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THE MEMBRANE BOUND RETINOL DEHYDROGENASE FROM BOVINE ROD OUTER SEGMENTS

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SUMMARY - Retinol dehydrogenase from bovine rod outer segments was solubilized in detergent and partially purified 25-fold through a combination of hydroxyapatite and retinyl-Sepharose chromatography. All-trans retinol solubilized in protein solutions of bovine serum albumin or β-lactalbumin was a better substrate for the enzyme than retinol solubilized in detergents or suspended in buffer. Retinol dehydrogenase was sensitive to the carbonyl reagent pyridoxal-5'-phosphate but was not inhibited by retinal followed by reduction with $NaBH_4$. The solubilized enzyme requires phospholipids to maintain enzymatic activity, as was evidenced by the inactivating effect of phospholipase A_2 on the partially purified enzyme.

Introduction - Retinol dehydrogenase, a membrane bound enzyme found in the rod outer segments of the retina, catalyzes the interconversion of retinol and retinal (1). The enzyme which under physiological conditions generates retinal, the form of vitamin A needed in the visual pigment rhodopsin, plays a central role in the visual cycle. Although the enzyme in detergent-free rod outer segment suspensions has been studied (2-4), characterization of a solubilized and partially purified form of the enzyme has not been reported.

In this paper, we report a successful partial purification scheme for retinol dehydrogenase. The establishment of the best conditions for the assay of the enzyme are discussed; and some of the reported properties of the enzyme in dispersed rod outer segments were reexamined. The role played by the phospholipid environment on the catalytic function of the enzyme is explored.

Experimental Procedures

Purification of retinol dehydrogenase. Rod outer segments from 20 bovine retinas were isolated according to the procedure of Papermaster and Dreyer

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(5). These rod outer segments were suspended in 10mM phosphate (pH 7.0) containing 1mM β-mercaptoethanol and 1% (w/v) sodium cholate and homogenized in a Dounce homogenizer. This homogenate was then sonicated for 10 minutes with a Heat Systems W-375 sonicator delivering 100w peak power. During sonication the homogenate was held in a water jacketed sample chanber at 4°C and flushed continuously with a stream of argon. The sonicate was centrifuged at 100,000 x g for one hour and the yellow supernatant was saved.

This supernatant was then applied to a hydroxyapatite column (2.6 x 10cm) prepared according to Levin (6) and previously equilibrated with 10mM phosphate (pH 7.0). The enzymatic activity was retained on the hydroxyapatite and was eluted with a linear gradient of phosphate (pH 7.0) containing 0.4% (w/v) sodium cholate. The enzyme eluted between 400 and 600mM phosphate; and was concentrated and washed in an Amicon PM-30 concentrator with 10mM phosphate (pH 7.0) containing 0.4% (w/v) sodium cholate.

The active fractions were then applied to a retinyl-sepharose column (10 x 0.9cm) equilibrated with 10mM phosphate (pH 7.0) containing 0.4% (w/v) sodium cholate. The enzyme is retained by the column and was eluted with 700mM KCl in 10mM phosphate (pH 7.0) containing 0.4% (w/v) sodium cholate. A purification scheme is included in Table I. The partially purified enzyme can be stored at -12°C without loss of enzymatic activity for 2 days.

Assay of retinol dehydrogenase. Retinal formation was monitored by following the increase in absorbance at 390nm in an Aminco DW-2 dual wavelength spectrophotometer. The assay mixture contained bovine serum albumin (5mg/ml), all-trans retinol (0.1mM), $NADP^+$ (0.1mM) and enzyme in 0.1M sodium pyrophosphate (pH 8.6).

Source of materials used. Sepharose derivatized with N-(w-aminohexyl)-retinyl was prepared by reacting the "spacer group" (w-aminohexyl) of Sepharose AH (Pharmacia) with all-trans retinal at pH 9. One gram of Sepharose AH was suspended in 10ml of 0.1M sodium bicarbonate, mixed with 1ml of all-trans retinal (2mM) in methanol, brought to pH 9 by addition of 0.1M NaOH, and allowed to react at 37°C for 2 hours. The resulting Schiff's base was reduced by addition of solid sodium borohydrade. The derivatized Sepharose was washed with 0.1M sodium acetate (pH 5) containing 0.5M NaCl.

Bee venom phospholipase A_2 and phospholipase C obtained from Sigma were free of contaminating proteolytic enzymes.

TABLE I
PARTIAL PURIFICATION SCHEME FOR RETINOL DEHYDROGENASE

	Purification Step	Volume ml	Protein mg/ml	Units nmoles/min	Specific Activity units/mg
1)	Crude Homogenate Supernatant from ROS	3.5	21.4	5.35	.250
2)	Hydroxyapatite Chromatography	3.0	0.80	1.74	2.18
3)	Retinyl-Sepharose	2.5	0.16	1.00	5.62

1 unit = 1 nmole Retinal formed per min. at 25°C.

Results

Properties of retinol dehydrogenase. When all-trans retinol is added to an aqueous solution (0.1M pyrophosphate buffer, pH 8.6), it forms aggregates which can be separated into two fractions after gel filtration through a Sephadex G-200 column (1.6 x 40cm). One class of aggregates is excluded by the column, whereas the second class of aggregates is retained and eluted with 0.1M pyrophosphate buffer (pH 8.6).

The excluded fraction was found to have a weight average molecular weight higher than 5 x 10^6, whereas the smaller aggregate has a molecular weight of 120,000 when examined by light scattering. Zimm's plot for the aggregate of 120,000 molecular weight is given in Figure 1.

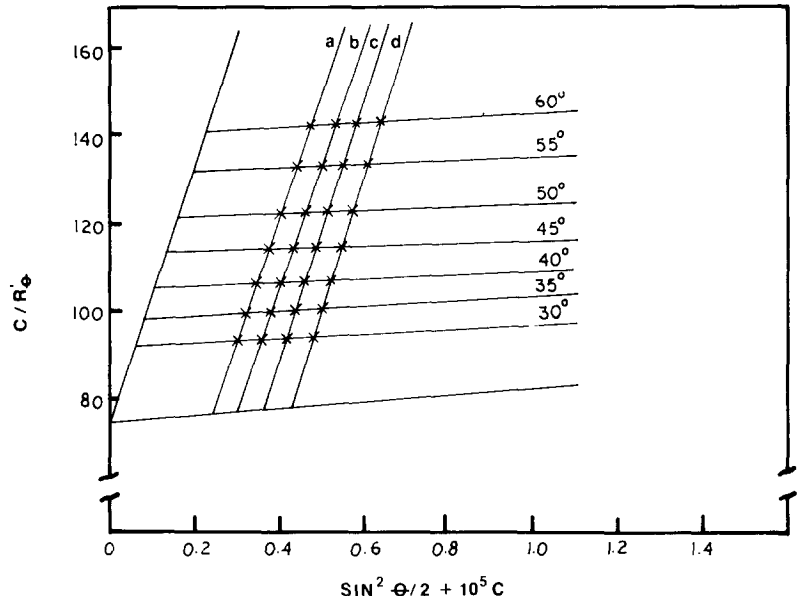

Figure 1. Zimm's plot of the light scattering data obtained with retinol aggregates (Fraction II) after gel filtration through Sephadex G-200. The vertical lines, labeled a, b, c and d represent 2.5, 2.9, 3.5 and 4.4 μgrams of all trans-retinol per milliliter, respectively. For the light scattering measurements the excitation wavelength was 546nm in a Brice Phoenix photometer (series 2000).

TABLE II
RELATIVE ACTIVITIES OF RETINOL DEHYDROGENASE TOWARDS ALL-TRANS RETINOL SOLUBILIZED BY VARIOUS TECHNIQUES

Substrate	Relative Maximum Velocity
1. Retinol (10^{-4}M) in buffer	
A) Retinol Aggregates (Fraction I)	0%
B) Retinol Aggregates (Fraction II)	0%
2. Retinol (10^{-4}M) in detergents	
A) Cholate (0.4%)	17%
B) Cholate (0.4%) + 5mg/ml BSA	25%
C) Lubrol (0.1%)	40%
D) Lubrol (1.0%)	17%
E) Digitonin (0.1%)	35%
F) Digitonin (1.0%)	11%
3. Retinol (10^{-4}M) + protein carrier (5mg/ml)	
A) Bovine serum albumin	100%
B) β-lactoglobulin	90%

(Specific activity of enzyme = 1.0 nmoles/min/mg)

When the partially purified retinol dehydrogenase was assayed for enzymatic activity using as substrates retinol aggregates, it was found that no conversion of retinol into retinal takes place even in the presence of excess $NADP^+$. However, retinol solubilized in either a solution of bovine serum albumin or β-lactoglobulin is a good substrate of retinol dehydrogenase as shown by the results included in Table II.

The presence of detergents in the assay mixture containing bovine serum albumin and retinol must be minimized in order to observe maximum specific activity (Table II). Sodium cholate at concentrations higher than 0.1% tends to displace retinol bound to serum albumin as revealed by fluorescence measurements of bound retinol conducted in the presence of the detergent. It appears that the binding of retinol to bovine serum albumin prevents the formation of substrate aggregates.

Since retinol solubilized in bovine serum albumin yields reproducible rate constants during enzymatic assays, all the measurements designed to determine the kinetic parameters of retinol dehydrogenase were performed in the presence of bovine serum albumin as substrate carrier. The Michaelis

TABLE III
MICHAELIS CONSTANTS FOR THE SUBSTRATES OF RETINOL DEHYDROGENASE

Substrate	Km (μM)
ALL-TRANS Retinol	9.0
ALL-TRANS Retinal	4.0
$NADP^+$	6.0
NADPH	3.5

constants determined graphically from double reciprocal plots are given in Table III. The reaction catalyzed by retinol dehydrogenase is freely reversible and it is characterized by an equilibrium constant of $1.1 \times 10^7 M^{-1}$ at pH 6.5, 25°C.

As shown in Table III, $NADP^+$ and NADPH are substrates of retinol dehydrogenase. The partially purified enzyme is reversibly inactivated by pyridoxal 5-P, but the addition of all-trans retinal followed by reduction with $NaBH_4$ has no effect on the catalytic properties of the enzyme.

Phospholipase inactivation of retinol dehydrogenase. The partially purified enzyme was sensitive to phospholipase A_2. The inactivation by phospholipase A_2 was extremely rapid and always resulted in a loss of approximately 80% of the enzymatic activity. Phospholipase C had little effect on the enzymatic activity and when added to the enzyme in conjunction with phospholipase A_2 resulted in no additional enzymatic inactivation beyond that of phospholipase A_2 alone. Additionally, free fatty acids and lysophospholipids had no effect on the activity of the enzyme. Figure 2 gives the time course of phospholipase inactivation of the dehydrogenase when various mixing ratios of phospholipases are added to the enzyme.

Attempts to reconstitute the enzyme were met with partial success. Crude soy bean phospholipids sonicated with the inactivated dehydrogenase after removal of the lipases could restore approximately 30% of the lost activity.

Discussion. Retinol dehydrogenase has been purified 25 fold from the rod outer segments of bovine retina.

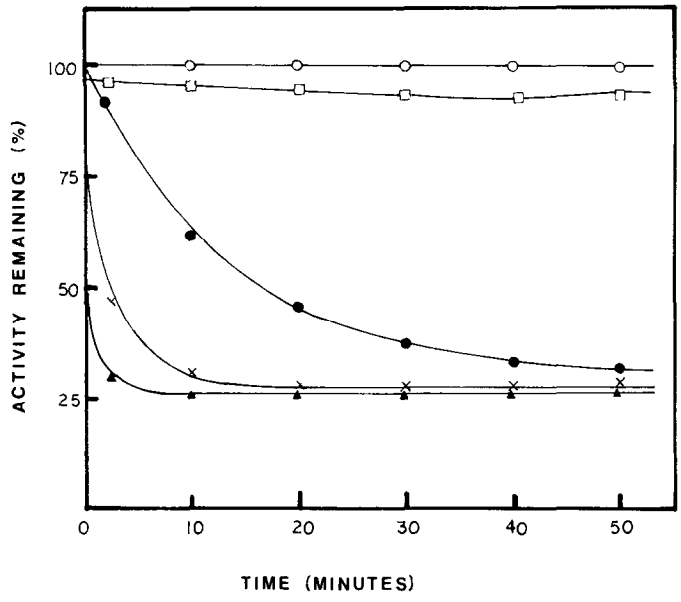

Figure 2. Inactivation of partially purified retinol dehydrogenase by phospholipase A_2 and phospholipase C. Results obtained when 1mg of enzyme was incubated with phospholipase C (5μg) (□), and with phospholipase A_2 (0.2μg) (○), 1μg (X) and 50μg (△) at 37°C. Aliquots removed at the specified time intervals were immediately assayed for enzymatic activity. The results obtained with the enzyme alone preincubated at 37°C are also included in the figure (○).

The finding that retinal solubilized in a protein carrier is the best substrate for the dehydrogenase might reflect the true physiological substrate requirement of the enzyme. Retinal binding proteins have been reported in many tissues (7,8), and recently an 11-cis retinal binding protein has convincingly been demonstrated to exist in bovine rod outer segments (9). The observation that retinol dehydrogenase prefers as a substrate retinol bound to a binding protein, suggests the *in vivo* substrate for the enzyme may be retinol bound to a binding protein. Thus, a molecule of retinol could enter the rod outer segment and be transported by the binding protein to the dehydrogenase, where the chromophore is oxi-

dized to retinal; and subsequently be transported to another acceptor, such as opsin.

The partially purified enzyme is sensitive to the carbonyl reagent pyridoxal-5'-phosphate. This result is consistent with the work of Bonting on whole rod outer segments (10). However, we are unable to inactivate the emzyme with $NaBH_4$ in the presence of excess retinal. We therefore are unable to verify the existence of a retinyl-enzyme intermediate as postulated by Bonting (4).

The importance of the phospholipid environment surrounding retinol dehydrogenase is clearly demonstrated by the sensitivity of the enzyme to phospholipase digestion. Although the enzyme has been removed from its membrane environment, it can still be surrounded by phospholipids in a mixed protein-lipid-detergent micelle (11). The ability of phospholipase A_2 to inactivate this dehydrogenase, while phospholipase C had little effect on enzymatic activity, suggest that the fatty acyl side chains are important in stabilizing dehydrogenase activity.

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