Retinol dehydrogenase from Bovine Retinal Rod Outer Segments

KINETIC MECHANISM OF THE SOLUBILIZED ENZYME*

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Retinol dehydrogenase solubilized by Lubrol 12A9 from bovine retinal rod outer segments forms mixed micelles of Stokes radius 8.5 nm. The kinetic properties of the solubilized retinol dehydrogenase were examined and retinaldehyde reduction and retinol oxidation were seen to proceed at pH 8.3 by a sequential Ordered Bi Bi mechanism. This conclusion was supported by bisubstrate initial velocity studies, dead-end and product inhibition.

The kinetic mechanism of retinol dehydrogenase is not altered by the effect of Lubrol until a concentration of 2 mM is reached, at which the detergent lowers the values of the Michaelis and dissociation constants. The catalytic rate of the retinol dehydrogenase is significantly lowered by detergent in the range of pH 3 to 9.

Retinol dehydrogenase plays an important role in the visual excitation process. Although considerable information about this enzyme has been accumulated (1-8), it has not been possible to accomplish purification of a stable form of the enzyme (9). Retinol dehydrogenase is a structural component of rod outer segments (7, 8); therefore, it cannot be easily separated from the membrane.

We report the principal kinetic parameters of the retinol dehydrogenase solubilized with nonionic detergent Lubrol 12A9. A classic kinetic model for soluble enzymes is considered for retinol dehydrogenase and substrates in micellar phases. The influence of pH and detergent on the kinetic constants obtained is also investigated.

EXPERIMENTAL PROCEDURES

RESULTS

To separate retinol dehydrogenase from membrane components, nonionic and ionic detergents were tested. Lubrol 12A9 appears to be an excellent detergent to solubilize rod outer segment proteins and to extract retinol dehydrogenase in the concentration range of 1.0 to 1.5% (ratio of Lubrol to protein is 0.5 to 0.8).

In the same concentration range Triton X-100 and sodium deoxycholate were less efficient in solubilizing rod outer segment proteins. The activity of retinol dehydrogenase is decreased by 70 to 90% with respect to Lubrol-enzyme at a detergent concentration of 1.5%. Other detergent, sodium dodecyl sulfate, and cetyl trimethylammonium bromide had a dramatic inhibitory effect even at low concentrations.

Physical Properties—In the chromatographic pattern on Bio-Gel A-5m two enzyme peaks were usually observed, a first peak in the void volume together with a major peak of protein and a second peak with the highest retinol dehydrogenase specific activity. The elution position of the smallest aggregate corresponds to an observed Stokes radius of 8.5 nm (Fig. 1). A similar radius value is found on Bio-Gel A-1.5 m.

The sedimentation pattern in sucrose gradients showed a major peak of retinol dehydrogenase between malate dehydrogenase (4.32 S) and alcohol dehydrogenase (5.39 S). The observed sedimentation coefficient calculated by the method of Martin and Ames (22) was 4.5 S. The electropherogram of the enzyme from Bio-Gel A-5m shows three bands, one of which is identified as retinol dehydrogenase by binding of 14C-retinol in the presence of NADP prior to SDS treatment.

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The abbreviation used is: SDS, sodium dodecyl sulfate.
The relative mobility of retinol dehydrogenase compared to standard proteins indicates an enzyme molecular weight of 70,000 (Fig. 2). Locating the enzyme on gel tetrazolium salt staining (23) is not feasible, the retinol dehydrogenase being inactivated by SDS.

Kinetic Studies—The kinetic constants obtained from the initial velocity analysis (Figs. 3 and 4) were in agreement with those calculated from product inhibition data (Tables I and II). As expected for a sequential ordered mechanism, the coenzyme product was a competitive inhibitor of its like substrate (Fig. 5a). Other product inhibitions, namely NADP versus retinaldehyde, were of the noncompetitive type (Fig. 5b).

Dead-end inhibition analysis with retinoic acid shows that a ternary complex E-NADPH-retinoic acid is formed. The dead-end inhibitor is competitive versus retinaldehyde (Fig. 6a) and uncompetitive versus NADPH (Fig. 6b). Retinoic acid is also uncompetitive versus t-retinol in the reverse direction (Fig. 6c) showing that it cannot bind to the E-NADP form. The apparent inhibition constants for dead-end inhibitor obtained from the data are shown in Table II.

pH and Detergent Effects—Retinene reductase activity is lost at both high and low pH (Fig. 7). Changes in pH influence both the catalytic constant and the Michaelis constant $K_a$ and $K_b$. The pH dependence is illustrated in Fig. 9 in the form of log $K_a$ or log $K_b$ versus pH plot with minimum observed $K_a$ or $K_b$ at pH 8 to 9. At 0.65 mM Lubrol two molecular dissociation constants for the free enzyme and for the enzyme substrate complex were determined from Dixon-Webb plots (24). At higher detergent concentration, namely 1.8 mM, no slope variations appeared in the log $V$ and in the log $V/K$ graphs (Figs. 7 and 8). A slight increase of pKel values from 3.8 to 4.15 is observed when the log $V/K$ profile is studied at 1.8 mM Lubrol. The hydrophobic environment created by the detergent can stabilize the unionized form of the enzyme groups (25).

The catalytic constant at all pH values is the parameter most affected by Lubrol 12A9. The detergent also affects the kinetic values for the forward reaction (Table I) but this for the reverse reaction is more difficult to demonstrate.

Rate Constants—Rate constants for retinol dehydrogenase were calculated from millimolar values of the kinetic parameters in Table I (high detergent concentration) as reported by Segel (26) on the assumption of the following Ordered Bi Bi mechanism.
Discussions

Solubilization of sonicated rod outer segments with nonionic detergent Lubrol 12A9 results in a transitory activation of retinol dehydrogenase. The detergent-solubilized retinol dehydrogenase is rather labile, losing its activity within a few hours in ionic and nonionic detergents used for its extraction as studied by earlier workers (7, 8, 27). The relative ability of the detergent to activate retinol dehydrogenase is due to its ability to solubilize the lipid substrate and substitute in part the native hydrophobic interaction of the enzyme.

Zachman and Olson (2) report that the effect of the detergent on hepatic soluble retinene reductase activity is correlated with the physical properties of the micelle Tween-retinaldehyde. The detergent principally affects the dispersion of the substrate rather than enzyme, and then detergent inhibition or retinene reduction by liver alcohol dehydrogenase must be due (increasing the detergent concentration) to a decreasing number of substrate molecules per micelle. The inhibitory effect by Triton X-100 on the activity of the soluble enzyme phospholipase A₂ has been discussed by Dennis (28) in terms of a surface dilution model. According to this model increasing the surfactant concentration dilutes the substrate on the surface of the mixed micelles, and this causes the decreased activity of the enzyme. These considerations have led to a kinetic scheme for membrane-bound phosphatidylserine decarboxylase in which the enzyme first binds to a mixed micelle, Triton-substrate. Then the enzyme becomes part of the mixed micelle and binds the substrate in its activation site (29). Enzymatic properties of retinol dehydrogenase were studied using both substrate and enzyme in the mixed micelle form with Lubrol 12A9. In this system the catalytic rate was strongly dependent on detergent retinaldehyde ratio and on the micellar status of the enzyme. The kinetic constants reported are determined at a presumed molar ratio of 10/1 for Lubrol/retinaldehyde micelles and at a ratio of 0.5 for Lubrol/protein, by weight.

Further increase in the level of Lubrol led to a substantial decrease in the rate of catalysis and to a minor decrease in the values of the kinetic parameters, while the sequential ordered enzyme mechanism remained unaltered.

Although the reduction of the kinetic constants may be interpreted as result of lowered substrate concentrations as a direct consequence of an enzymatic assay in which the catalytic capacity of the enzyme is diminished, we maintain that the substrate rather than enzyme, and then detergent inhibition or retinene reduction by liver alcohol dehydrogenase must be due (increasing the detergent concentration) to a decreasing number of substrate molecules per micelle. The inhibitory effect by Triton X-100 on the activity of the soluble enzyme phospholipase A₂ has been discussed by Dennis (28) in terms of a surface dilution model. According to this model increasing the surfactant concentration dilutes the substrate on the surface of the mixed micelles, and this causes the decreased activity of the enzyme. These considerations have led to a kinetic scheme for membrane-bound phosphatidylserine decarboxylase in which the enzyme first binds to a mixed micelle, Triton-substrate. Then the enzyme becomes part of the mixed micelle and binds the substrate in its activation site (29). Enzymatic properties of retinol dehydrogenase were studied using both substrate and enzyme in the mixed micelle form with Lubrol 12A9. In this system the catalytic rate was strongly dependent on detergent retinaldehyde ratio and on the micellar status of the enzyme. The kinetic constants reported are determined at a presumed molar ratio of 10/1 for Lubrol/retinaldehyde micelles and at a ratio of 0.5 for Lubrol/protein, by weight.

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![Figure 5a](image_url)  
![Figure 5b](image_url)

Fig. 5. a, product inhibition by NADP with NADPH as the varied substrate and t-retinaldehyde concentration 84 μM. Inhibitor concentrations were ○, 0.0 μM; ●, 80 μM; ▲, 160 μM; △, 200 μM. b, product inhibition by NADP with t-retinaldehyde as the varied substrate and NADPH concentration 110 μM. Inhibitor concentrations were ○, 0.0 μM; ●, 80 μM; ▲, 160 μM; △, 200 μM.
FIG. 7. pH variation of V profile for the retinene reductase activity at 0.65 mM Lubrol (○) and at 1.8 mM Lubrol (●). The t-retinaldehyde concentrations ranged from 25 to 113 μM. [NADPH] was 454 μM. pKes₁ and pKes₂, molecular dissociation constants for the enzyme-substrate complex.

The reduction of the kinetic constants is a result of optimum conditions of micellization of the enzyme and lipid substrate which promotes the formation of a more stable EAB complex. Under these conditions, an inhibitory equilibrium that leads to EAB transforming itself to EA and which dissociates to E + A would be avoided.

FIG. 8. pH variation of V/K profile for the retinene reductase activity at low and high detergent concentration. ○, 0.65 mM Lubrol; □, 1.8 mM Lubrol. The t-retinaldehyde concentrations were 25 to 113 μM. [NADPH] was 454 μM. pKe₁ and pKe₂, molecular dissociation constants for the free enzyme.

The proposal of a more detailed kinetic mechanism for retinol dehydrogenase in the micellar state from which the role of the detergent can be deduced must take into consideration the fact that Lubrol 12A9 is not an inert substrate, inactivating catalytic sites, nor an inhibitor inasmuch as it favors hydrophobic interaction at recognition sites.
FIG. 9. pH variation of Michaelis constant for substrate. a, pH variation of $K_a$, t-retinaldehyde was ranged from 25 to 200 µM at constant 80 mM t-retinaldehyde. b, pH variation of $K_b$, t-retinaldehyde was ranged from 25 to 113 µM at constant 0.300 M NADPH.

Acknowledgments—We are indebted to J. R. J. Sutherland for his collaboration in the writing and editing of the manuscript, M. C. Spinto for the careful preparation of the photoreceptor membranes, and M. Valenza for the statistical analysis of the data.

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**EXPERIMENTAL PROCEDURES**

**ROD OUTER SEGMENT PREPARATION**

- Frozen bovine retinas in 5% sucrose-100 mM TRIS-Cl pH 7.4 (w/v) were homogenized in a Potter-Elvehjem homogenizer. The suspension was added to tubes, overlayed with 100 mM Tris-Cl pH 7.4 and centrifuged at 30,000 x g for 10 min. The supernatant was removed.

- The resulting material at the interface was collected and resuspended in 5% sucrose, overlayed with Tris buffer and centrifuged at 20,000 x g for 30 min. The membrane pellets were suspended in 100 mM Tris-Cl pH 8.0, 0.6 M sucrose, and a sharp red band at the top of the gradient was taken, diluted and sedimented.

- All operations were performed in ice cold light.

- **PSEUDO-DENATURED** - Unsolubilized rod outer segments were resuspended in 10 mM phosphate buffer pH 7.0 and the mixture, kept on ice, was used throughout.

- **DETERMINATION OF ENZYME ACTIVITY AT OPTIMAL pH**

- The activity of soluble rod retinol dehydrogenase was assayed spectrophotometrically by monitoring the increase in absorbance at 340 nm due to NADPH oxidation at 25°C. The absorbance was measured in a Beckman DU-10 spectrophotometer.

- **DETERMINATION OF ENZYME ACTIVITY AT OPTIMAL pH**

- The activity of soluble rod retinol dehydrogenase was assayed spectrophotometrically by monitoring the increase in absorbance at 340 nm due to NADPH oxidation at 25°C. The absorbance was measured in a Beckman DU-10 spectrophotometer.

- **EFFECTORS**

- The effectors were incubated with the enzyme at optimal pH for 10 min before the addition of substrate.

- **RESULTS**

- The results are expressed as specific activity (nmol/min/mg protein) and are the average of at least three determinations.

- **DISCUSSION**

- The authors conclude that the solubilized retinol dehydrogenase is a highly active enzyme with a broad pH optimum, making it a good candidate for further study.

**REFERENCES**


