ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 8, pp. 1045-1051. © Pleiades Publishing, Ltd., 2010.
Published in Russian in Biokhimiya, 2010, Vol. 75, No. 8, pp. 1157-1165.

Proof of Oligomeric State of Frog Rhodopsin: Visualization of Dimer and Oligomers on Gels after BN- and HRCN-PAGE Using Antibodies to Rhodopsin and by Retinylopsin Fluorescence

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Received February 11, 2010

Abstract—Staining by antibodies to rhodopsin (Rh) and fluorescence of N-retinylopsin (RO) have shown that digitonin (DIG)- , dodecyl-β-D-maltoside (DM)- , and sodium dodecyl sulfate (SDS)-solubilized frog Rh after BN- and HRCN-PAGE is situated in the gradient gel in the state of dimer with a slight content of higher oligomers (trimer, tetramer, etc.). With increasing detergent harshness (DIG < DM < SDS), the proportion of higher oligomers in extracts becomes more prominent. Formation of RO in rod outer segments (ROS) in the presence of 0.7 M $NaBH_3CN$ at pH 5.0 occurs only when Rh is simultaneously photolyzed during reduction. Dithiothreitol at the concentration of 0.005 M failed to induce RO production. Formation of a stable C–N bond between all-*trans*-retinal and opsin in RO is accompanied by decrease in the dimer share and increase in the share of the higher oligomers due to secondary dissociation–aggregation of solubilized opsin. The position of the Rh dimer in relation to the anode during both native electrophoreses is determined not only by its molecular mass, but probably also depends on unfolding degree (or form): the harsher the detergent, the closer to the anode the dimer is located. Treatment of ROS by agents modifying the cholesterol component of lipid membrane (MβCD, filipin III, nystatin, saponin) did not change the character of Rh oligomerization, thus showing that integrity of the cholesterol component of photoreceptor membrane is not a crucial factor for oligomerization of opsin. It is supposed that the dimer–oligomer "portrait" of frog Rh, which has been found by two methods of native electrophoresis in three detergents with different degree of harshness, corresponds to a physiological state of this protein in native photoreceptor membrane.

DOI: 10.1134/S0006297910080146

Key words: frog rhodopsin, oligomerization, BN-PAGE, HRCN-PAGE, antibodies, fluorescence, retinylopsin, digitonin, dodecyl maltoside, SDS

The question whether native mammalian rhodopsin (Rh) in the photoreceptor membrane exists in the form of a monomer [1-4] or is present as oligomers [5-11], as many known G-protein-coupled receptors (GPCRs) [12-14] are, has not been finally solved. In our previous paper [15] it has been shown that after solubilization of frog rod outer segments (ROS) with mild detergents (digitonin (DIG), dodecyl-β-D-maltoside (DM), Chaps, Triton X-100) and subsequent 1D BN-PAGE (1D blue native electrophoresis in polyacrylamide gel), the position of Rh on the gradient gel does not match the monomer with M_{app} of 40 kDa but appears self-associated into aggregate (RhA) with molecular mass varying in different detergents from 85 to 125 kDa. After 1D BN-PAGE, besides the major band of RhA on the gel, the existence of other minor bands was observed. They could be higher oligomers of Rh, but their identification as Rh remained to be proved. But our previous data on frog Rh [15] suggested heteromeric state of Rh.

In this work, we returned to the question of Rh state in the native membrane of disks. Here we have applied a number of new approaches. They are as follows: i) a sparing method of ROS isolation, allowing their isolation in a relatively unfragmented state and with minimal content of protein impurities in detergent extracts of Rh; ii) two ways of identification of Rh and its oligomers on gels

Abbreviations: 6AA, 6-aminohexanoic acid; CBB, Coomassie Brilliant Blue G-250; 1D BN-PAGE, 1D blue native electrophoresis in polyacrylamide gel; 1D HRCN-PAGE, 1D high resolution clear native electrophoresis in polyacrylamide gel; DIG, digitonin; DM, dodecyl-β-D-maltoside; DOC, sodium deoxycholate; DTT, dithiothreitol; GPCRs, G-protein-coupled receptors; M_{app}, apparent molecular mass; MβCD, methyl-β-cyclodextrin; PVDF, polyvinylidene difluoride; Rh, rhodopsin; RhA, aggregate of Rh; RO, N-retinylopsin; ROS, rod outer segments; RPE, N-retinyl-phosphatidylethanolamine; TBS, Tris-buffered saline.

(staining by antibodies and registration of the fluorescence of N-retinylopsin (RO)); iii) in addition to already known and tried BN-PAGE [15-17], another method of native electrophoresis, HRCN-PAGE (1D high resolution clear native electrophoresis in polyacrylamide gel) [18], was applied; iv) to compare the state of Rh in mild and harsh detergents, simultaneous runs in DIG, DM, and denaturing SDS (sodium dodecyl sulfate) were performed.

The results show that solubilized frog Rh after BN- and HRCN-PAGE, irrespectively of degree of harshness of the detergent, is in a gradient gel as a dimer with slight contents of higher oligomers (trimer, tetramers, etc.). Formation of RO in bleached ROS suspensions under the influence of $NaBH_3CN$ is accompanied by decrease in the dimer share and increase in proportion of higher opsin oligomers, this being due to secondary dissociation–aggregation processes which we reported earlier [15]. These results unequivocally point to the presence of Rh dimers and higher oligomers in native frog disk membranes.

MATERIALS AND METHODS

All chemicals used in the work were purchased from Sigma-Aldrich (USA) and from Fluka, Merck, Serva, and Ferak (Germany). Grass frogs (*Rana temporaria*) were used as the experimental animals. The animals were kept in a refrigerator at 4°C in small volume of regularly replaced water without food. On the eve of an experiment, 1-2 animals were placed in a small plastic box with water and held for 3-4 h at room temperature under the conditions of usual light exposure of the laboratory. Then the animals were transferred to a light-proof box and adapted to darkness until the next morning in the presence of a small volume of water. All subsequent operations, including isolation and treatment of ROS and electrophoresis of extracts from dark-adapted ROS, were carried out under dim red light. The animals were decapitated, eyes were excised, and retinas were washed in Ringer solution (in mM): NaCl, 110; KCl, 2.5; $CaCl_2$, 1; $MgSO_4$, 1; $NaHCO_3$, 5; EDTA, 0.05; Glc, 10; Hepes (Na salt), 10. The saline had pH 7.5-7.6. The retinas were transferred to an Eppendorf vial containing 600 μl of 0.4 M sucrose in 0.05 M imidazole buffer, pH 7.0. The tissue was subjected to rotational–vibrational movement on a Sky Line vortexer (Elmer Ltd, Latvia) for 10 sec, which led to breaking off of ROS. The vial was left to stand on ice for 30 sec, thus allowing retinas to settle on the bottom. In advance two Eppendorf vials were prepared, one smaller and having a perforated bottom with 3 mm layer of glass wool. The small tube was put into the other. After the retinas settled on the bottom, the supernatant was removed and loaded on glass wool. The two vials were centrifuged at 300*g* for 30 sec using a C-1200 mini-centrifuge (National Labnet Co, USA). The formed filtrate was collected. To the remaining retinas, 600 μl fresh ice-cold mixture of sucrose/imidazole was again added and all steps described above were repeated 3-4 times. By the end of such treatment, under day light all retinas had white color because practically all red-colored ROS were broken off from the retinas. The combined filtrate containing floating intact dark-adopted ROS as well as their fragments and small amounts of black melanin granules was centrifuged at 20,000*g* at 4°C for 15 min (angle rotor N5404 of K280R centrifuge; Centurion, England). The pellet was vigorously homogenized three times with a pipet in 50 μl of solubilizing buffer A (in mM): NaCl, 50; imidazole, 50; 6-aminohexanoic acid (6-AA), 2; EDTA, 1; pH 7.0 [17], each time centrifuging at 20,000*g* for 10 min to precipitate insoluble material. Finally, the pellet (~220 μg), purified from water-soluble proteins, was then suspended in 600 μl of buffer A, divided into three parts, pelleted at 20,000*g* for 15 min, and then used for 1D BN-/1D HRCN-PAGE or for the determination of protein.

To visualize Rh and its oligomers on the gel, we reduced the protonated retinal Schiff base linkage with the ε-amino group of a Lys residue in Rh using $NaBH_3CN$ (Sigma, USA) ([19]. The product of bleaching of Rh in the presence $NaBH_3CN$ (or $NaBH_4$) is not retinol, but the stable secondary amine, N-retinylopsin (RO), in which retinal is still bound to denatured opsin at the chromophore site [20]. The fluorescence of the retinyl group, which has its excitation maximum at 330 nm and emission maximum at about 480 nm, could be observed in solutions and on various carriers during exposure to UV light [20]. In our experiments all ROS obtained from 1-2 frogs were suspended in 500 μl of mixture of 0.25 M acetate buffer with 0.7 M $NaBH_3CN$, pH 5.0 [19], and incubated 1 h in the light (or in the dark) at room temperature. Formation of RO was monitored by disappearance of absorbance at 500 nm and by appearance of absorbance near 330 nm. Optical monitoring was done in microcuvettes of a Nicolet Evolution 300 spectrophotometer (France). After finishing the reaction, the suspension was sedimented at 20,000*g* for 15 min and washed three times with 0.05 M imidazole buffer, pH 7.0, using the same conditions of centrifugation. The final pellet was immediately solubilized, the extract was loaded onto a gel, and after electrophoresis the fluorescence of RO on the gel was viewed and documented.

In a number of experiments we studied whether a preliminary treatment of lipid microdomains of ROS using cholesterol-modifying agents changes the character of Rh oligomerization [21]. The suspension of ROS (70-100 μg protein) was incubated for 1 h at 25°C in the presence one of the following substances: MβCD (methyl-β-cyclodextrin) (15.3 mM), filipin III (2 μg/ml), saponin (0.2%), or nystatin (100 μg/ml) (all these reagents were purchased from Sigma). Then the ROS suspension was

washed three times with 0.05 M imidazole buffer, pH 7.0, and solubilized as described below.

To solubilize intact or treated ROS (70-80 μg protein), membranes were mixed with 10 μl of solubilizing buffer A (see above) and 5 μl 20% (w/v) solution of one of the detergents (DIG from Merck, DM and SDS from Sigma) prepared in buffer A. The samples were transferred to a thermostat, incubated for 1 h at 25°C, and centrifuged at 20,000*g* for 20 min. The supernatant (13-15 μl) was supplemented with 5 μl 50% (w/v) glycerol/0.05 M imidazole, 5 μl 5% (w/v) Coomassie Brilliant Blue G-250 (CBB)/0.05 M imidazole (1D BN-PAGE), or 5 μl 0.1% Ponceau S (Sigma)/0.05 M imidazole (1D HRCN-PAGE). The mixture was vortexed and then centrifuged for 5 sec using a microcentrifuge. After that the aliquots (2-15 μl, 10-40 μg protein) were loaded under the cathode buffer onto the sample gel using a Guide ReadyGel Sample loading device (Bio-Rad, USA) and a syringe from Hamilton (USA). The electrophoretic chamber was placed in a refrigerator on a magnetic stirrer, a standard stir bar was put in the cathode compartment, and current was turned on. The technique of separation of membrane proteins and Rh using 1D BN-PAGE was described earlier [15, 17]. The application of HRCN-PAGE for separation of membrane proteins was described by Wittig et al. [18]. In brief, the principles of the method are as follows. Solubilized with mild detergents membrane proteins are subjected to electrophoresis in a gradient gel system containing in the cathode buffer mixed micelles of the anionic detergent sodium deoxycholate (DOC) and one of two nonionic detergents (DM or Triton X-100). It was concluded that the presence of these detergents in the buffer keeps membrane proteins solubilized, prevents their aggregation, imposes a negative charge shift on the proteins, and increases resolution considerably [18]. CBB renders a similar action on solubilized membrane proteins under BN-PAGE [17].

After electrophoresis, the sample gel was cut off, and the rest of the gel, depending on the task, was subjected to: i) fixation and protein staining [22]; ii) electroblotting of proteins onto membrane and subsequent staining of bands with antibodies to Rh; iii) exposure to UV light (λ_{max} 365 nm) to reveal RO fluorescence.

The electroblotting apparatus and power supply were the Mini Trans-Blot Electrophoretic Transfer Cell and Power Pac HC (250 V, 3 A, 300 W), respectively (both from BioRad). Polyvinylidene difluoride (PVDF) membranes (BioRad) that had briefly (~3 min) been incubated in 100% (v/v) methanol were used. The transfer buffer was 0.025 M Tris/0.192 M glycine, pH 8.3 (no HCl was added). Before beginning the transfer, the gel and the PVDF membrane were equilibrated in transfer buffer for 10 min using an OS-20 orbital shaker (Biosan, Latvia). Electroblotting was run overnight in a refrigerator (0-4°C) at 10 V and 0.02 A. After finishing transfer, the PVDF membranes were briefly (~3 min) washed in 100% (v/v) methanol to destain background and protein bands (BN-PAGE) as well as to increase resolution (HRCN-PAGE). Then the membranes were air dried and kept in a refrigerator between two strips of filter paper.

To visualize Rh with antibodies, dried membranes were placed for 2 h in 20 ml of blocking buffer consisting of a mixture of Tris-buffered saline (TBS) (in mM: Tris-HCl, 50; NaCl, 138; KCl, 2.7) and 5% (w/v) BSA (Sigma), pH 8.0. This operation and the following incubation and washing steps were carried out at room temperature on the orbital shaker platform. After incubation, the blocking buffer was removed and membranes were shaken for 2 h in 10 ml of TBS with 1% (w/v) BSA and monoclonal antibody to Rh (10 μl, titer 1 : 1000, mouse, clone 1D4; Sigma). Then the membranes were washed four times for 5 min in 20 ml of TBS/0.05% Tween 20 (Sigma) mixture. The washed membranes were shaken for 1 h in 30 ml of TBS, 0.05% Tween 20, and anti-mouse IgG (1 μl, titer 1 : 30,000). Antigen-specific goat secondary antibodies containing conjugated alkaline phosphatase (Sigma) were used. The membranes were washed four times by TBS/Tween 20 and three times by TBS alone and then transferred into 20 ml of enzyme-revealing substrate mixture containing (in mM): nitroblue tetrazolium, 0.48; disodium salt of 5-bromo-4-chloro-3-indolyl phosphate, 0.56; Tris-HCl, 10; $MgSO_4$, 59. The pH of the mixture was 9.5. The membranes were incubated in the substrate mixture for 10-30 min until color development, and then they were washed with water, air-dried, and kept in a refrigerator in a light-proof box.

Protein bands on the gels and membranes after staining with CBB and antibodies were viewed in scattered white light of a TFX-L/WL transilluminator (Vilber Lourmat, France). To visualize the fluorescence of RO on the gels, they were exposed to scattered UV light (λ_{max} 365 nm) emitted by the same device. The images were taken by a Lumix DZ-2 camera (Panasonic, Japan). The M_{app} for Rh and its oligomers as well as for protein in the samples were determined as described earlier [15].

RESULTS AND DISCUSSION

In this work a rapid and gentle method of isolation of frog ROS has been developed. The method does not contain a step of disruptive homogenization and is based on the principle of breaking off ROS from the retina during short-term vibration and rotation generated by a vortex. The method yields a mixture of intact and fragmented ROS from 1-2 frogs in quantities (~0.1 mg protein/retina) sufficient for electrophoretic analysis immediately on the day of isolation. Active 2-3-fold washing of the ROS with the solubilizing buffer A [17] removes most water-soluble protein impurities from the membrane fraction and allows more precise identification of Rh and its oligomers on gels using staining with antibodies and RO fluores-

cence. The method is faster and easier compared with an earlier described method [15], but its disadvantage is the presence in the ROS fraction of a small number of black melanin granules.

The arsenal of all methods used has shown that after 1D BN-PAGE and 1D HRCN-PAGE in all detergents, independent of their stringency (SDS > DM > DIG), the major band is identified as a dimer of Rh (Figs. 1 and 2). In the previous paper [15] this band was called an aggregate of Rh, i.e. RhA. In addition to dimer, a small proportion of trimer and higher oligomers was observed also, but the latter was seen mainly in DM and SDS extracts using 1D HRCN-PAGE. As it is obvious from the presented data, dissociation of oligomers to monomeric form did not proceed under our conditions even in the case of the harsh detergent SDS. Of note, the possibility of dissociation of oligomers into monomer under conditions of SDS-PAGE was demonstrated earlier [15].

The M_{app} of Rh dimer in extracts of DIG, DM, and SDS was on average 85.3 ± 2.5, 80.4 ± 1.1, and 76.9 ± 3.5 kDa, respectively. The M_{app} of trimer in the same detergents was 125.6 ± 5.9, 122.8 ± 6.0, and 109.3 ± 2.3 kDa, respectively. Sensitivity of the method of staining Rh by antibodies in comparison with CBB seems to be higher. That gave the possibility to discern on the blots of DM and SDS extracts oligomers of higher order than dimer and trimer (Fig. 1b). It should be mentioned that due to squeezing of the gel inside a sandwich during the blotting procedure, the actual size of the print was somewhat distorted (Fig. 1b). As seen from Figs. 1 and 2, all detergent extracts contain in addition to Rh oligomers also protein impurities (tagged as x, y, and z), which are small in proportion, have low molecular mass (they run before the dimer), and do not give positive reaction with antibody or fluorescence.

We failed to see fluorescence of RO on unfixed gels after BN-PAGE because CBB adsorbed on the protein bands and on the gradient gel as a background completely quenched fluorescence of RO (data not shown). On the other hand, after HRCN-PAGE prominent RO fluorescence was seen in all Rh aggregates (Fig. 2c). Our experiments showed that the production of RO in frog ROS proceeds only if the suspension is photolyzed in the presence of $NaBH_3CN$ (Fig. 2c). In the dark-adapted suspension of ROS, the formation of RO does not proceed even during 24-h incubation at room temperature (data not shown). It is interesting that unbleached bovine Rh, solubilized by DIG, Triton X-100, and emulfogen, catalyzes rapid (within minutes) formation of RO [19]. This indicates that some aspects of the native opsin structure in ROS shield the protonated Schiff base linkage from reactions with $NaBH_3CN$.

The formation of RO in bleached ROS in the presence of $NaBH_3CN$ is accompanied by decrease in share of dimer and increase in share of higher oligomers. It should be noted that this re-proportioning was found in all detergents and using of both kinds of electrophoresis (Figs. 1 (a and c) 2 (a and b)). Surprisingly, a milder

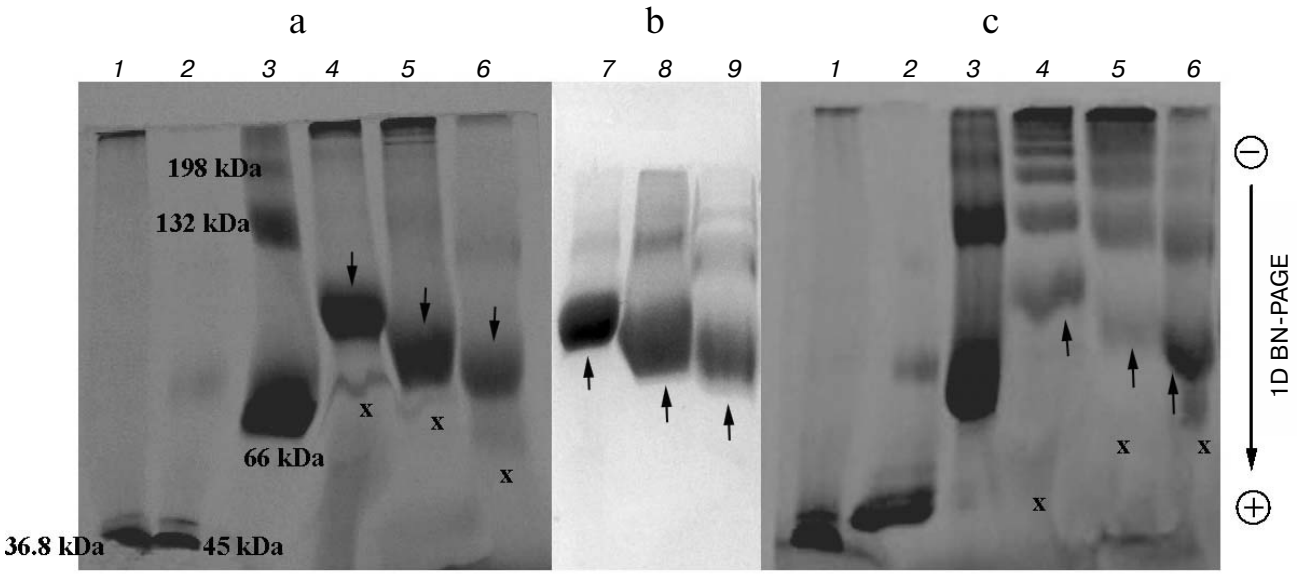

Fig. 1. 1D BN-PAGE of extracts obtained by solubilization of ROS with various detergents. Gel (a): *1-3*) markers β-lactoglobulin, ovalbumin, and BSA (monomer, dimer, trimer), respectively; *4-6*) dimers (oligomers) of Rh solubilized by DIG, DM, and SDS, respectively, and stained with CBB. Gel (b): *7-9*) dimers (oligomers) of Rh solubilized by DIG, DM, and SDS, respectively, transferred onto PVDF membrane and stained by the product of the alkaline phosphatase reaction due to interaction of the primary (specific to Rh) and secondary (specific to IgG) antibodies. Gel (c): same as on gel (a), but before detergent extraction the suspension of ROS was subjected to photolysis with white light for 1 h at pH 5.0 and in the presence of 0.7 M $NaBH_3CN$. Arrows show dimers of Rh, "x" indicates unidentified protein impurities. The loaded sample contained 20-25 μg protein in 15 μl. Data of one typical experiment are presented.

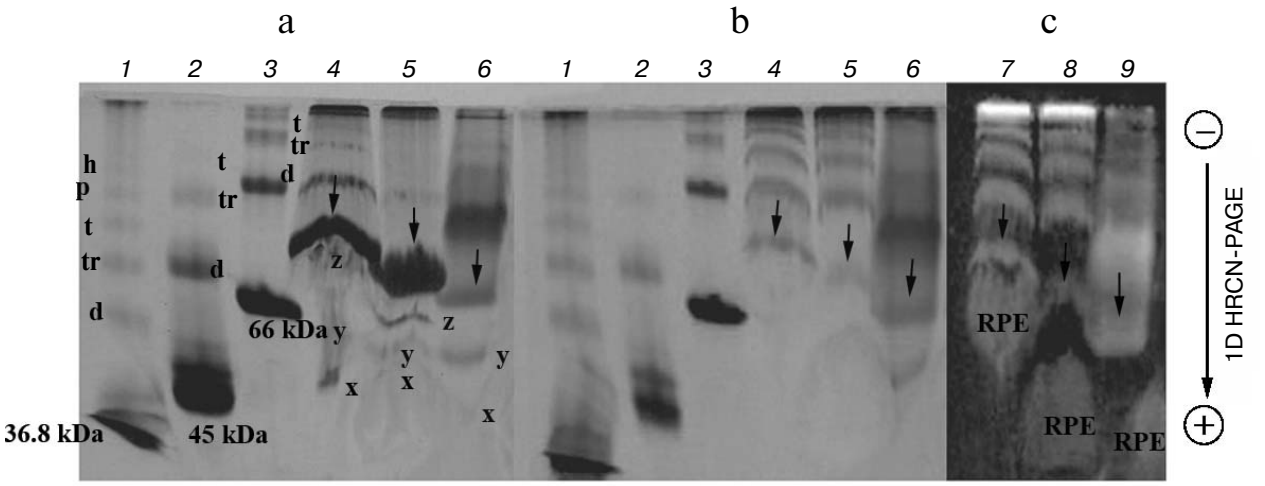

Fig. 2. 1D HRCN-PAGE of extracts obtained by solubilization of ROS with various detergents. Gel (a): *1-3*) markers β-lactoglobulin, ovalbumin, and BSA, respectively; *4-6*) dimers (oligomers) of Rh solubilized by DIG, DM, and SDS, respectively, and stained with CBB. Gel (b): same as gel (a) but before detergent extraction the suspension of ROS was subjected to photolysis with white light for 1 h at pH 5.0 in the presence of 0.7 M $NaBH_3CN$. Proteins were stained by CBB. Gel (c): *7-9*) same as lanes *4-6* on gel (b) but immediately after electrophoresis the unfixed gel was exposed to UV light and a picture was taken. Arrows designate Rh dimers; d, tr, t, p, and h correspond to dimer, trimer, tetramer, pentamer, and hexamer of corresponding markers; x, y, and z designate unidentified protein impurities; RPE, N-retinyl-phosphatidylethanolamine. The volume and quantity of the sample proteins are the same as in Fig. 1. Data of one typical experiment are presented.

reducer (DTT) at rather high concentration (5 mM) did not have a similar effect on Rh with either kind of PAGE (data not shown). The changes observed after treatment of bleached ROS by $NaBH_3CN$ are obviously consequences of a process of secondary dissociation–aggregation of Rh that has been noted by us earlier [15]. This process might allow the formation of additional (not characteristic for native Rh) disulfide cross-linking in the presence of high concentrations of the reducer. It is also possible that $NaBH_3CN$ somehow changes the character of the usual hydrophobic interactions between Rh dimers.

Figure 2c shows that in addition to fluorescence of RO in all oligomer bands, relatively intensive fluorescence is observed as a diffuse spot ahead of the Rh dimer. This spot represents a compound with relatively low molecular mass which is not stained by protein-specific CBB. A possible interpretation of these results might be that irradiation of ROS in the presence of a surplus of $NaBH_3CN$ at pH 5.0 also induces formation of retinyl group between retinal and lipid of ROS (phosphatidylethanolamine), thus forming N-retinyl-phosphatidylethanolamine (RPE). A probable substrate for this reducing reaction is N-retinylidene phosphatidylethanolamine, whose accumulation in ROS during Rh photolysis at acid pH was shown earlier [23-25].

Of note, under conditions of 1D HRCN-PAGE (Fig. 2a, *1* and *2*) the protein markers (β-lactoglobulin and ovalbumin) for some reason become able to oligomerize, thus mimicking the behaviour of BSA, which under conditions of both types of electrophoresis is always in a monomer–oligomer state (Figs. 1 (a and c, lane *3*) and 2 (a and b, lane *3*)). There is not yet any explanation for such behaviour.

From the data of Figs. 1 and 2 it is seen that the dimer of Rh solubilized with DIG after running in both types of electrophoresis is close to the cathode, but after SDS extraction it is shifted toward the anode. Similar differences in position on gel between Rh dimers extracted by mild detergents DIG and Chaps, respectively, were noted in our previous paper [15]. Mixing DIG or DM extracts of Rh with equal volumes of SDS extracts or with equal volume of 20% (w/v) SDS solution led, predictably, to bleaching of Rh. In this case oligomers of Rh in the mixed extracts behaved like they were extracted by SDS alone, i.e. they were maximally shifted to the anode (Fig. 3, a and b, lanes *4-6*).

It is well known that Rh in SDS is completely denatured and unfolded. This is accompanied by loss of its red colour (it becomes bleached) and capacity to regenerate into the initial dark state in the presence of exogenous 11-*cis*-retinal. These specific properties (light absorption and regeneration) indicating a roughly native state of visual pigments remain unaltered after solubilization of Rh with DIG [26] or DM [27]. As follows from theory of BN- and HRCN-PAGE [16-18], Rh dimer solubilized by SDS must run in the electrical field due to net negative charge produced by molecules of SDS and CBB (BN-PAGE) or SDS, DOC, and DM (HRCN-PAGE) bound to opsin. The position of this dimer on the gradient gel should be determined only by its size (molecular mass) through the molecular sieve effect. As it is obvious from our experiments, the same membrane protein (e.g. Rh) in different

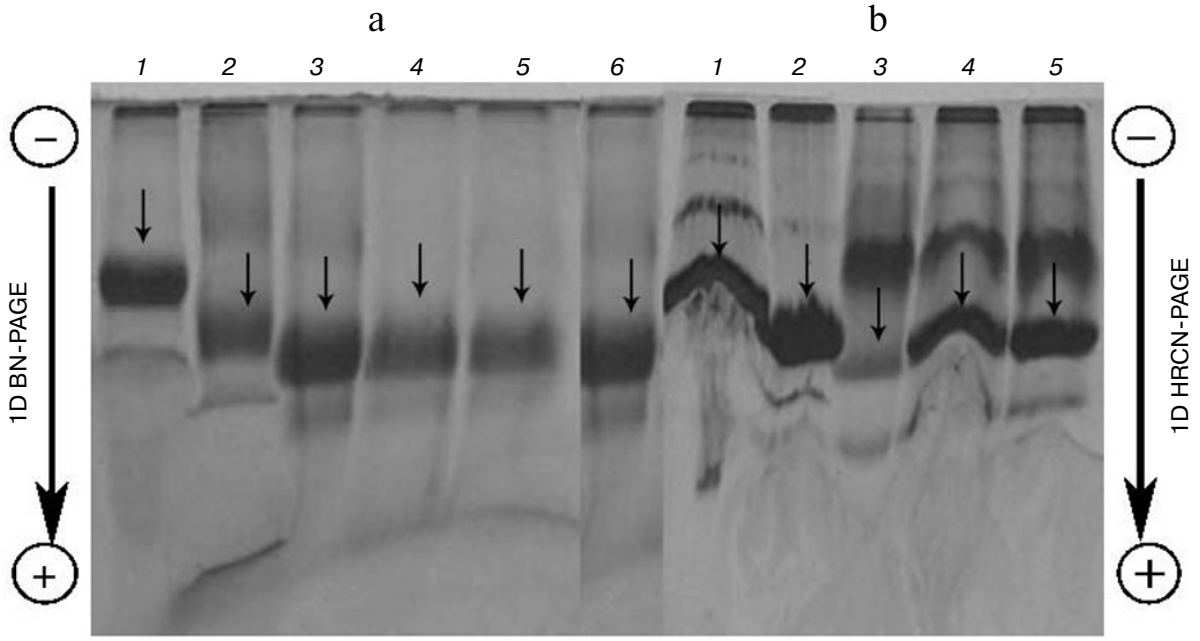

Fig. 3. 1D BN-PAGE (a) and 1D HRCN-PAGE (b) of extracts obtained by solubilization of ROS with various detergents and by their subsequent mixing. Gel (a): *1-3*) dimers (oligomers) of Rh solubilized by DIG, DM, and SDS, respectively; *4*) mixture of DIG and SDS extracts (1 : 1 v/v); *5*) mixture of DM and SDS extracts (1 : 1 v/v); *6*) mixture of DIG extract and 20% SDS solution (1 : 1 v/v). Gel (b): *1-5*) same as lanes *1-5* on gel (a). A sample containing 25-30 μg protein in 15 μl aliquots was loaded. Arrows show dimers of Rh. Data of one typical experiment are presented.

detergents and having different degree of unfolding can occupy in BN- and HRCN-PAGE different positions in the gradient gel and can formally show different M_{app} although the length of the polypeptide chain is identical in all detergents. Denatured and completely unfolded in SDS dimer of Rh probably acquires a favorable conformation for operation of the molecular sieve effect (it probably changes its shape or becomes more compact) and as a result dimer is closer to the anode than Rh dimer in relatively native state. To what extend such behavior will relate to other membrane proteins remains an open question.

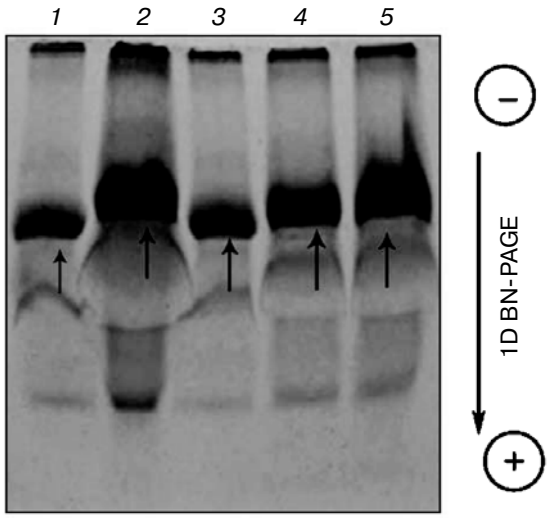

Fig. 4. 1D BN-PAGE of DIG extracts obtained from ROS that were preliminarily treated by cholesterol-modifying agents: *1*) no treatment (control); *2-5*) treated by saponin, nystatin, filipin III, and MβCD, respectively. A sample containing 25 μg of protein in 15 μl aliquots was loaded. Arrows show dimers of Rh. Data of one typical experiment are presented.

Taking in account numerous indications that the lipid environment of the rod disk membrane is not homogeneous [21] and that neither the polar moiety nor diglyceride part of the phospholipids in ROS are essential for the spectral integrity of Rh [28], we have studied how the state of aggregation of Rh varies in ROS treated with agents modifying or removing cholesterol from the lipid bilayer. Figure 4 shows that preliminary treatment of ROS membranes with methyl-β-cyclodextrin (MβCD), saponin, nystatin (which solubilize and remove cholesterol) as well as filipin III (which binds and forms cholesterol aggregates [21]) did not influence the Rh dimer or its oligomers. Apparently the integrity of the cholesterol component of the membrane is not a key factor in the mechanisms of aggregation Rh into its oligomers.

Thus, application of various approaches for identification of Rh state clearly removed our remaining doubts about the fact that the observed protein heterogeneity on electrophoregrams illustrated the aggregated state of Rh in ROS and is not caused by the presence of protein

impurities in the studied detergent extracts. Equal prevalence of the dimeric forms of Rh with a small proportion of higher oligomers in all detergents, differing in varying degrees of harshness, and changes in proportion of oligomers in experiments only after exposure ROS to $NaBH_3CN$ is evidence that native Rh in the photoreceptor membrane is located in a relatively stable state of the dimers and oligomers. Although the dimer-oligomer model for most GPCRs is considered already proved (see reviews [29-31]), the monomeric [32, 33] and oligomeric [34, 35] state of mammalian Rh continued to be an item of discussion.

This work was supported by the Russian Foundation for Basic Research (grant No. 09-04-00691-a) and by the Program of the Russian Academy of Sciences (Devision of Biological Sciences) (grant No. 1OB-05).

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