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RETINOID METABOLISM AND CONVERSION OF RETINOL TO DEHYDRORETINOL IN THE CRAYFISH (*PROCAMBARUS CLARKII*) RETINA

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Abstract

1. Activities of retinylester hydrolase (REHase) and retinol dehydrogenase (RDHase) were detected in the crayfish retina which has two visual pigment chromophores and three stored retinoids.
2. RDHase and 11-*cis* REHase activities were localized to rhabdomal fraction and enzymes were specific to 11-*cis* isomers of substrates. Overall reaction from retinylester to retinal was highly specific to 11-*cis* isomers and this reaction was not selective between retinol and dehydroretinol.
3. When 11-*cis* [³H]-retinol was incubated with retina homogenates, radioactivity was detected in 11-*cis* dehydroretinol.
4. These results suggest that retinol is converted to dehydroretinol after hydrolysis of retinylesters.

INTRODUCTION

The crayfish, *Procambarus clarkii* is a unique invertebrate that has the rhodopsin–porphyropsin visual pigment system (Suzuki *et al.*, 1984; Hariyama and Tsukahara, 1988; Zeiger and Goldsmith, 1989). The proportion of porphyropsin increases in response to low temperature and disappears at high temperature (Suzuki *et al.*, 1985). 3,4-Didehydroretinal (dehydroretinal), the chromophore of porphyropsin is derived from dehydroretinol stored as esters in the retina (Suzuki *et al.*, 1988), and this dehydroretinol may be synthesized from retinol in the retina. Additional retinoid, 3-hydroxyretinol, was found in the retina and it is probably an intermediate of the retinol–dehydroretinol conversion (Suzuki and Miyata, 1991). The proportion of dehydroretinal in the chromophores is far higher than that of dehydroretinol in stored esters (Suzuki *et al.*, 1988, 1991). This discrepancy of retinoid

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composition might be explained by one of two possible mechanisms; (1) enzymes producing the chromophores favor the formation of dehydroretinoids, (2) retinol is converted to dehydroretinol after hydrolysis of retinyl esters. In order to examine these possibilities, we investigated two enzymes, retinylester hydrolase (REHase) and retinol dehydrogenase (RDHase). We also studied the conversion of retinol to dehydroretinol.

MATERIALS AND METHODS

The crayfish (*Procambarus clarkii*) were purchased from a local dealer and kept at 10°C in the dark until use. These conditions induced the accumulation of dehydroretinol and porphyropsin in the retina. The eyestalks were cut from animals, and the retinas were isolated from the crystalline cone layer, homogenized in distilled water and used for enzyme assay. In the experiment of enzyme localization, retinal tissue was separated into three fractions (soluble, rhabdomal and precipitate) by the centrifugation of retina homogenate on a sucrose-density gradient (10–60%) as reported previously (Suzuki *et al.*, 1988).

Enzyme assay

All-*trans* and 11-*cis* retinyl [³H]-palmitates were synthesized by reacting retinols with the anhydride of [³H]-palmitic acid as described (Prystowsky *et al.*, 1981) and purified by high-performance liquid chromatography (HPLC). For REHase assays the substrate concentration was adjusted to 2 nmol/10 µl ethanol (sp. act. 20.3 nCi/nmol for all-*trans* and 13.2 nCi/nmol for 11-*cis* retinyl [³H]-palmitate). The standard incubation mixtures consisted of 20–40 µg protein of retina homogenate, 10 µM substrate and 50 mM Tris–maleate buffer (pH 8.0) in a final reaction volume of 200 µl. Incubations were conducted at 25°C for 1 hr under dim light. After the incubation, [³H]-palmitic acid liberated by enzyme reaction was extracted as described by Belfrage and Vaughan (1969). One milliliter of the extract was mixed with 5 ml of Aquasol 2 (Dupont) and radioactivity was measured with a liquid scintillation counter (Packard 2500TR).

Retinols and dehydroretinols (11-*cis* and all-*trans*) were purified by HPLC after reducing the corresponding retinals by NaBH₄. The concentration of retinols was adjusted to 2 nmol/10 µl ethanol. The RDHase activity was assayed by the method which converts aldehydes to oximes (Terakita *et al.*, 1991). The standard incubation mixture consisted of 50 mM Tris–maleate buffer (pH 8.0), 100 mM NH₂OH, 10 µM substrate and 60–70 µg protein of tissue homogenate in final volume of 200 µl. The mixture was incubated at 25°C for 1 hr under dim light and the reaction was terminated by addition of 0.5 ml cold ethanol. Retinaloximes were extracted with hexane and quantified with HPLC as previously described (Makino-Tasaka and Suzuki, 1986).

Non-radioactive retinyl (and dehydroretinyl) palmitates were synthesized from corresponding alcohols and palmitoyl chloride and purified by HPLC. Overall reaction (REHase + RDHase) was assayed in the same reaction mixture as for RDHase using retinyl and dehydroretinyl palmitates (10 µM) as substrates instead of retinols. The products, retinaloximes, were extracted and analyzed as above.

Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Conversion of retinol into dehydroretinol and 3-hydroxyretinol

11-*Cis* [^3H]-retinol was prepared from all-*trans* [11,12(n)- ^3H]-retinol (Amersham, Bucks, UK) using the isomerase activity of retinochrome (Hara and Hara, 1984). All-*trans* [^3H]-retinol (100 $\mu\text{Ci}/20\text{ nmol}$) was dissolved in 1 ml hexane and passed through an activated MnO_2 (1 g) column to oxidize to retinal. The retinal solution was evaporated and the residue was dissolved in 25 μl ethanol. The retinal was dispersed in 1 ml of 0.5% digitonin solution containing *ca* 0.2 nmol retinochrome, 50 mM phosphate buffer (pH 6.5) and 100 mM ascorbate and irradiated with orange light. The photoproducts (main component 11-*cis* isomer) were reduced with NaBH_4 , extracted with petroleum ether, and 11-*cis* retinol was purified by HPLC. The concentration of 11-*cis* [^3H]-retinol was adjusted to 10 nmol/10 μl (sp. act. 23 nCi/nmol) by addition of non-radioactive 11-*cis* retinol.

Incubation mixture was 100 μl of 100 mM Tris-HCl (pH 8.0, final 50 mM), 80 μl tissue homogenate (about 0.5 mg protein), 10 μl of 20 mM NADPH and 10 μl of 1 mM 11-*cis* [^3H]-retinol in ethanol in a final volume of 200 μl . The mixture was incubated at 25°C for 3 hr under dim light and the reaction was terminated by addition of cold 0.5 ml methanol. Non-radioactive standard mixture (1.0 nmol each of all-*trans* retinol, 11-*cis* retinol, all-*trans* dehydroretinol, 11-*cis* dehydroretinol, all-*trans* 3-hydroxyretinol, 11-*cis* 3-hydroxyretinol) was added to the sample and, after addition of 1 ml distilled water, total retinoids were extracted with petroleum ether three times. The extracts were combined, evaporated under a stream of N_2 , and the residue dissolved in 200 μl HPLC solvent. Retinoids were separated by HPLC as reported previously (Suzuki and Miyata, 1991). A 100 μl aliquot was injected into HPLC equipped with a silica gel column (Waters) and retinols and dehydroretinols were separated with 6% dioxane/hexane at flow rate of 1.5 ml/min. Absorbance at 330 nm was monitored with a UV-detector and recorded. HPLC eluates for every 15 sec were collected into 4 ml mini-vials and mixed with 3 ml Aquasol 2, and radioactivity was determined with a liquid scintillation counter. 3-Hydroxyretinols were separated with an elution solvent of 20% dioxane/1% ethanol/hexane with the same HPLC using a residual 100 μl aliquot of the extract, and UV-absorbance and radioactivity were determined as above.

RESULTS

Characterization of REHase and RDHase

The first step of retinoid metabolism for chromophore formation is the hydrolysis of stored retinyl ester to retinol. In order to eliminate the contribution of retinylester synthetase reaction, we used retinyl [^3H]-palmitate as substrate and liberated [^3H]-palmitic acid was determined.

The retina REHase showed interesting pH-dependencies. When all-*trans* retinyl palmitate was used as substrate, the enzyme activity showed a single peak and the optimum pH was 5. On the contrary, two activity peaks were found when 11-*cis* retinyl palmitate was used as substrate, around at pH 5 and pH 8 (Table 1). Hydrolysis was abolished by heat treatment of

homogenate (90°C, 3 min). These results suggest that the preparation is the mixture of two species of REHase, one active at pH 5 may be non-specific ester hydrolase and another with activity peak at pH 8 is specific to retinoid metabolism.

Rhabdomyosarcoma and associated membranes are structures important to retinoid metabolism, because oil droplets containing retinylesters closely associated with the rhabdomyosarcoma structure (Suzuki *et al.*, 1988). Three subcellular fractions were obtained from retina homogenates, and REHase was determined for 11-*cis* retinyl palmitate at pH 8.0. The highest specific activity was found in the rhabdomyosarcoma fraction (Table 2). The isolated rhabdomyosarcoma were treated with small volume of hypotonic buffer (20 mM Tris-HCl, pH 8.0) and further divided into two fractions (membranous and soluble protein fractions) by centrifugation at 100,000 g for 60 min. High REHase activity (18.5 nmol/mg/hr) was found in the precipitate but no detectable activity in the supernatant. These results indicate the REHase is a membrane-associated protein localized to rhabdomyosarcoma and related membranes.

Dehydrogenation (oxidation) of retinols is the next step of retinoid metabolism for chromophore production. Hydroxylamine was used to trap the products, retinals, to prevent backward reaction and to reduce stereoisomerization which easily occurs without NH₂OH. Effects of NADP, co-factor of dehydrogenases, on the reaction were investigated. Only a slight acceleration effect was observed in the presence of 1 mM NADP under the present experimental conditions. Therefore, the dinucleotide was not added to the reaction medium.

The RDHase showed a single broad peak of activity, maximum at pH 8.0, which had nearly the same optimum pH as REHase. The activity was lost after the heat treatment of homogenate (90°C, 3 min). Substrate specificity of RDHase was studied using 11-*cis* and all-*trans*-retinyl palmitates. RDHase was specific to 11-*cis* isomer (Table 1).

Subcellular distribution of RDHase was studied by preparing three fractions; water soluble, rhabdomyosarcoma and heavy precipitate fractions. The highest specific activity was found in rhabdomyosarcoma fraction, indicating that RDHase is concentrated in rhabdomyosarcoma and related membranous structures (Table 2).

Overall reaction (REHase + RDHase)

Substrate selectivity of two successive reactions (REHase and RDHase) was examined by using retinyl palmitates as substrates and then determining retinaloximes as final products. The results are shown in Fig. 1. The overall reaction was highly specific to 11-*cis* isomers and activity for all-*trans* isomers was hardly detected. However, there was not a significant difference in specificity between 11-*cis* retinyl and 11-*cis* dehydroretinyl palmitates.

Incorporation of radioactivity to dehydroretinol and 3-hydroxyretinol

In order to examine the conversion of retinol to 3-hydroxyretinol and/or dehydroretinol, 11-*cis* [³H]-retinol was incubated with retina homogenate and products were separated by HPLC. Figure 2 shows the radioactivities of HPLC eluates. During the incubation and extraction procedures, considerable amount of 11-*cis* retinol was isomerized to 13-*cis* and all-*trans* isomers. This stereoisomerization of retinol was observed also in the control experiments using heat-denatured preparation. Considerable radioactivity was found in the

fractions of 11-*cis* dehydroretinol when 11-*cis* [³H]-retinol was incubated with retinal homogenate but this was not observed in control using heat-denatured proteins. Time course of the radioactivity of 11-*cis* dehydroretinol was examined in additional experiments, and it was found that the activity increased with time up to 3 hr (data not shown). These results indicate that retinol was converted to dehydroretinol by the action of an enzyme.

3-Hydroxyretinols were separated by HPLC and radioactivities were determined as above. Small amount of radioactivity was found at the position of 11-*cis* 3-hydroxyretinol and the peak was observed to be thermally labile. The amount of radioactivity associated with 3-hydroxyretinol remained constant during the incubation up to 3 hr. The level of radioactivity, however, was too low to establish unequivocally the formation of 3-hydroxyretinol.

DISCUSSION

In the crayfish retina, retinol and dehydroretinol are stored in the esterified form and most of them are in the 11-*cis* configuration (Suzuki *et al.*, 1988). Chromophores of visual pigments, retinal and dehydroretinal, must be derived from these retinyl esters by two enzyme reactions governed by REHase and RDHase. In the present study we detected both enzyme activities which are specific to 11-*cis* isomers of retinoids. Little was known about these enzyme activities in invertebrate retina in contrast to many studies in retinal pigment epithelium (RPE) of vertebrate.

In bovine RPE, Berman *et al.* (1985) reported that REHase is localized to the lysosomal fraction and that the activity was highest at pH 4.0–4.5 with low specificity for retinyl- and fatty acyl-esters. Blaner *et al.* (1987) used human RPE homogenate, and reported that REHase has an activity peak at pH 8.5 and was specific to 11-*cis* isomer. REHase activities at pH 8 were reported also in bovine RPE and neural retina by Tsin and Malsbury (1991). The different results reported on REHase are probably due to the different preparation and different assay conditions. Mammalian RPE seems to contain two esterhydrolases, one specific to retinoid metabolism and another lysosomal, non-specific esterhydrolase. If the crayfish retina had lysosomal, non-specific esterhydrolase, besides REHase, specific to 11-*cis* retinyl esters, the two peaks of REHase activity at pH 8 and 5 could be reasonably explained.

The properties of RDHase activity in the crayfish retina are similar to those reported in vertebrate retina and RPE; specific to 11-*cis* isomer and pH optimum near 8. (Lionet *et al.*, 1975; Saari and Bredberg, 1982; for review, Bridges, 1984). In the present experiment, NADP had little effect on RDHase activity in contrast to the analogous reaction in vertebrate RPE. In our experiment, hydroxylamine in a high concentration was used to trap retinals produced by enzymatic reaction. This is one of the possible reasons for dinucleotide independence.

Both REHase and RDHase showed highest specific activity in rhabdomal fraction (Table 2), and further, in sub-rhabdomal membrane fraction. As reported previously, oil droplets containing retinyl esters are closely associated with rhabdom structure in the retinula cell

(Suzuki *et al.*, 1988). The structural relationship between stored sites of esters and enzyme localization seems to be reasonable for efficient formation and transport of the chromophores of the visual pigment.

An important property noticed is the substrate specificity to retinol and dehydroretinol, because the proportion of dehydroretinal in the chromophores is far higher than that of dehydroretinol in stored ester. The results of Fig. 1 show that the reactions producing chromophores from stored esters are selective for 11-*cis* isomers but not specific towards dehydroretinol. Therefore, the discrepancy of retinoid composition between that in chromophores and that in stored ester cannot be explained simply by the substrate specificity of the two enzymes.

A possible mechanism to explain the discrepancy of retinoid composition is the conversion of retinol to dehydroretinol after the hydrolysis of retinyl esters. The results of Fig. 2 show that retinol was converted to dehydroretinol by enzyme action of retina homogenate.

3-Hydroxyretinol found in the stores esters was considered, for *in vivo* studies, to be an intermediate in the retinol-dehydroretinol conversion (Suzuki and Miyata, 1991). In the present study, the radioactivity incorporated into 3-hydroxyretinol was very low and the result was not completely conclusive. However, if the 3-hydroxyretinol was indeed the intermediate, it is reasonable to expect this low activity level of hydroxyretinol. The conversion of retinol to dehydroretinol was also observed in tadpole retina (Tsin *et al.*, 1985), but there has been no information on the involvement of 3-hydroxyretinol in the retinol-dehydroretinal conversion in the vertebrate retina.

In conclusion, the chromophores of visual pigments in the crayfish retina are produced by the actions of two enzymes, REHase and RDHase, which show no selectivity for dehydroretinol. Retinol is converted into dehydroretinol after the hydrolysis of stored retinyl esters and this conversion may be responsible for the difference in retinoid composition between that in chromophores and that in stored esters. 3-Hydroxyretinol is probably the intermediate of the retinol-dehydroretinol conversion.

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Fig. 1. Substrate specificity of overall reaction (REHase + RDHase). These activities were determined at pH 8.0 using retinyl palmitates as substrates and retinaloximes as final products. Cis1, 11-*cis* retinyl palmitate; Cis2, 11-*cis* dehydroretinyl palmitate; AT1, all-*trans* retinyl palmitate; AT2, all-*trans* dehydroretinyl palmitate. Mean $\pm$ SD ($N = 3$) are shown.

Fig. 2.
HPLC profiles of radioactive retinoids derived from [^3H]-retinol. 11-*cis*[^3H]-retinol was incubated with retina homogenate at pH 8.0 for 3 hr, and the products were separated by HPLC. Boiled homogenate (90°C, 3 min) was used as control (-♦-). One representative is shown. Elution positions of standard retinoids are indicated by arrows: 11₁, 11-*cis* retinol; 13₁, 13-*cis* retinol; 11₂, 11-*cis* dehydroretinol; AT₁, all-*trans* retinol; AT₂, all-*trans* dehydroretinol.

Table 1

Activities of REHase and RDHase in crayfish retinal homogenate

	Substrate isomer		
	PH	11- <i>cis</i>	All- <i>trans</i> (pmol/mg/hr)
REHase	5.0	5447 ± 149	6098 ± 277
	8.0	9155 ± 513	408 ± 144
RDHase	8.0	1239 ± 89	310 ± 19

Enzyme activities were assayed using retinyl [³H]-palmitates for REHase and retinols for RDHase as substrate as in Materials and Methods. Mean ± SD (*N* = 3) are represented.

Table 2

Subcellular distribution of REHase and RDHase

	Soluble	Rhabdomal	Heavy precipitate (pmol/mg/hr)
REHase	1884 ± 217	11341 ± 616	1768 ± 166
RDHase	144 ± 24	3695 ± 103	566 ± 117

Three fractions were obtained by sucrose-density gradient centrifugation and specific activities were determined at pH 8.0 using 11-*cis* retinyl[³H]-palmitate for REHase and 11-*cis* retinol as substrate as in Materials and Methods. Mean ± SD (*N* = 3) are represented