Purification and partial characterization of a rat retina alcohol dehydrogenase active with ethanol and retinol

Pere JULIÀ, Jaume FARRÉS and Xavier PARÉS

*Departament de Bioquímica, Facultat de Ciències i Institut de Biologia Fonamental 'Vicent Villar Palasí',
Universitat Autònoma de Barcelona, Bellaterra, Spain*

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Homogeneous alcohol dehydrogenase (ADH) from rat retina was obtained by chromatography on DEAE-Sephadex and AMP–hexane–Sephadex. The enzyme is a dimer of $M_r \approx 80\,000$ and oxidizes ethanol using NAD^+ as a cofactor. Careful activity determinations demonstrate unambiguously that rat retina ADH is active with retinol as a substrate. This result opens the question about the role of retina ADH in the visual cycle.

Rat retina exhibits a significant alcohol dehydrogenase specific activity as compared with other rat organs (Raskin & Sokoloff, 1972). The presence in the retina of a characteristic alcohol dehydrogenase (ADH) different from the hepatic enzyme was first indicated with the use of starch-gel electrophoresis and ethanol-activity staining (Koen & Shaw, 1966). A partial purification and characterization of the rat retina ADH was reported by Watkins & Tephly (1971).

Since retinol is a good substrate for rat liver ADH (Reynier, 1969), it could be inferred that the retina enzyme would also oxidize vitamin A alcohol. Evidence *in vivo*, based on the effect of pyrazole and ethanol on rhodopsin regeneration in the albino-rat retina, support the involvement of rat retina ADH in retinol mechanism (Raskin *et al.*, 1976). However, studies with a 7.4-fold-purified enzyme preparation detected no activity with retinol when five different enzymic assays were used (Watkins & Tephly, 1971).

We report here the use of affinity chromatography (Parés & Vallee, 1981) to obtain homogeneous rat retina ADH. Enzymic and molecular studies indicate that rat retina ADH has properties similar to those of other known mammalian alcohol dehydrogenases (Brändén *et al.*, 1975). In addition, it is clearly demonstrated that the enzyme catalyses the oxidation of retinol to retinal.

Materials and methods

Purification of rat retina ADH

Sprague–Dawley rats (150–250 g) were used.

Abbreviations used: ADH, alcohol dehydrogenase; SDS, sodium dodecyl sulphate; 'retinol' refers to the all-*trans* isomer; χ -ADH, human liver alcohol dehydrogenase isoenzymes described by Parés & Vallee (1981).

Rats were decapitated and the eyes rapidly removed. Retinas were separated by dissection from cornea, iris, lens and humours and stored at -15°C . In a typical preparation 200 retinas were homogenized with distilled water (1:3, w/v). The homogenate was centrifuged in a Sorvall RC-5B centrifuge at $27\,000g$ for 1 h at 4°C . The supernatant was dialysed against 10 mM-Tris/HCl, pH 7.9, and applied to a column (2 cm $\times$ 18 cm) of DEAE-Sephadex CL-6B equilibrated with the same buffer. The column was washed with 700 ml of 10 mM-Tris/HCl, pH 7.9, and 1 litre of a linear gradient containing from 0 to 0.15 M-NaCl in the same buffer. Fractions exhibiting ADH activity were combined, concentrated, dialysed extensively against 10 mM-Tris/HCl, pH 7.8, and applied to a column (1 cm $\times$ 21 cm) of AMP–hexane–Sephadex equilibrated with the same buffer. The column was washed with 250 ml of this buffer and the fractions showing ADH activity were concentrated and rechromatographed on a second AMP–hexane–Sephadex column under identical conditions. Finally, rat retina ADH was concentrated, dialysed against 0.1 M-Tris/HCl, pH 8.5, and stored at 4°C . The purity of the enzyme preparation was determined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), the silver-stain technique being used to detect the protein bands (Merril *et al.*, 1981). Total protein was measured by the Coomassie Blue method (Bradford, 1976). The M_r was determined by means of chromatography on a column (2.3 cm $\times$ 8.9 cm) of Sephadex G-200 (superfine) (Lange *et al.*, 1976).

Enzymic assay

ADH activity was measured spectrophotometrically at 340 nm by monitoring the production of NADH at 25°C with a Cary 219 instrument.

Standard assay conditions were 0.1M-glycine/NaOH, pH 10.0, containing 1M-ethanol and 4mM-NAD⁺ (grade III; Sigma, St. Louis, MO, U.S.A.) in a total volume of 3 ml. Activities are expressed as units (μmol of substrate transformed/min). Retinol dehydrogenase activity was measured by two different methods.

(a) *Spectrophotometric method.* Retinol (Sigma) was dissolved in 0.1M-glycine/NaOH (pH 10.0)/0.02% Tween 80/4mM-NAD⁺. The enzymic formation of retinal was monitored by recording the increase in A_{400} (Reynier, 1969). Concentrations of retinol were determined from their A_{328} (Reynier, 1969).

(b) *Thiobarbituric acid method.* A reaction mixture was prepared with 0.1M-glycine/NaOH, pH 10.0, 0.02% Tween 80, 107.6 μM -retinol, 2 μg of rat retina ADH, 4mM-NAD⁺, 150 μg of lactate dehydrogenase (Boehringer-Mannheim, Mannheim, Germany) and 5.5mM-sodium pyruvate (Boehringer-Mannheim) in 3.275 ml, and it was incubated at 37°C. A blank, containing all the components except rat retina ADH, was also prepared and incubated at 37°C. At indicated times a 0.2 ml sample was taken from the blank and the reaction mixture and the amount of retinal was measured by the thiobarbituric acid method (Futerman & Saslaw, 1961; Daemen *et al.*, 1970). The spontaneous production of retinal was corrected for by subtracting the blank from the amounts of retinal produced by the enzymic reaction. All-*trans*-retinal (Sigma) served as a standard.

Results and discussion

Purification and molecular properties of rat retina ADH

The results of a typical purification procedure of rat retina ADH are presented in Table 1. The enzyme can be successfully isolated under conditions similar to those used for the purification of human χ -ADH isoenzymes (Parés & Vallee, 1981). However, in contrast with the χ -ADH behaviour, rat retina ADH does not bind strongly to the Sepharose-hexane-AMP column, but it is only retarded with regard to the major protein peak (Fig. 1).

When the active fractions are rechromatographed on the same affinity column and under identical conditions, a pure preparation is obtained. Only a single band was observed on SDS/polyacrylamide-gel electrophoresis (Fig. 2, inset), and a single protein peak was detected on Sephadex G-200 chromatography (result not shown).

Like all known mammalian alcohol dehydrogenases, rat retina ADH has an M_r of about 80 000 as indicated by gel filtration (Fig. 2). Gel electrophoresis under denaturing conditions demonstrates that the enzyme is a dimer with subunits of M_r about 40 000 (Fig. 2, inset).

Subcellular localization of rat retina ADH

Rat retinas (23 in all) were homogenized in 16 ml of 0.25 M-sucrose/10mM-Tris/HCl, pH 7.6 and the suspension was centrifuged in a Beckman L8-70 ultracentrifuge at 100 000g and 4°C for 1 h; 90% of the ADH activity was detected in the supernatant, demonstrating that rat retina ADH, like liver ADH, is a soluble enzyme.

Rat retina ADH activity with ethanol and retinol as substrates

Spectrophotometric methods were used to monitor the ethanol dehydrogenase and retinol dehydrogenase activities through the purification procedure. Both activities co-purified in all separation steps (Fig. 1). The enzyme required NAD⁺ as a cofactor for activity with both substrates. NADP⁺ was not effective under the same conditions.

Since other authors have indicated that rat retina ADH was not active with retinol (Watkins & Tephly, 1971), it was necessary to confirm our observations in order to rule out any possible artefact in the measurement of absorbance variations. Therefore we showed that the spectrophotometric change observed was directly proportional to the amount of enzyme added to the reaction mixture over the concentration range used (5–31.5 nM), with a substrate concentration of 84.7 μM -retinol (results not shown). The thiobarbituric acid method was used as an additional system to determine the retinol dehydrogenase activity of the enzyme.

As indicated in the Materials and methods section, a reaction mixture was prepared with

Table 1. *Purification of rat retina alcohol dehydrogenase*
Alcohol-oxidizing activity was determined by using the standard assay described in the Material and methods section.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Supernatant	233.75	11	0.047	1	100
DEAE-Sepharose	8.78	4.65	0.53	11.3	42.2
First Sepharose-hexane-AMP	0.51	2.7	5.29	112.5	24.5
Second Sepharose-hexane-AMP	0.046	1.4	30.43	647.4	12.7

Fig. 1. First chromatography of rat retina ADH on Sepharose-hexane-AMP

The major protein peak and the activity peak were eluted separately by washing with 10mM-Tris/HCl, pH 7.8. Fractions (10ml) were collected. The standard assay was used to measure ethanol dehydrogenase activity (●). Retinol dehydrogenase activity (○) was determined by the spectrophotometric method with 150 μ M-retinol. ▲, A_{280} .

Fig. 2. M_r determination of rat retina ADH by chromatography on Sephadex G-200

Proteins were eluted with 0.1M-Tris/HCl buffer, pH 7.5 at 4°C (Lange *et al.*, 1976); the inset shows the silver-stained rat retina ADH after SDS/polyacrylamide-gel electrophoresis.

enzyme, retinol and NAD^+ at pH 10.0. To accelerate the reaction, the oxidation of retinol was coupled to the reduction of pyruvate through the addition of lactic dehydrogenase and pyruvate to oxidize NADH (Futerman & Saslaw, 1961). Under these conditions the thiobarbituric acid method shows the formation of retinal as a function of the incubation time (Fig. 3). The amount of retinal produced was linear with time for the first 6 h. Later, a decrease in rate was observed, probably because of the accumulation of products or denaturation of the enzyme.

Fig. 3. Oxidation of retinol by rat retina ADH

A reaction mixture containing retinol and enzyme was incubated under the conditions indicated in the Materials and methods section. At intervals 0.2ml samples were removed and the amount of retinal was determined by the thiobarbituric acid method.

The results shown demonstrate unambiguously that retinol is a substrate of rat retina alcohol dehydrogenase. Therefore the enzyme may play a role in the visual-cycle reactions that involve oxidation of retinol to retinal, i.e. the synthesis of the visual chromophore from blood retinol (Bridges & Yoshikami, 1969) and the regeneration of retinal in the darkness after bleaching illumination (Dowling, 1969; Raskin *et al.*, 1976).

Our findings agree with the previous detection of a NAD-dependent retinol dehydrogenase in rat retina homogenate (Reading & Sorsby, 1966; Koen & Shaw, 1966), and they clearly support the results of experiments *in vivo* performed by Raskin *et al.* (1976). The absence of retinol-oxidizing activity in rat retina ADH preparations, previously reported by other authors (Watkins & Tephly, 1971), can be explained by the low purity of their enzyme, and by the low activity detected with retinol as compared with ethanol (Fig. 1).

In conclusion, we have purified a soluble NAD^+ -dependent alcohol dehydrogenase from rat retina that oxidizes retinol. The enzyme is different from the better-known $NADP^+$ -dependent and membrane-linked retinol dehydrogenase (Futerman, 1963; Lion *et al.*, 1975; Blaner & Churchich, 1980; Nicotra & Livrea, 1982). The two enzymes may have complementary roles in the visual-cycle reactions. However, the relative importance of the retina ADH function will only be understood after an extensive study of its kinetic properties.

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