.1% sodium dodecyl sulfate, 40 mM Tris-HCl, 20 mM sodium acetate, and 2 mM EDTA. After electrophoresis at 0.5 mA/cm gel length, the gels were fixed in 50% methanol/5% acetic acid and then stained with 2.5% Coomassie blue. The gels were destained and scanned at 550 nm using a Beckman ACTA 111 equipped with a gel scanner. The scanned gel was sliced into 1-mm-thick disks, incubated overnight at 37°C in a scintillation vial with 10 ml of 3% Protosol (NEN) in Econofluor (NEN) to elute the tritiated protein from the gel and counted.

RESULTS

Phosphorylation of Rhodopsin in the Disks Derived From Different Parts of Rod Outer Segment

Elution profiles of phosphorylated pigment from a column of epichlorohydrin triethanolamine cellulose were essentially identical with those published previously [4]. The protein eluted first (pigment I) was unphosphorylated pigment and the second protein was phosphorylated pigment (pigment II). Figure 1 shows a plot of the time after injection versus the ratio of (^{3}H)-leucine radioactivity of phosphorylated pigment (pigment II) to that of unphosphorylated pigment (pigment I) determined at different time intervals after injection of (^{3}H)-leucine. The ratio (open symbols) was high in rods in which labeled disks were associated with the basal region (proximal to the inner segment). In other words, (^{3}H)-radioactivity was primarily associated with phosphorylated pigment rather than un-

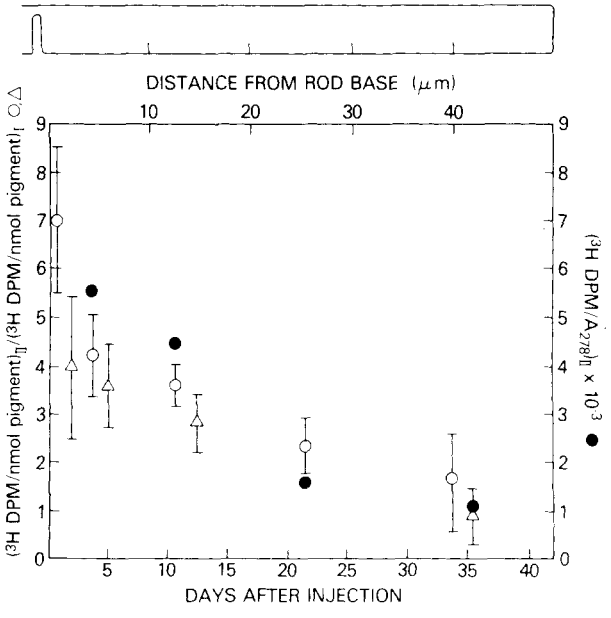


Fig. 1. Distribution of rhodopsin phosphorylation reaction in frog rod outer segment. Pigment I is nonphosphorylated pigment and pigment II is phosphorylated pigment. Open circles and open triangles are the ratio of specific radioactivity of pigment II to that of pigment I and represent two different sets of experiments. Bars indicate the range of deviation from the mean values. Filled circles are (^{3}H)-radioactivity per phosphorylated pigment protein (A_{278}).

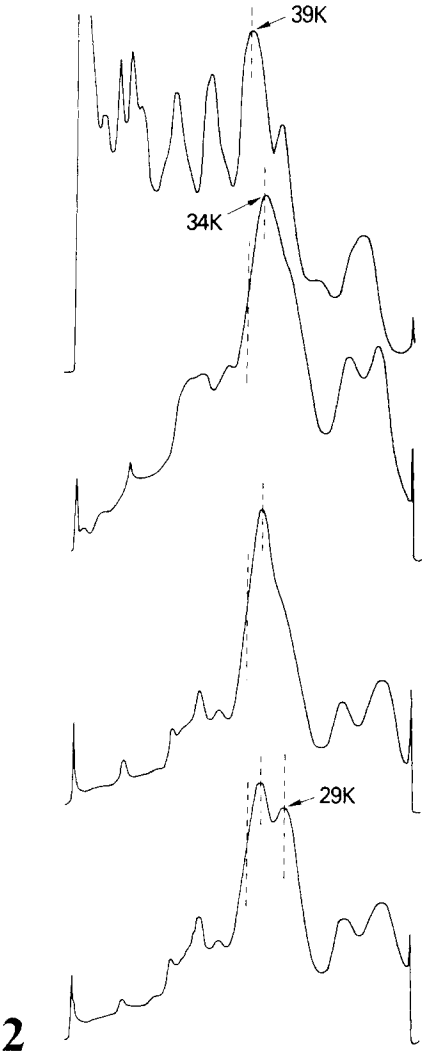
phosphorylated pigment in these rods. The ratio decreased gradually as the labeled disks moved toward the apical (distal) region of the segment. The changes in the ratio were not due to a greater increase in (^{3}H)-radioactivity in unphosphorylated pigment than in phosphorylated pigment because the (^{3}H)-radioactivity of phosphorylated pigment itself (filled circles) showed a decreasing tendency. Therefore, it is evident that in the rods in which (^{3}H)-radioactivity is associated with the disks in the distal region, the radioactivity is mainly associated with unphosphorylated pigment. To correlate the data with the distance of labeled disks from the base, there is also shown in Figure 1 a measure of the longitudinal axis of outer segments that was calculated from the frog rod renewal rate (6 weeks [7]).

Susceptibility of Disk Proteins to Papain

In order to investigate whether age has any effect on the susceptibility of disks to proteolytic enzymes, (^{3}H)-labeled outer segments collected 3.5 days and 21.5 days after injection of (^{3}H)-leucine were subjected to papain digestion. A protein with a molecular weight of 39,000 (39K protein, mostly rhodopsin) was first converted by papain to peptide A (molecular weight 34,000, 34K protein) which, in turn, gave rise to peptide B (molecular weight 29,000, 29K protein). The digestion profile, shown in Figure 2 as scans of Coomassie blue-stained protein bands, is essentially in agreement with those reported previously [6, 9, 10], although different investigators give somewhat different values for the size of peptide fragments formed. The gels were sliced in thin sections, and changes in the (^{3}H)-radioactivity of the digestion products with incubation time were followed. A comparison between rods 3.5 days after injection and rods 21.5 days after injection showed markedly different digestion profiles in terms of changes in (^{3}H)-radioactivity (Fig. 3). During papainolysis of rod segments collected 21.5 days after injection, the specific radioactivity of 39K protein remained virtually constant and the radioactivity of peptides A and B increased only slightly (Fig. 3B). In contrast, the specific radioactivity of 39K protein in the rod outer segments 3.5 days after injection decreased considerably in 5 min of digestion, with a concomitant rise in the radioactivity of peptide A (Fig. 3A). Decay of radioactive peptide A resulted in a subsequent rise in the radioactivity of peptide B. The relative levels of radioactivity of 39K protein, peptide A, and peptide B did not indicate a quantitative conversion of 39K protein to these products. That was possibly because of the presence in the individual Coomassie-stained bands of other proteins and peptides with similar molecular weights that appeared (or disappeared) transiently at different times of proteolysis. Nevertheless, the results are consistent with a sequential conversion of 39K protein to peptide B via peptide A.

DISCUSSION

Autoradiography of the eye of a vertebrate animal that received an injection of the radioactive amino acids shows that the radiolabeled disks newly assembled at the base migrate as a distinct band toward the apical region of outer segment [7]. This occurs because disks are continuously assembled and replace older disks. If the phosphorylation reaction took place uniformly and to the same extent throughout the outer segment, phosphorylated rhodopsin should have constant radioactivity. On the other hand, if the reaction occurred in limited regions of the segment, the radioactivity of phosphorylated rhodopsin would become higher while the labeled disks pass through the regions. It is evident from the present



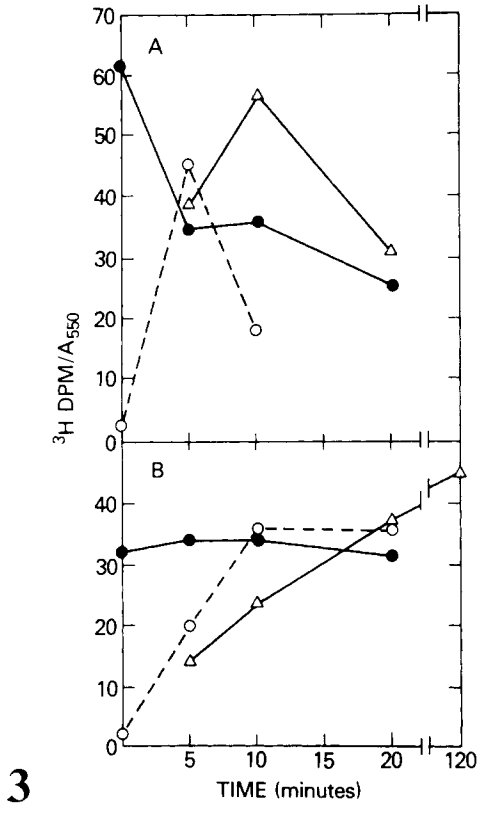


Fig. 2. Typical scanning patterns of gels containing rod proteins digested with papain. Time of digestion (from top to bottom) was: 0, 5 min, 10 min and 20 min. Because the rods were not extensively washed, a number of high-molecular-weight proteins (possibly, peripheral proteins) are noted which disappear in 5-min proteolysis.

Fig. 3. Changes with incubation time in (^{3}H)-radioactivity of 39K protein (•), peptide A (23K protein) (○), and peptide B (29K protein) (△). A: rod outer segments 3.5 days after injection; B: rod outer segments 21.5 days after injection. The range of deviation for three experiments was less than ± 8% of the mean for all points.

study that the latter is the case. In other words, the result shown in Figure 1 indicates that the newer the disks, the higher the radioactivity of phosphorylated rhodopsin. It is therefore concluded that rhodopsin phosphorylation occurs preferentially in the newer disks in the basal region, and hence also in the plasma membrane that is continuous with the newly assembled disks. It is not clear whether the decreased rhodopsin phosphorylation in older disks is due to a loss of rhodopsin kinase or to a modification of opsin conformation during the aging process. This is the first biochemical evidence that rod disks are not homogeneous. Heterogeneities of disks are also suggested by the finding that proteins in newer disks are more susceptible to proteolytic enzymes than in older disks. Older disks may contain lower concentrations of cis-unsaturated fatty acids because of oxidative degradation during aging and may have tighter (ie, less flexible) packing of lipids [11]. As a result, rhodopsin in older disks would become less susceptible to proteolytic enzymes than in newer disks.

The heterogeneity of the rod membrane system has been noted also by other techniques. The intrinsic birefringence of frog rod outer segments is high in the basal region and decreases gradually as the point of measurement moves away from the basal end [12]. Whatever the origin of birefringence changes is, the finding is highly suggestive of inhomogeneities of rod segments. Electron microscopic observations on freeze-fractured photoreceptor membranes of several species also reveal structural inhomogeneities of rod membranes; particle-free patches are found in basal disks and the plasma membrane but not in older disks [13]. Cholesterol has been implicated as a component of patches. Heterogeneities in the disks seem to be indicated in vitamin A-deficient rats [14]. The structural disruption of photoreceptor outer segments in rats that had undergone 23 weeks of deficiency was localized initially in disks of the distal third. Electrophysiologic evidence exists that suggests functional heterogeneities of the rod system [15]. When local light stimuli were applied to different regions of an outer segment, the electric response was faster for illumination near the base than for illumination of the distal tip. These results, taken together, suggest that newer disks in the basal region are functionally more important than older disks. It remains to be determined whether the localized phosphorylation reaction reported here is related to the differences in structural and electrophysiologic properties between newer and older disks.

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