ISOELECTRIC FOCUSING OF ROD OUTER SEGMENT MEMBRANE PROTEINS

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Received 27 August 1973

1. Introduction

Isoelectric focusing has been employed extensively as a high resolution technique for the separation of water-soluble proteins [1]. The problems inherent in the solubilization of biological membranes, however, have limited the applicability of this method in the fractionation of membrane-bound proteins. In several such studies [2–4] membranes solubilized and focused in the presence of 6–8 M urea (and/or detergent) were resolved into a number of protein bands. Under such conditions which promote protein denaturation, however, identification of the proteins is difficult due to lack of suitable assay methods. Recently, using mild non-ionic detergents which preserve some of the 'functional' characteristics of membrane proteins the isoelectric focusing of *Rhodopseudomonas spheroides* Y reaction center [5] on polyacrylamide gels and the *Torpedo* acetylcholine receptor protein [6] on density columns have been described. Heitzmann and Richards [7] have mentioned the focusing of fluorescent derivatives of opsin.

In this report we describe a procedure for polyacrylamide gel isoelectric focusing of bovine rod outer segment membranes solubilized in the non-ionic detergent, Emulphogene (an alkoxypoly(ethylenoxy) ethanol). Under the conditions used the spectral characteristics and the light-dependent bleaching of rhodopsin are maintained. Futhermore, unbleached rhodopsin which focuses at pH 6.0 can be separated from opsin (bleached rhodopsin) which focuses at a lower pH. The finding that isoelectric focusing can separate different charged species of the same membrane protein points to the potential application of this method for the fractionation of proteins derived from other more complex membrane systems.

2. Materials and methods

Acrylamide, N,N' methylene-bis-acrylamide (Bis). N,N,N',N'-tetramethylethylenediamine (TMEDA) and ammonium persulfate (APS) were obtained from BioRad. The non-ionic detergent Emulphogene BC-720 was obtained from GAF. Ampholine, 40%, pH 3.5–10 (batch 1) was obtained from LKB-Produkter. All other chemicals were reagent grade.

2.1. *Rod outer segments (ROS)*

ROS were prepared from frozen bovine retinas (Hormel & Co., Austin, Minn.) under dim red light according to the procedure of McConnell et al. [8] with some modifications [9, 10]. The final ROS membrane suspensions at 6–8 mg/ml protein concentration and in 0.2 M sucrose were stored frozen at −20°C in small light-tight vials. Protein concentration was determined by amino acid analysis using a Beckman Model 121 Analyzer. Using a molar extinction coefficient at 500 nm of 4.05×10^4 and a mol. wt. of 3.9×10^4 [11], concentrations of unbleached rhodopsin were estimated from the absorbance measurements at 500 nm after solubilization of ROS in 2% Emulphogene and centrifugation at 20 000 *g* for 10 min. The purity of ROS preparations was verified by absorbance ratios: $A_{400}:A_{500}$ and $A_{280}:A_{500}$.

2.2. *Sodium dodecyl sulphate (SDS) gel electrophoresis*

SDS gel electrophoresis was performed by the method of Fairbanks et al. [12].

2.3. *Isoelectric focusing*

All solutions were freshly made using deionized distilled water. Manipulations involved in rhodopsin sample preparation and focusing were carried out under dim red light.

2.3.1. Gel preparation

For 12–13 gels, 1.5 g acrylamide, 0.05 g Bis, 0.075 ml Emulphogene were dissolved in 26.4 ml water. The solution was degassed for 5 min by stirring under vacuum. After the solution was chilled in an ice bath, 1.5 ml Ampholine, 1.0 ml 1.5% (w/v) APS and 1.0 ml 0.5% (v/v) TMEDA were added with stirring. The gel solution was quickly pipetted into acid-washed Pyrex gel tubes (125 X 8 mm i.d.), the lower ends of which were sealed with rubber grommets, and were overlayed with 0.05 ml of water. Gel tubes were filled to a height of 8 cm. Rapid polymerization necessitated fast working in pouring and overlaying the gel solutions to ensure flat gel tops. It was noticed also that Ampholine, without addition of TMEDA or APS, will initiate and propagate polymerization. The gels were stored overnight at room temperature before use.

This procedure yielded a polyacrylamide gel containing 5% acrylamide (with 1:30 Bis cross-linkage), 0.25% detergent and 2% Ampholine concentration. A slight shrinkage of the gels occurred during polymerization resulting in a final gel length of 7.8 cm. The gels had background A_{280} of 0.4 and were permeable to β-galactosidase (mol. wt. 530 000).

2.3.2. Sample preparation

ROS samples used for isoelectric focusing were diluted with an equal volume of water and pelleted at 20 000 *g* (Sorvall RC-2B centrifuge, SS-34 rotor, 13 000 rpm) for 10 min. The pellet was solubilized in a carrier solution at pH 8 such that the final concentrations were 1% detergent, 2% Ampholine and 0.1–1.0 mg/ml protein. The solution was incubated at 4°C for 45 min and centrifuged at 25 000 *g* for 40 min to pellet any unsolubilized material. A few crystals of sucrose (Mann ultrapure) were dissolved in a known volume of the supernatant in order to increase the density of the solution and thus facilitate layering onto gels.

In some experiments the samples were bleached for varying lengths of time in a quartz cuvette at a distance of 7 cm from a 100 W light source equipped with an infrared filter. Fifty percent bleaching was obtained after 3–4 sec. The percentage of rhodopsin bleached was determined from absorbance difference at 500 nm.

2.3.3. Electrophoresis

Gels were placed in an electrophoresis apparatus with 0.01 M phosphoric acid (anolyte) in the lower reservoir and 0.02 M sodium hydroxide (catholyte) in the upper reservoir. A Buchler Model 3–1014A voltage and current regulated power supply was used. The gels were electrophoresed at 1 mA/gel for 1 hr to remove residual ammonium persulfate and to initiate formation of the pH gradient. Samples of vol. 0.01–0.1 ml were then applied to the tops of the gels. Focusing was performed at 12°C with $\frac{1}{2}$ mA constant current per gel until a voltage of 30 V/cm of gel was reached, at which point the voltage was kept constant at 30 V/cm of gel. The current was observed to drop to a small constant value (about 0.25–0.35 mA/gel). After 10 hr under these conditions and protein loadings, focusing was terminated and gels were removed and tattooed with a 25G needle dipped in India ink at 2 cm and 1 cm from top and bottom of gels, respectively.

2.3.4. Gel staining

Protein gels were stained by a solution containing 20% 5-sulfosalicylic acid, 25% isopropyl alcohol, and 0.025% Coomassie Blue R-250 (Schwarz Mann) for 35 hr and then destained in 20% acetic acid. Staining patterns were similar when gels were fixed in 20% 5-sulfosalicylic acid to elute out detergent and Ampholine and then stained by the method of Fairbanks et al. [12]. This latter method gave a higher background staining than the former. Both 10% trichloroacetic acid, when used as fixatives, gave excessively high background staining. Total staining and destaining time required was 45 hr. The pH of each band was estimated by normalization of all gel lengths to that of the pH gel.

2.3.5. pH gel and gel scanning

The pH gel (loaded with 25–50 μl blank carrier solution) was tattooed at 3 mm intervals and hand-sliced into 3 mm pieces with a clean razor blade. Each piece was further sliced into quarters and soaked in 1 ml 0.1 M potassium chloride for 1 hr with frequent shaking. The pH of each piece was then determined to ± 0.01 pH unit using a Radiometer Model 25 pH meter with a scale expander.

Gels were initially scanned at 280 nm (protein absorption), 380 nm (bleached rhodopsin absorption),

and 500 nm (unbleached rhodopsin absorption), using a Gilford 2000 spectrophotometer with a gel scanning attachment, then bleached for 10 min at 16 cm from a GE 100 W frosted bulb, and finally scanned at 500 nm and 380 nm.

2.4. *Proton change measurements*

One vol of ROS (1 mg protein) was mixed with 1 vol of water and the suspension was centrifuged at 20 000 *g* for 10 min. The pellet was rewashed with an additional 2 vol of water. After solubilization in 1.2 ml of 2% Emulphogene and centrifugation at 20 000 *g* for 10 min, an aliquot of the solution was used to measure the rhodopsin concentration and a known volume was pipetted into a reaction vial at 4°C. The pH was adjusted to the desired value with 0.01 N NaOH solution. The ROS solution was bleached and the pH change was recorded. A quantitative estimate of the proton uptake or release as a result of bleaching was determined from the volume of standardized titrant required to back titrate the solution to its original pH value.

3. Results

3.1. *Equilibrium conditions for isoelectric focusing*

Under the conditions described proteins migrated to their final positions by 4–6 hr. Considerable band-sharpening occurred until 10 hr when equilibrium was considered to have been reached. The pH gradient, formed early during focusing, extended from about pH 4 to pH 8 with a reproducible linear range from pH 4.5 to pH 7.5. The final pH at which the protein complexes focused (their apparent isoelectric point) varied less than ± 0.2 units between different experiments and less than ± 0.1 units in duplicate gels within a given focusing experiment.

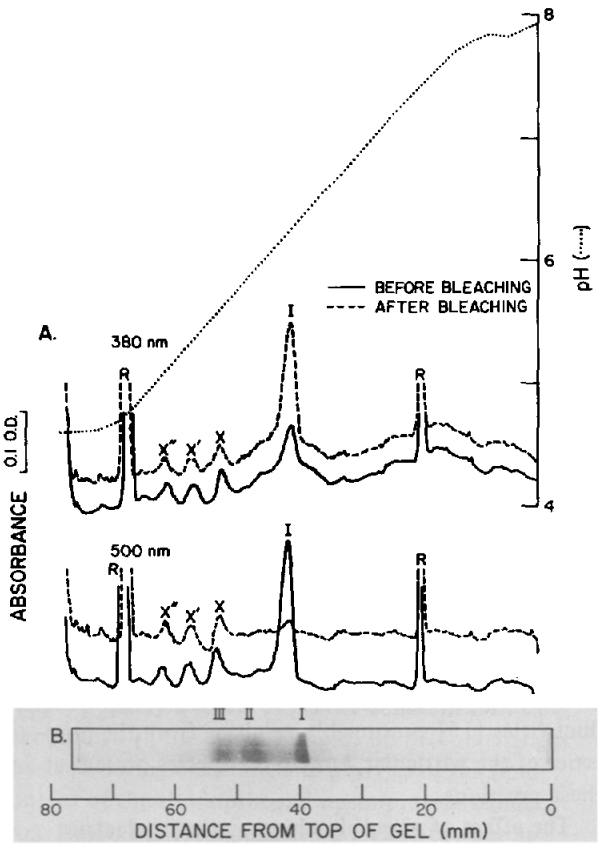

Fig. 1. Isoelectric focusing of unbleached ROS solubilized in 1% Emulphogene. A: pH gradient in the gel and spectrophotometric scanning traces of a gel before staining at 380 nm (bleached rhodopsin) and 500 nm (unbleached rhodopsin) before and after illumination. Small peak at 40 mm in 380 nm scan before bleaching is due to slight bleaching when gel was positioned for scanning. Bands II, III and minor bands are not detected by scanning. India ink reference marks (R) appear as sharp peaks at 20 mm and 70 mm. B: The same gel stained with Coomassie Blue. In this experiment bands I, II and III are at pH 6.2, 5.7 and 5.4, respectively.

3.2. *Purity of ROS membranes*

Bovine rod outer segment preparations used in the present study had absorbance ratios A_{280}: A_{500}= 2.3–2.6 and A_{400}: A_{500}= 0.24–0.26 after solubilization in 2% Emulphogene or 2% cholate detergent. In one preparation, comparison of the rhodopsin concentration by spectral measurements with the total protein concentration determined by amino acid analysis indicated that greater than 85% of the protein in ROS constitutes unbleached rhodopsin. Isoelectric focusing substantiated previous reports on the high content of rhodopsin in ROS membranes [11, 13, 14]. The electrophoretic pattern of ROS preparations on SDS-polyacrylamide gels is similar to that previously described [9] with rhodopsin exhibiting a relative mobility corresponding to an apparent mol. wt. 33–35 000.

3.3. *Isoelectric focusing of ROS proteins*

Rod outer segment membranes solubilized in the non-ionic detergent, Emulphogene, and focused in the dark on gels containing detergent displayed a band pattern shown in fig. 1. In the stained gels, one intense band (I) and two less intense bands, (II, III) were observed at pH 6.03 (S.D. 0.11), 5.62 (S.D. 0.13), and 5.26 (S.D. 0.14), respectively. Six faint bands were observed over the pH range 6.6–5.3. No bands were observed above pH 6.6.

The sharp major band I was identified as the visual receptor protein, rhodopsin, by spectrophotometric scanning of the gels before and after illumination prior to staining. This band was found to absorb light at 500 nm and to undergo bleaching upon exposure to light (fig. 1), resulting in the loss of absorption at 500 nm and an increase in absorption at 380 nm. The more diffuse band II did not absorb either at 500 nm or 380 nm.

The reproducible absorption-like peaks (X,X′,X″, figs. 1 and 2) in the pH range 4.2–5.8 resulted from regions of constriction and swelling in the gel after focusing and also appeared in blank gels. It was found that these gel distortions were due to local low conductivities [15] presumably resulting from the properties of the particular Ampholine species present at these positions.

The effect of photobleaching on the isoelectric focusing pattern of ROS proteins is depicted in fig. 2. After focusing comparison of both the absorbance scans at 280 nm and the stained bands for unbleached, partially bleached (approx. 50%) and bleached ROS samples indicate that band I progressively decreases in intensity with increasing amount of bleaching, whereas the opposite is true for band II. On the basis of these results, we conclude that band II is opsin. The failure of this band to absorb 380 nm light is presumably due to the separation of the retinal group from opsin during electrophoresis. The isoelectric focusing patterns of a ROS sample which has been bleached 50% was the same as that for a sample containing an equal mixture of bleached and unbleached ROS.

No difference in protein band pattern was observed between ROS freshly prepared and ROS membranes stored at −20°C in the dark for over 9 months. In one ROS preparation, however, for which A_{280}/A_{500} was greater than 5, and additional intensely stained band was noticed at pH 5.0.

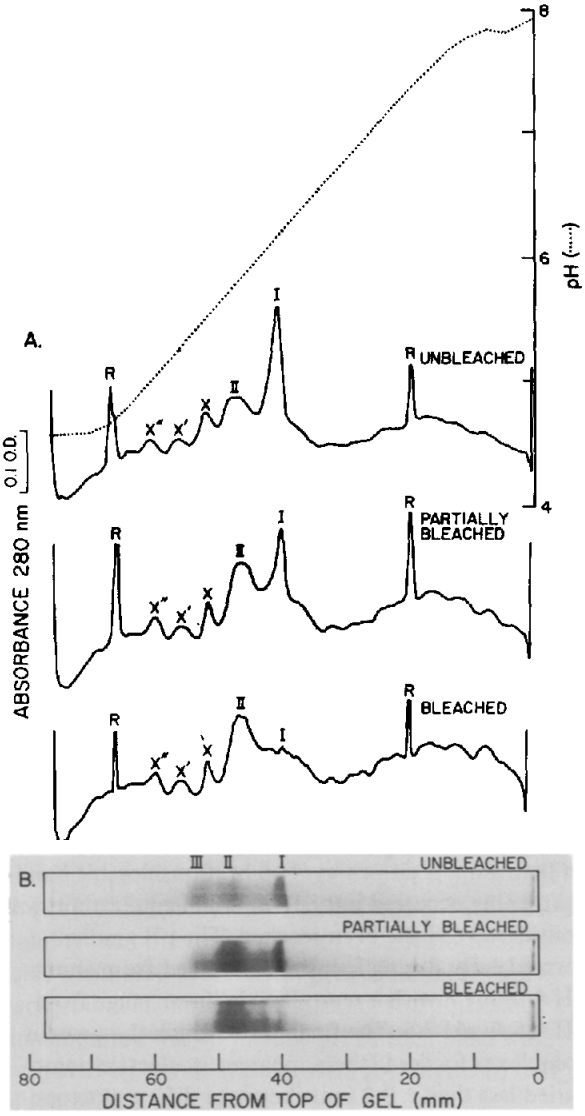

Fig. 2. Isoelectric focusing of unbleached, partially bleached (approx. 50%), and bleached ROS solubilized in 1% Emulphogene. A: pH gradient in the gels and spectrophotometric scanning traces of gels at 280 nm (proteins) before staining. Band III and minor bands are not detected by scanning. India ink reference marks (R) appear as sharp peaks at 20 mm and 70 mm. B: The same gels stained with Coomassie Blue. In this experiment bands I, II and III are at pH 6.2, 5.7 and 5.4 respectively.

3.4. *SDS-gel electrophoresis*

Bands I and II, eluted from the focusing gels and electrophoresed on SDS-gels, showed similar relative

mobilities to that of rhodopsin. These results support the conclusion that the major species of unbleached rhodopsin has an apparent isoelectric point of pH 6.0 and that after illumination it is lowered to pH 5.6.

From the loss in intensity of the stained band III on bleaching (fig. 2), it appears that this species is light-dependent, but its nature and significance are as yet unclear. The concentration of this species was too low to be measured by absorbance. The SDS-gel electrophoresis of this band when eluted showed a very faint band similar to rhodopsin in relative mobility. In all cases of SDS-gel electrophoresis Ampholine ran with the dye front and was stained.

3.5. *Proton change upon bleaching.*

At pH 8 a decrease of 0.35 pH units was observed upon photobleaching. This corresponded to a release of 2.5 protons per molecule of rhodopsin bleached. At pH 6 there was a 0.05 unit increase of pH. These data agree well with those of McConnell et all. [16] for ROS solubilized in Triton X-100.

4. Discussion

ROS preparations having demonstrated purity by spectral criteria have been focused on polyacrylamide gels. The results indicate that rhodopsin, the major component of ROS membranes, focuses at pH 6.0 in the presence of Emulphogene and can be resolved from opsin which focuses at pH 5.6 and other minor membrane proteins which band in the range pH 5–6.6. In this system the 500 nm absorption and light-dependent bleaching of rhodopsin are preserved.

The difference in apparent isoelectric points between unbleached rhodopsin and opsin is consistent with the presence of a greater net negative charge on the latter above pH 5.6. Two possible explanations for this effect are: 1) a release of protons due to a protein conformational change during bleaching in the detergent, and 2) a difference in the number of acidic phospholipids or other anions bound to the two forms of the protein. Quantitative measurements of proton changes during bleaching of detergent-solubilized ROS show a release of protons from rhodopsin bleached at pH 8 but a slight uptake of protons at pH 6. From this data and from the data of McConnell et al. [16], the observed proton change at pH 6 does not account for the observed difference in isoelectric points on polyacrylamide gels. The apparent discrepancy between these two measurements may be a result of a difference in the nature of the protein complex before and after focusing. After solubilization of rhodopsin by nonionic detergents, different amounts of lipids can be further removed depending on the extraction procedure [17]. During focusing it is apparent that all-trans retinal is dissociated from opsin; it is also possible that lipids may be separated from rhodopsin and/or opsin, thus affecting their conformational states and the net change in protons upon bleaching. Related to this is the observation by McConnell et al. That upon bleaching *ROS membranes* exhibit an uptake of protons above pH 6, whereas *Triton X-100-solubilized ROS* show a release of protons. Heitzmann and Richards [7] reported that *N*-retinyl opsin, delipidated by a Brij 35-Triton X-100 mixture and subjected to isoelectric focusing, had an apparent isoelectric point of pH 5.4, similar to our results for opsin (pH 5.6). The more diffuse nature of the opsin band also suggests a heterogeneity in net charge, possibly a result of partial denaturation. Further studies on the effect of lipids on proton changes observed during bleaching and analysis of the lipid content of the rhodopsin and opsin protein bands from isoelectric focusing gels may yield further insight on these points.

The procedure of isoelectric focusing described here suggests the potential use of this technique for resolving phosphorylated forms of rhodopsin and thus for gaining information on the number and nature of the phosphorylated sites [9]. Furthermore, this technique may be useful in the fractionation and characterization of proteins of other membrane systems under mild conditions through appropriate choice of non-ionic detergents.

Acknowledgements

This work was supported by USPHS grant GM 065 95. HVH is a Danforth Fellow and RSM is supported by an American Cancer Society Postdoctoral Fellowship.

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