Vol. 90, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 12, 1979 Pages 58-64

THE PRESENCE OF TWO MAJOR PROTEIN COMPONENTS IN THE BOVINE PHOTORECEPTOR DISC MEMBRANE

R. Uhl[1], T. Borys[1], N. Semple[2], J. Pasternak[2], and E.W. Abrahamson[1]

1. Department of Chemistry, University of Guelph, Guelph, Ontario, Canada, N2G 2W2

2. Department of Biology, University of Waterloo, Waterloo, Ontario, Canada, N2L 3G1

Received July 16,1979

SUMMARY

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of thoroughly washed rod outer segment membrane preparations from bovine retinae revealed two major membrane-bound components and not one as has been generally thought. The higher molecular weight peak (≈38500 molecular weight) contains a carbohydrate component and is covalently bound to the retinylidene chromophore. Moreover, this material is extensively phosphorylated in vitro upon illumination. Therefore, this component (peak H) is rhodopsin. The nature and function of the other photoreceptor disc membrane component (peak L, ≈34500 molecular weight) remains to be determined.

The photoreceptor disc membrane is considered to be unique, in part, because the pigment molecule rhodopsin is believed to comprise more than 80% of the total protein content of the membrane (1,2). Recently, Siebert et al. (3) reported that purified disc membrane preparations after sodium dodecyl sulfate[1] (SDS) gel-electrophoresis revealed three protein components within the molecular weight range of 30000 to 42000. They tentatively concluded that (a) the largest (≈42000 molecular weight) of these components was glycosylated but not opsin, (b) the 37000 molecular weight component was opsin and (c) the lightest (≈33000 molecular weight) band was a loosely bound protein that could be removed readily from the disc membrane preparation by washing with low ionic strength buffer. Moreover, Siebert et al. (3) estimated that the putative opsin comprised only 50% of the total protein in the membrane. Certainly, the presence of a major disc

---

1. The abbreviations used are: SDS (sodium dodecyl sulfate); ROS (rod outer segment); PAS (periodic acid-Schiff test); PIPES (1,4-piperazinediethane sulfonic acid) and EDTA (ethylenediamine tetracetic acid).

0006-291X/79/170058-07$01.00/0

protein in addition to rhodopsin compromises current models of disc membrane organization. As well, the question of whether the second protein component is functionally coupled to rhodopsin during visual transduction is raised. For these reasons, we have investigated further the protein components of bovine rod outer segment (ROS) disc membranes, in detail, using SDS-gel electrophoresis.

MATERIALS AND METHODS

Rod outer segment fragments were prepared by density gradient centrifugation (4). The A_{280}/A_{500} ratio for these samples was between 2.3 and 2.5. To remove soluble proteins, ROS preparations were washed with 3 mM Tris-HCl (pH 7.3), 1.5 mM PIPES buffer and 1 mM EDTA. After centrifugation and resuspension, the membrane preparations had a A_{280}/A_{500} ratio of about 2.00. Intact isolated discs were prepared by a modified version of the procedure of Smith et al. (5). Briefly, isolated ROS fragments were suspended into 5% ice-cold Ficoll, in 5 mM Tris-HCl (pH 7.3) and stirred for 60 min. After a 60 min centrifugation (30000 rpm in a SW-27 rotor) intact discs were harvested from the Ficoll-5 mM Tris interface. The yields from this preparation were 85 to 90% with a A_{280}/A_{500} ratio of 1.85 to 1.90. All steps in the isolation procedure were conducted at 4°C under argon to minimize damage due to oxidation. In some cases, the ROS were delipidated with chloroform: methanol (2:1). Membrane proteins were solubilized in electrophoresis sample buffer (6) at room temperature by vigorous vortexing. The resulting solution was kept at -60°C until electrophoresis. Reduction of the retinal - opsin Schiff base linkage in rhodopsin entailed either treatment of native rhodopsin with 200 mM $NaCNBH_3$ at pH 4.2 for 10 min at 20°C (7) in the presence of 1% Emulphogene or incubating the membrane with 400 mM $NaCNBH_3$ at pH 4.2 for 30 min at 20°C.

Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed in a disc gel apparatus (6). Commercial acrylamide and N, N-bis-methylene acrylamide were recrystallized before use (8). Gel cylinders containing 7.5% acrylamide were pre-run at 2 mA/gel at 6°C for 60 min before electrophoresis of protein samples at 4 mA/gel at 6°C. Gels, with $NaCNBH_3$-reduced samples, were scanned at 325 nm after fixation in 25% isopropanol and 10% acetic acid for 16 h which was followed by treatment with 10% isopropanol and 10% acetic acid for 8 h. These gels were subsequently stained for carbohydrate with periodic acid-Schiff (PAS) reagent (9). After scanning at 560 nm, the same gels were stained with Coomassie blue and then scanned at 550 nm after destaining in 10% acetic acid. Protein standards were phosphorylase a (100000 molecular weight), bovine serum albumin (66000 molecular weight), aldolase (40000 molecular weight) and α-chymotrypsinogen-A (25700 molecular weight).

For in vitro phosphorylation, rod outer segment preparations were incubated with 60 mM Tris-HCl (pH 7.3), 0.5-1.0 mM $MgCl_2$, 1 mM ATP and ≃ 40 μCi [γ-^{32}P] ATP in a total volume of 600 μl in the dark for 10 minutes at room temperature. A 300 μl aliquot was placed into 25 ml of ice-cold 3 mM Tris-HCl (pH 7.3), 1.5 mM PIPES and 1 mM EDTA for 20 min and subsequently retained as a control. The remaining reaction mixture was illuminated for 1 min with light and then returned to the dark for 30 min. The reaction was terminated by adding ice-cold 3 mM Tris-HCl (pH 7.3), 1.5 mM PIPES and 1 mM EDTA. After 20 minutes, the membranes were collected by centrifugation, suspended in electrophoresis sample buffer and stored at -60°C until use.

RESULTS AND DISCUSSION

Sodium dodecyl sulfate-gel electrophoresis of purified rod outer segment preparations often revealed two major peaks with apparent molecular weights

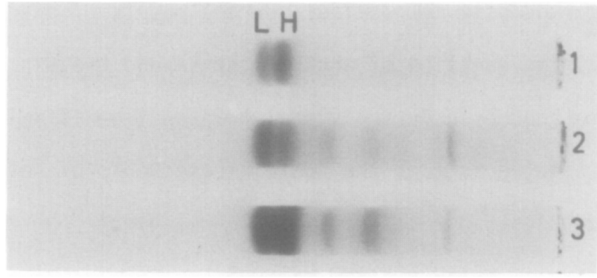

Fig. 1. Electrophoretic analysis with sodium dodecyl sulfate-polyacrylamide gels of purified rod outer segments. Direction of migration is from right to left. Prior to treatment the initial protein concentration was 25 µg. Preparations for gels 1, 2 and 3 were untreated, delipidated and isolated in the presence of 1% Emulphogene respectively.

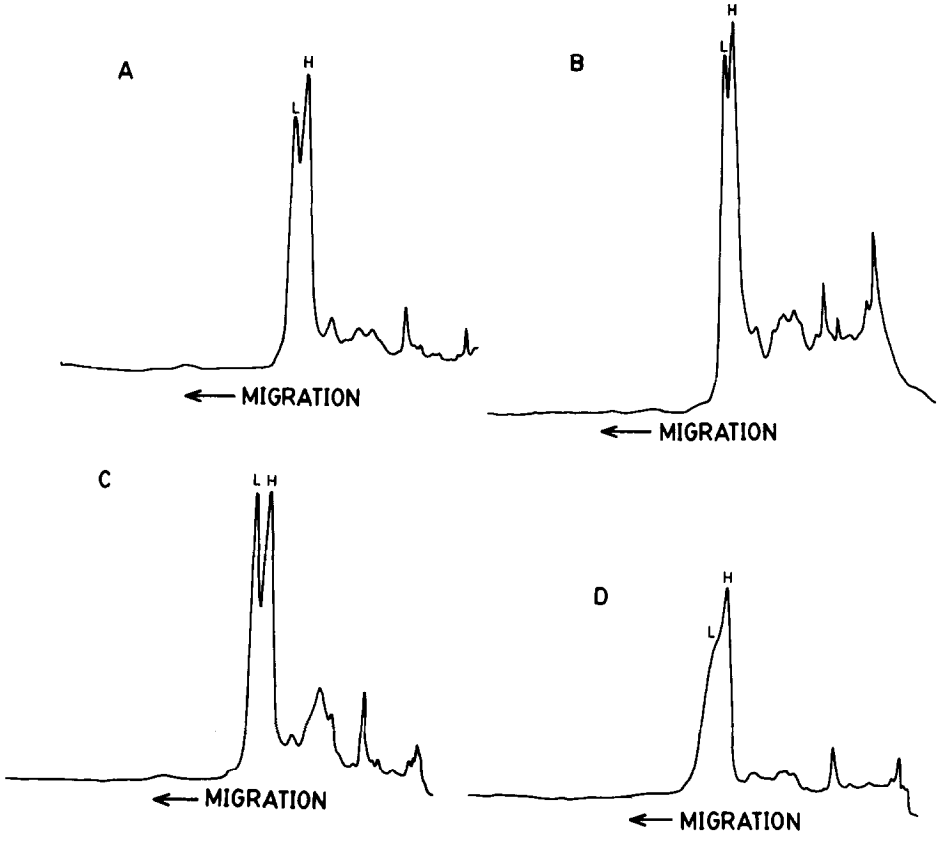

Fig. 2. Densitometric scans of Coomassie blue-stained SDS-gels from various photoreceptor membrane preparations. A: 25 µg, delipidated rod outer segment preparation; B: 50 µg, delipidated disc membrane preparation; C: 33.5 µg, rod outer segment preparation; D: 25 µg, rod outer segment preparation. Migration is from right to left.

of about 38500 (peak H) and 34500 (peak L) (Fig. 1). Defatting of either rod outer segment or disc membrane preparations enhanced the separations of peaks H and L (Figs. 2A and B). In a number of instances rod outer segments that were not defatted revealed peaks H and L (Fig. 2C); whereas, more generally, untreated samples tended to show a shoulder on the light side of a major peak (Fig 2D). Optimal resolution of peaks H and L was dependent upon the concentration of protein that was placed on top of the gels. Usually, 25 μg protein revealed two major peaks. A decrease in this amount of protein often resulted in obscuring the clear separation of the two peaks. With 50 and 100 μg of input protein, the presence of two components was often evident after extensive delipidation. In some defatted preparations, peak L appeared as a diffuse band migrating ahead of peak H but not clearly separated from it

Since opsin is purported to be a glycoprotein, it should be possible to localize the uptake of glycoprotein staining reagent (periodic acid-Schiff test reagent) to one or both of peaks H and L. After staining with PAS, the H peak alone, of the two major bands, took up the dye (Fig 3B). With gels that

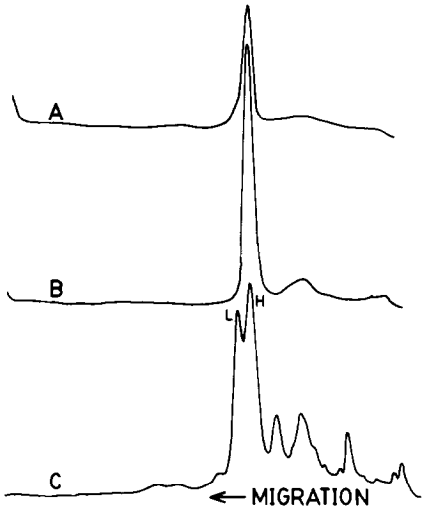

Fig. 3. Densitometric scans of a SDS-gel from a washed rod outer segment preparation. The preparation was reduced with $NaCNBH_3$ prior to electrophoresis. After electrophoresis the gel was scanned at 325 nm (A) subsequently the gel was stained with PAS reagent and scanned at 560 nm (B) and then the gel was superstained with Coomassie blue and scanned at 550 nm (C). The Rm for the 325 nm-absorbing band, the PAS-positive material and peak H was 0.39. The Rm for peak L was 0.42. Migration is from right to left.

were superstained with Coomassie blue, the PAS band coincided precisely with peak H when changes of gel length during the staining procedures were taken into account. To substantiate that the glycoprotein component is indeed rhodopsin, the Schiff base linkage between retinal and opsin was reduced and subsequently identified after electrophoresis by scanning at 325 nm. In these cases, the absorption peak at 325 nm coincided with peak H (Fig. 3A). In a series of experiments (n=7) when the same gels were scanned at the appropriate wavelength after (i) running reduced material, (ii) staining with PAS reagent and (iii) superstaining with Coomassie blue, the single peaks that were observed at 325 nm and after treatment with PAS reagent aligned with peak H and not peak L (Fig. 3C). Thus, peak H is rhodopsin. Siebert et al. (3) tentatively concluded that their peak 12 (≈42000 molecular weight) was glycosylated whereas peak 13 (≈37000 molecular weight) was reduced and therefore the latter peak was considered to be rhodopsin. It is difficult to reconcile unequivocally our observations with those originally reported by Siebert et al. (3) because of the differences in the apparent molecular weight values of the two tightly bound membrane components. In the work of Siebert et al. the heavier peak (peak 12; 42000 molecular weight) gave a positive reaction to PAS; whereas, the lighter component (peak 13; 37000 molecular weight) seemed to carry the chromophore. Consequently, Siebert et al. suggested that peak 13 and not peak 12 was opsin. However, the essential discrepancy between the conclusions of Siebert et al. and our results can be resolved readily if it is assummed that peak 12 of Siebert et al. is opsin and not peak 13 as they originally speculated. Thus, both the location of the reduced chromophore in the gels and the calibration of the molecular weights of the two major rod outer segment components may not have been well resolved in the original study of Siebert et al. (3). The nature and role of peak L, which may be equivalent to peak 13 of Siebert et al. (3), remains unknown. By spectrophotmetric criteria, the degree of purity of our samples (washed ROS, A_{280}/A_{500} ≈2.0; disc membranes, A_{280}/A_{500} ≈1.85) indicates that peak L is not due to a unique, adventitious component. At present, additional experiments are

TABLE I

Light-induced in vitro phosphorylation of rod outer segment membranes.

CONDITIONS	PEAK H	PEAK L
dark only with [γ-^{32}P]ATP, 10 min	45 cpm	31 cpm
light (1 min) followed by dark with [γ-^{32}P]ATP for 30 min	2243[a] cpm	262 cpm

[a]In twelve experiments peak H contained, on the average, 85% of the total counts.

required to establish the stoichiometries of peaks H and L in rod outer segment preparations. In the present study, the relative amount of Coomassie blue positive material in peaks H and L, without prior defatting, was about 2:1; whereas this relationship was variable after the preparations had been delipidated. Rhodopsin (peak H) may be differentially removed during defatting (cf., gels 2 and 3 in Fig. 1).

Upon illumination, rhodopsin can be phosphorylated in vitro (10). In a number of experiments (n=12) rod outer segments were phosphorylated in the presence of [γ-^{32}P]ATP. After electrophoresis and staining with Coomassie blue both peaks H and L were cut out of the gel. The slices were solubilized in 0.3 ml 30% H_2O_2 at 50°C overnight and counted in a liquid spectrometer. If rhodopsin alone can be phosophorylated, then we would have expected peak H to contain most of the labelled material. This contention was upheld (Table I).

ACKNOWLEDGEMENT

This work was supported by grants of the National Research Council of Canada to E.W.A. and J.P.

REFERENCES

1. Heitzman, H. (1972) Nature New Biol. 235, 114.
2. Papermaster, D.S. and Dreyer, W.J. (1974) Biochemistry 13, 2438-2444.
3. Siebert, F., Schmid, H. and Mull, R.H. (1977) Biochem. Biophys. Res. Comm. 75, 1071-1077.
4. Uhl, R., Hofmann, K.P. and Kreutz, W. (1978) Biochemistry 17, 5347-5352.
5. Smith, H.G., Stubbs, G.W. and Litman, B.J. (1975) Expt. Eye Res. 20, 211-217.

6. Laemmli, U.K. (1970) Nature 227, 680-685.
7. Fager, R.S., Gentilcore, P.C. and Abrahamson, E.W. (1978) Vision Res. 18, 483-488.
8. Loening, U.E. (1967) Biochem. J. 102, 251-257.
9. Fairbanks, E., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617
10. Shichi, H. and Somers, R.L. (1978) J. Biol. Chem. 253, 7040-7046.