Biophys. Struct. Mechanism 3, 199–203 (1977)

Biophysics of Structure and Mechanism

Isoelectric Focusing of Phosphorylated Cattle Rhodopsin*

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Abstract. ^{32}P-rhodopsin was partially separated by isoelectric focusing into several fractions of different phosphorylation extent. It was found that the incorporated phosphate is not uniformly distributed in a population of rhodopsin molecules. In a preparation with an average phosphorylation extent of 2.4 moles of phosphate per mole of rhodopsin, most of the ^{32}P-phosphate was found in fractions where 4–5 phosphates are bound per rhodopsin, whereas a large fraction of the total rhodopsin was not phosphorylated at all. The maximum number of phosphate binding sites in rhodopsin appears to be at least five.

Key words: Rhodopsin – Isoelectric focusing – Phosphorylation – Membrane proteins.

Absorption of light by the visual pigment, rhodopsin, leads, among other molecular changes, to a phosphorylation reaction in which the terminal phosphate group of ATP is transferred enzymatically to the protein moiety of rhodopsin (Kühn and Dreyer, 1972; Bownds et al., 1972). The phosphate is covalently bound to serine (and to a lesser extent threonine) residues in a very stable phospho-ester linkage (Kühn and Dreyer, 1972). Up to 2.4 moles of phosphate incorporated per mole of rhodopsin (P/Rh)[1] have been found in purified cattle rod outer segments (ROS) (McDowell and Kühn, 1977), and up to 4 P/Rh in crude frog ROS (Miller and Paulsen, 1975). This raises the question about the maximum number of phosphate binding sites in the rhodopsin molecule. We used the method of isoelectric focusing on polyacrylamide gels (Huang et al., 1973) to separate phosphorylated and unphosphorylated rhodopsin.

* Presented in part at the EMBO workshop on "Transduction Mechanism of Photoreceptors", held in Jülich, Germany, on 4–8 October, 1976

[1] Abbreviations used: P/Rh = moles of phosphate per mole of rhodopsin, and ROS = rod outer segments

Methods

ROS membranes from fresh cattle eyes were prepared essentially according to the method of Papermaster and Dreyer (1974), except that the buffer used was 70 mM Na-phosphate (pH 7.0), containing 1 mM $MgCl_2$, and 1 mM dithiothreitol. ROS membranes containing 29 nmoles of rhodopsin (determined spectrophotometrically, assuming $\varepsilon_{500} = 41{,}000$) were incubated in the presence of 1.3 mM γ-^{32}P-ATP (specific activity 52,000 cpm/nmole), 3.5 mM $MgCl_2$, and 8.7 mM tris-HCl (pH 7.3) for 90 min at 30° C in orange light (> 550 nm). Then a three-fold molar excess of 11-cis retinal was added in the dark and regeneration was allowed to proceed for 1 h at 30° C. The membranes were sedimented, washed with water, and resuspended in water. The phosphorylation extent was measured on aliquots that had been acid-precipitated and washed on Millipore filters and was found to be 2.4 P/Rh. The regeneration yield after incubation with 11-cis retinal was determined spectrophotometrically to be 110% of the rhodopsin originally present in the unbleached preparation. In agreement with earlier measurements (Kühn et al., 1973), the regeneration procedure with 11-cis retinal did not change the amount of ^{32}P bound to the protein. SDS-polyacrylamide gel electrophoresis showed that rhodopsin (opsin) is the only protein phosphorylated in this preparation.

Isoelectric focusing was performed on cylindrical gels (11 × 0.5 cm) at 4° C in the dark. The gels contained 5% (w/v) acrylamide, 0.17% N,N′-methylene bisacrylamide, 0.5% (v/v) Triton X-100, 4% (v/v) Servalyt pH 2–11 (Serva, Heidelberg), and 1.7% (v/v) Servalyt pH 4–6. The anode buffer was 0.01 M H_3PO_4, and the cathode buffer was 0.02 M NaOH. The ROS samples were solubilized at final concentrations of 3.3% Triton X-100, 1.3% Servalyt (pH 2–11; neutralized to pH 7.0), 27% sucrose, and 0.15% rhodopsin, for 1 h at 20° C. The gels were pre-focused in the absence of the ROS samples for 1 h at 1 mA per tube. Then the samples were applied to the tops (= cathode) of the gels, and focusing was performed at 0.25 mA per tube for 16 h. The pH gradient was determined by slicing and extracting blank gels. The rhodopsin containing gels were scanned at 500 nm both before and after bleaching. The relative amounts of rhodopsin were determined from the areas under the light-sensitive peaks in the scans. After scanning, the gels were either stained with Coomassie Blue (Huang et al., 1973), or put on X-ray film at −30° C for autoradiography, or cut into 1 mm slices using a Mickle gel slicer. The radioactivity in each slice was measured by liquid scintillation counting in the presence of NCS (tissue solubilizer, Nuclear Chicago).

Results and Discussion

With unbleached, unphosphorylated ROS membranes dissolved in Triton X-100, we obtained results similar to those reported by Huang et al. (1973) for ROS dissolved in the detergent emulphogene, i.e. rhodopsin focused as one predominant band at pH 6.0[2]. The same result was obtained with ROS suspensions which had been bleached

[2] In some ROS preparations, we found additional rhodopsin bands which focused at pH 5.5 and at pH 5.6. But the amount of rhodopsin in these bands was always small as compared to the main band at pH 6.0. The particular ROS preparation used in this report did not contain detectable amounts of these additional rhodopsin bands (in the un-phosphorylated state)

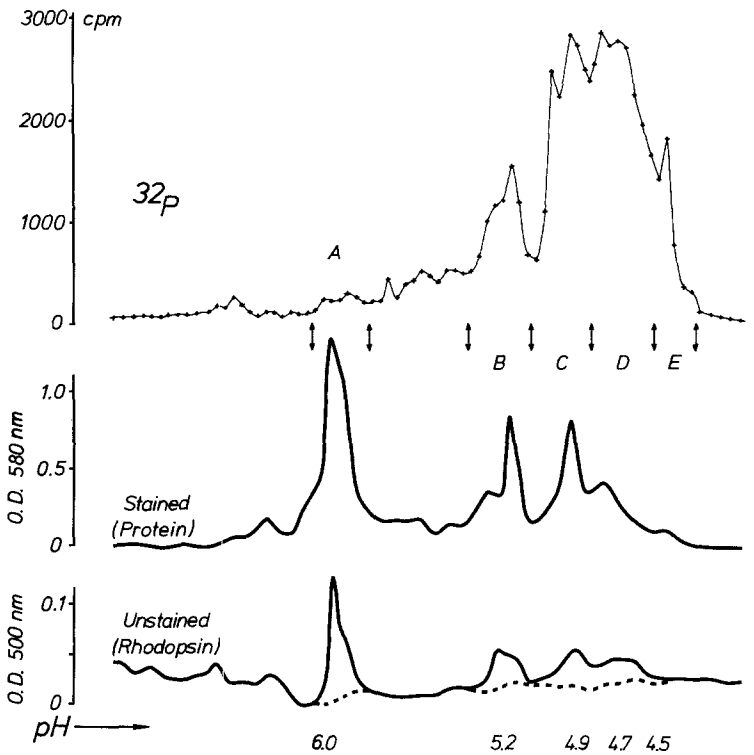

Fig. 1. Distribution of ^{32}P and rhodopsin after isoelectric focusing of ^{32}P-rhodopsin in 0.5% Triton X-100. The lower trace shows the absorption scan at 500 nm of a dark-kept gel before (solid line) and after bleaching (dotted line). The same gel was then cut into 1 mm slices and the radioactivity in each slice was measured (upper trace). The middle trace shows the absorption scan at 580 nm of a Coomassie Blue stained gel which had been run under the same conditions. Each gel contained 43 μg rhodopsin; the average phosphorylation extent was 2.4 moles phosphate per mole rhodopsin. The pH gradient was determined from separate gels run simultaneously

and then regenerated with 11-cis retinal before they were dissolved in Triton. Thus, bleaching and subsequent regeneration did not change the isoelectric properties of rhodopsin. However, ROS suspensions, which were bleached *in the presence of* $AT^{32}P$ such that the bleached rhodopsin became phosphorylated, behaved quite differently: When the ^{32}P-opsin was regenerated with 11-cis retinal to ^{32}P-rhodopsin, then dissolved in Triton and focused on the pH gradient, *several* red (light-sensitive) rhodopsin bands appeared on the gels (Fig. 1, lower trace). In addition to the main red band which still focused at pH 6, a number of red bands appeared in the acidic region between pH 4.5 and 5.3. We tested various ROS preparations which were phosphorylated to different extents and observed that the relative amount of rhodopsin focused in the acidic region was roughly proportional to the amount of phosphate incorporated.

The particular ROS preparation shown in Figure 1 had a rather high average phosphorylation extent (2.4 P/Rh). However, 40% of the total rhodopsin still focused at the position of unphosphorylated rhodopsin (pH 6).

Figure 1 shows that the main rhodopsin peak (at pH 6) contains almost no ^{32}P-phosphate and that most of the radioactivity is accumulated in the acidic rhodopsin bands (pH 4.5–5.3). If five fractions are taken at the positions indicated by the arrows in Figure 1, and the phosphorylation extent is calculated for each fraction, the following approximate numbers are obtained: The phosphorylation extent of the main rhodopsin band (A, pH 6) is 0.18 P/Rh, and of the acidic bands is 2.0 P/Rh (B, at pH 5.2), 3.7 P/Rh (C, at pH 4.9), 5.0 P/Rh (D, at pH 4.7) and 6.8 P/Rh (E, at pH 4.5), respectively. The observed shift of the higher phosphorylated rhodopsin species to lower isoelectric pH values corresponds to their expected increase in negative net charge due to the binding of phosphate groups. Unfortunately, complete separation of the phosphorylated rhodopsin species was not obtained, so the fractions probably contain mixtures of differently phosphorylated species. It is therefore possible that the maximum number of phosphate binding sites is even higher than 6–7, since even the fraction E at pH 4.5 may contain a mixture of higher and lower phosphorylated species. The number of 6.8 P/Ph in this fraction is not very accurate because of the uncertainties in evaluating such low amounts of material in the absorption scans. It is clear, however, that beyond the peak D at pH 4.7 containing 5 P/Ph, there is some rhodopsin with an even higher phosphorylation extent than 5.

We conclude that in phosphorylated rod outer segment membranes, the phosphate is highly unhomogeneously distributed among the rhodopsin molecules. This is in qualitative agreement with the findings of Shishi et al. (1974), but it does not necessarily confirm his hypothesis that "most rhodopsin molecules are not phosphorylated", since we found more than 50% of the rhodopsin to be in the "phosphorylated" (acidic) fractions. Our findings would agree with his hypothesis only in the case that the acidic fractions (pH 4.5–5.3) contain unphosphorylated rhodopsin in addition to the phosphorylated species.

Acknowledgements. We thank S. Bader for expert technical assistance, P. Carl for the gift of 11-cis retinal, and C. N. Rafferty for critical reading of the manuscript. One of us (J. H. M.) received a fellowship from Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 160 ("Biologische Membranen").

Note added in proof

Recently, Plantner and Kean (1976) reported isoelectric separation of "native" rhodopsin, after column chromatographic purification in the detergent emulphogene, into three species present in equal amounts having isoelectric points of 5.91, 5.33 and 4.99. This corresponds roughly to our species A, B and C, respectively, of which we find only A in our "native" (unphosphorylated) preparations. At the present time we cannot explain the apparent discrepancy since we do not really know what the principle of separation is, in either our or their system. Separation may not only be due to intrinsic charge differences in the rhodopsin molecule itself, but may also depend on other factors like, for instance, the binding of ampholytes or the presence of rhodopsin oligomers within the micelles. In recent experiments we found that the presence of *phospholipids* in the detergent solution has no influence on the isoelectric properties of either rhodopsin or ^{32}P-rhodopsin. With lipid-free preparations, purified by affinity chromatography on Concanavalin A-Sepharose, we obtained the same pattern as shown in Figure 1 for ^{32}P-rhodopsin, and only one band at pH 6 for unphosphorylated rhodopsin.

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Received October 28, 1976