Purification of Retinol Dehydrogenase from Bovine Retinal Rod Outer Segments*

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We purified retinol dehydrogenase from bovine rod outer segments using polyethylene glycol precipitation and hydroxylapatite, concanavalin A-Sepharose CL-4B, and Sepharose CL-6B column chromatography in the presence of NADP. We obtained 13-fold purification of retinol dehydrogenase with specific activity of 61.8 nmol/min/mg and 3.8% recovery. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that retinol dehydrogenase had a molecular mass of 37,000 daltons. The K_m values of purified retinol dehydrogenase for all-trans retinol and all-trans retinal were 6.6 mM and 0.085 mM, respectively. The purified enzyme reacted with the all-trans retinal but not with 13-, 11-, and 9-cis compounds.

In addition, we prepared antibody to retinol dehydrogenase using rat. The anti-retinol dehydrogenase antibody precipitated retinol dehydrogenase activity and was confirmed to bind to 37-kDa protein by Western blotting. We also found that anti-retinol dehydrogenase antibody bound to bovine rod outer segments specifically by immunohistochemical technique. The molar ratio of retinol dehydrogenase to opsin in rod outer segments estimated by enzyme-linked immunosorbent assay was 1:140.

In the visual cycle, all-trans retinal from bleaching rhodopsin is converted to all-trans retinol by all-trans-specific retinol dehydrogenase in the rod outer segments of photoreceptor cells (1). All-trans retinol derived from the bleaching photoreceptor cells (2), and the choroidal blood supply (3) is delivered to the retinal pigment epithelial (RPE) cells and is converted to 11-cis retinal through esterification (4-6), de-esterification (7), isomerization (8-10), and oxidation by 11-cis-specific retinol dehydrogenase (1, 11). The 11-cis retinal formed in the RPE cells is delivered to rod outer segments (ROS) to regenerate rhodopsin (2, 12-14).

Retinol dehydrogenase in ROS catalyzes the interconversion of all-trans retinol and all-trans retinal. The reaction, however, is directed to form all-trans retinol using NADPH in the ROS (15) during light adaptation. The enzyme binds to membrane and is labile when dissolved by detergents (1, 16, 17). Therefore, purification of the enzyme has not yet been accomplished. We found that retinol dehydrogenase, even solubilized with detergents, is not so labile in the presence of NADP. We purified the enzyme from bovine ROS and partially characterized it.

**MATERIALS AND METHODS**

**Preparation of Retinal Isomers—**Photoisomerization of retinal was performed according to the method of Bridges and Alvarez (18). Retinal isomers were isolated with high-pressure liquid chromatography using Chemcosorb 5Si column (4.6 × 250 mm) according to Tsuchida et al. (19). Mobile phase is diethyl ether/n-hexane (8/92) at 1 ml/min. Peaks were detected at 365 nm. All-trans, 9-cis, 11-cis, and 13-cis compounds were collected separately and were kept under argon at −30 °C. Experiments involving the retinoids were carried out in dim red light.

**Assay of Retinol Dehydrogenase Activity—**Retinol dehydrogenase activity was measured by the modification of Lion et al. (1). The oxidation reaction was started by mixing 50 μl of enzyme solution and 50 μl of substrate solution containing 10 mM all-trans retinal, 6 mM NADP, 1.2% Tween 80, 12% aceton, and 0.4 M Tris-HCl buffer, pH 8.0. Incubation was carried out at 37 °C. The reaction was stopped with the addition of 0.5 ml of ethanol, and the retinol formed was assayed by adding 0.2 ml of thioarane reagent and 0.2 ml of thiobarbituric acid reagent according to Futterman and Saalaw (20). After 30 min, samples were centrifuged at 10,000 rpm for 1 min. Color developed was measured at 520 nm. To avoid precipitation of salts, 50 μl of distilled water was added to the supernatant before the measurement was performed.

The reduction reaction was started by mixing 50 μl of enzyme solution and 50 μl of substrate solution containing 10 nmol of all-trans retinal, 5 mM NADPH, 1.2% Tween 80, 12% aceton, and 0.2 M sodium acetate buffer, pH 5.0. Incubation was carried out at 37 °C, and the reaction was stopped with the addition of 0.5 ml of ethanol. Retinal that was decreased in 2 min was assayed by the thiobarbituric acid method, as described.

**Purification of Retinol Dehydrogenase—**Bovine eyes were obtained from a local slaughterhouse. The ROS from 300 bovine retinas were prepared in the light, according to the method of Papernost and Dreyer (21). The purified ROS were suspended with 24 ml of 5 mM phosphate buffer, pH 7.0. To the ROS suspension, 10.72 ml of distilled water, 0.24 ml of 1 M phosphate buffer, pH 7.0, 0.8 ml of 60 mM NADP, 0.24 ml of 300 mM diethyloletritol, 9.6 ml of glyceral, and 2.4 ml of 20% sodium cholate were added, in that order. Final concentration of the suspension was 1 mM NADP, 1 mM diethyloletritol, 20% glyceral, 10 mM phosphate buffer, and 1% sodium cholate. Ammonium sulfate (6.91 g) was added to the suspension (48 ml) to make 25% saturated solution for dissolving retinol dehydrogenase from ROS. The pH of the crude enzyme solution was adjusted to 7.0 with NaHCO3 powder (crude extract).

The same volume of 20% (w/v) polyethylene glycol 6000 (about 51 ml) was added to the crude extract. After 10 min, precipitated proteins were removed by centrifugation at 14,000 rpm for 20 min (polyethylene glycol supernatant).

The supernatant volume (about 96 ml) of buffer A (0.5% sodium cholate, 0.3 mM NADP, 1 mM diethyloletritol, 20% glyceral, 10 mM phosphate buffer, pH 7.0) containing 10% saturated ammonium sulfate was added to the supernatant. The sample was applied onto hydroxylapatite column (50 × 20 cm, 15 g of hydroxylapatite, Bio-Gel HTP, Bio-Rad) equilibrated with buffer A containing 10% saturation of am-

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‡ The abbreviations used are: RPE, retinal pigment epithelial; ROS, rod outer segment; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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monium sulfate (buffer B). Then retinol dehydrogenase was eluted with a linear gradient of 0-40% ammonium sulfate in buffer A at a flow rate of 1.5 ml/min. Total gradient volume was 600 ml. Fraction size was 10 g/tube. Aliquots (50 μl) of the fractions were used to assay retinol dehydrogenase activity. The oxidation reaction was performed for 10 min at 37°C. The fractions containing unbound retinol dehydrogenase were concentrated to approximately 4 ml. The concentrated sample was dialyzed overnight against 200 ml of buffer A containing 0.15 M NaCl (buffer C) at 4°C.

The dialysate was applied onto concanavalin A-Sepharose CL-4B column (0.3 × 12 cm) equilibrated with buffer B at a flow rate of 0.5 ml/min. Unbound retinol dehydrogenase was eluted with 100 ml of buffer C. Fraction size was 10 g/tube. After eluting retinol dehydrogenase, 100 ml of buffer C containing 0.25 mM methyl-α-D-mannopyranoside was used to elute the bound retinol dehydrogenase. Aliquots (50 μl) of the fractions were used to assay retinol dehydrogenase activity. The oxidation reaction was performed for 10 min at 37°C. The fractions containing retinol dehydrogenase were concentrated to approximately 4 ml.

Final purity of the retinol dehydrogenase was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli (25), with 10% acrylamide gel. After electrophoresis, proteins were detected with a silver staining kit (Bio-Rad).

Protein concentrations were measured according to Bensadoun and Weinstein (23) using sodium cholate with bovine serum albumin as the standard.

Preparation of Antibodies—Rabbit anti-bovine opsin antibody was prepared, as described previously (24).

We prepared anti-bovine retinol dehydrogenase antibody using rat. Retinol dehydrogenase was partially purified by polyethylene glycol, hydroxylapatite, and Sepharose CL-6B column chromatography. As the column chromatography of concanavalin A-Sepharose CL-4B decreased the recovery, we removed this step. Retinol dehydrogenase was further purified by SDS-PAGE with 10% gel from the partially purified sample. We stained the gel with Coomassie Brilliant Blue R-250 (25) and eluted the 37-kDa protein electrophoretically using the electrode buffer of Laemmli (22). The eluted sample was concentrated by lyophilization.

We mixed 20 μg (200 μl) of the electrophoretically purified retinol dehydrogenase with the same volume (200 μl) of complete adjuvant and injected the emulsified sample intraperitoneally into rat two times. Incomplete adjuvant and 50 μg of protein were used for the third injection. Plasma was collected 3 days after the final injection.

Western Blotting—Western blotting was performed according to Towbin et al. (26). After electrophoretic transfer of proteins to nitrocellulose membrane, the membrane was treated with 3% gelatin for 1 h at 37°C. We used 1:100 diluted rat anti-retinol dehydrogenase plasma and 1:50 diluted goat anti-rat IgG antibody conjugated with peroxidase. The antibodies were diluted with phosphate-buffered saline (0.14 M NaCl and 10 mM phosphate buffer, pH 7.4) containing 1% bovine serum albumin and 0.05% Tween 20. Incubation time was 1 h at room temperature. Washing was performed after each step with phosphate-buffered saline containing 0.05% Tween 20. Color development with d-iodosinadine was carried out for 20 min at room temperature.

Light Microscopic Immunohistochemistry of Retinol Dehydrogenase—Bovine retinas were prepared with periodate-lysine-paraffin-alkyde fixative (27) at 4°C overnight and embedded in paraffin. Indirect staining was performed, as reported previously (24), except that 1:200 diluted rat anti-retinol dehydrogenase plasma as first antibody and peroxidase-conjugated goat anti-rat IgG antibody as second antibody were used. We prepared control sections treated with anti-retinol dehydrogenase plasma absorbed with excess antigen (10 μg/ml) or omitted the step of first antibody reaction.

Enzyme-linked Immunosorbent Assay of Opsin and Retinol Dehydrogenase—Crude extracts of rod outer segments were prepared, as described before, and diluted with 1% Triton X-100 containing 1% bovine serum albumin, 0.14 M NaCl, and 10 mM phosphate buffer, pH 7.4. Opsiin content and retinol dehydrogenase content were measured by competitive enzyme-linked immunosorbent assay, as reported previously (24).

RESULTS

The stabilizing effect of NADP on retinol dehydrogenase was determined using 1, 0.1, 0.01, and 0.001 mM in the presence of 20% glycerol, 0.1 M NaCl, 10 mM phosphate buffer, pH 7.0, and 1% sodium cholate. After 30 min, we measured the oxidation reaction of retinol dehydrogenase in a linear range of activity and obtained 73, 69, 58, and 21% activity compared with undissolved ROS, respectively. When glycerol was removed, the remaining activity decreased markedly. We used at least 0.3 mM NADP for the purification of the enzyme. We performed column chromatography at pH 7.0 because retinol dehydrogenase is rather stable from pH 5.0 to 7.5.

We found that retinol dehydrogenase dissolved with 1%
TABLE I

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<tr>
<th>Purification of retinol dehydrogenase from bovine rod outer segments</th>
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<td>Purification step</td>
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<td>Crude extract</td>
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<td>Polyethylene glycol</td>
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<td>Hydroxylapatite</td>
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<tr>
<td>Concanavalin A-Sepharose</td>
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<td>Sepharose CL-6B</td>
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FIG. 2. SDS-PAGE of purified retinol dehydrogenase and characterization of rat anti-bovine retinol dehydrogenase antibody by Western blotting and immunostaining techniques. Lanes a-d, SDS-PAGE silver staining. Lane a, molecular weight standards. Lane b, purified retinol dehydrogenase (1 μg, arrow). Lane c, electrophoretically purified retinol dehydrogenase (0.5 μg, arrow). Lane d, crude extract (7.5 μg). Lane e, Western blot of crude extract (7.5 μg) reacted with anti-bovine retinol dehydrogenase (described under “Materials and Methods”). The antibody reacts with a single molecular species that corresponds with the migration of the retinol dehydrogenase.

sodium cholate, sodium deoxycholate, Triton X-100, and Nonidet P-40 retained its enzyme activity, but dodecyltrimethylammonium bromide inactivated the enzyme even in the presence of NADP. As the enzyme bound to hydroxylapatite firmly and eluted with high concentration of ammonium sulfate (Fig. 1a), we selected sodium cholate.

As shown in Table I, concanavalin A-Sepharose column chromatography (Fig. 1b) sometimes decreased recovery of the enzyme, and we obtained only 3.8% in final recovery. However, without this step, we could not obtain highly purified retinol dehydrogenase.

Retinol dehydrogenase was larger than thyroglobulin by Sepharose CL-6B column chromatography (Fig. 1c), whereas its molecular mass was 37,000 daltons estimated by SDS-PAGE (Fig. 2). Dimer molecules of retinol dehydrogenase were noted (Fig. 2e).

We carried out nondenaturing polyacrylamide gel electrophoresis to obtain further information about the active form.
present in the rod outer segments (arrow in panel b), whereas no immunoreactivity was observed in the control sections, for which the step of first antibody reaction was omitted (panel a). OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Bar indicates 50 μm.

Fig. 6. Immunohistochemical demonstration of retinol dehydrogenase in bovine retina. Marked immunoreactivity was present in the rod outer segments (arrow in panel b), whereas no immunoreactivity was observed in the control sections, for which the step of first antibody reaction was omitted (panel a). OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Bar indicates 50 μm.

Fig. 7. Competitive enzyme-linked immunosorbent assay of opsin and retinol dehydrogenase. Panel a, standard curves of retinol dehydrogenase (●) and opsin (○) show similar results. Concentration of purified retinol dehydrogenase is calculated based on $M_r = 37,000$. Panel b, content of retinol dehydrogenase (●) and opsin (○) in the crude extract is calculated by its % bound. Lines were obtained by a least-squares approximation. Molar ratio of retinol dehydrogenase to opsin was 1:140.

The results obtained herein constitute the first purification of retinol dehydrogenase to homogeneity from bovine ROS. The purified enzyme has stereospecificity for all-trans retinal. The results show that ROS retinol dehydrogenase does not convert 11-cis retinal delivered from RPE cells to 11-cis retinol. The 11-cis retinol is present in the interphotoreceptor matrix (14), but its origin is unknown.

Substrate specificity also distinguishes retinol dehydrogenase purified in the present study from another retinol dehydrogenase in RPE. Stereospecificity of retinol dehydrogenase in RPE is directed toward 11-cis compound (1, 30), while retinol dehydrogenase purified in this study has specificity for all-trans compound.

We found that anti-retinol dehydrogenase antibody bound to ROS specifically and had no cross-reactivity toward retinol dehydrogenase in RPE. Also of immunohistochemical interest was whether all-trans-specific retinol dehydrogenase is present in Müller cells or not. Müller cells have cellular retinal-binding protein (31, 32) and cellular retinol-binding protein (33), and it has been suggested that the glial cells play some role in vitamin A metabolism in the retina. All-trans-specific retinol dehydrogenase, however, was not found by our immunohistochemical technique. The reduction reaction of all-trans retinal to all-trans retinol seems to be a specific reaction in the ROS in the eye.

Opps and retinol dehydrogenase, being membrane proteins, have a marked tendency to exist as a large micellar complex with many molecules of the detergent. Nicotra and Livrea (17) showed that a major peak of retinol dehydrogenase solubilized with nonionic detergent Lubrol 12A9 corresponds to Stokes radius of 8.5 nm which is close to thyroglobulin molecule on Bio-Gel A-5 m. In the present study, we also...
observed a large micellar complex on a gel filtration column chromatography (Fig. 1c).

We obtained the molecular mass of 37,000 daltons for retinol dehydrogenase by SDS-PAGE. Nicotra and Livrea (17) showed that the molecular weight of retinol dehydrogenase was 70,000 by SDS-PAGE using [14C]retinol binding capacity. A molecular weight of 70,000 is almost equivalent to the dimer of our molecule. At present, it is unknown whether the active form of retinol dehydrogenase requires the dimer structure or not.

Retinol dehydrogenase constitutes about 0.6% of ROS protein since the molar ratio of retinol dehydrogenase to opsin is 1:140 (Fig. 7), and rhodopsin comprises over 80% of the protein in ROS (21). Highest $V_{\text{max}}$ value of our fully purified retinol dehydrogenase for all-trans retinol was 58.1 nmol/min/mg. The turnover number (molecular activity) can be calculated based on this specific activity and 37,000 Da as the molecular mass, i.e. $(58.1 \text{ nmol}/60 \text{ s})/(1,000,000 \text{ ng}/37,000) = 0.036 \text{ s}^{-1}$. This means that the conversion of 140 molecules of all-trans retinol released from 140 molecules of photobleached rhodopsin by one molecule of retinol dehydrogenase in ROS requires about 65 min to end at constant velocity. If the fully purified material contains inactive enzyme to some extent, the turnover number increases and the conversion finishes earlier. Retinal content of the rat retina practically reaches a level 10-15% after 30 min in the light (2). This result was obtained using different species, but the time required for the conversion seems to be not markedly different from the result of our calculation (15% after 55 min).

Finally, we purified labile, membrane-bound retinol dehydrogenase from bovine ROS. The enzyme plays an important role in the visual cycle in the eye. A deficiency of the enzyme may cause the accumulation of a potentially toxic all-trans retinal from bleached photoreceptor cells to RPE cells. Thus, it seems likely that a deficiency of ROS retinol dehydrogenase causes photoreceptor degeneration. Further molecular analysis of retinol dehydrogenase may provide an important clue in understanding the disorders caused by abnormality in vitamin A metabolism.

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REFERENCES