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Source: Zoological Science, 19(2) : 191-195
Published By: Zoological Society of Japan
URL: https://doi.org/10.2108/zsj.19.191
Identification of 3,4-Didehydroretinal Isomers in the *Xenopus* Tadpole Tail Fin Containing Photosensitive Melanophores

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**ABSTRACT**—It is well characterized that melanophores in the tail fin of *Xenopus laevis* tadpoles are directly photosensitive. In order to better understand the mechanism underlying this direct photosensitivity, we performed a retinal analysis of the tail fins and eyes of *Xenopus* tadpoles at stages 51-56 using high performance liquid chromatography (HPLC). Following the extraction of retinoids by the formaldehyde method, a fraction containing retinal and/or 3,4-didehydroretinal isomers from the first HPLC analysis were collected. These isomers were then reduced by sodium borohydride to convert retinal and/or 3,4-didehydroretinal isomers into the corresponding retinol isomers to prepare for a second HPLC analysis. Peaks of 11-cis and all-trans 3,4-didehydroretinol were detected in the eyes and tail fins containing melanophores, but they were not detected in the tail fins without melanophores. The amounts of 11-cis and all-trans 3,4-didehydroretinol were 27.5 and 5.7 fmol/fin, respectively, and the total quantity of 3,4-didehydroretinal was calculated at approximately 5×10⁶ molecules/melanophore. These results strongly suggest the presence of 11-cis and all-trans 3,4-didehydroretinal in melanophores of the tadpole tail fin, which probably function as the chromophore of photoreceptive molecules.

**Key words:** *Xenopus*, tadpole tail fin, photosensitive melanophore, chromophore of photoreceptive molecule, 3,4-didehydroretinal

**INTRODUCTION**

Lower vertebrates capable of changing their apparent body color, have many melanophores in the dermis of the skin (Bagnara and Hadley, 1973; Weber, 1983). This response on color change is based on the aggregation and dispersion of melanosomes, which occur as a result of hormonal influences, in the dermal melanophores of amphibians (Bagnara, 1960; Bagnara, 1963; Bagnara and Hadley, 1973; Rollag, 1988). However, melanophores in the tail fin of the late-stage (stages 51–56) *Xenopus* tadpole, respond to light directly; melanosomes aggregate with exposure to light even when the tail fins are isolated from the bodies, (Bagnara, 1957; van der Lek et al., 1958; Bagnara and Hadley, 1973; Moriya et al., 1996) or when the melanophores are cultured *in vitro* (Seldenrijk et al., 1979). Lythgoe and Thompson (1984) suggested the presence of a porphyrin-like photopigment in the isolated tail fin of the *Xenopus* tadpole based on the shape of the action spectrum, and Moriya *et al.* (1996) reported a peak in the action spectrum of the melanosome aggregation at 500 nm. In contrast to the melanophores in the tail fin of the late-stage tadpole, cultured pigment cells derived from the *Xenopus* embryo showed a light-induced dispersion of melanosomes with a peak in the action spectrum at approximately 460 nm (Dan-
HPLC analysis of retinoids

The extraction of retinoids was carried out using the formaldehyde method (Suzuki et al., 1986) with a minor modification according to Seki et al. (1987, 1994). Briefly, the isolated tissues were homogenized with a high-speed homogenizer (Physocotron, Nichion Irikkakai, Tokyo, Japan) in an equivalent volume of 20 mM Tris-HCl (pH 7.4) containing 2 volumes of formaldehyde. The homogenate was mixed with dichloromethane using a pipette, kept at room temperature for 5 min, and mixed with isopropanol and n-hexane. The mixture was centrifuged at 1,500 g and 4°C for 10 min, and a vacuum was used to evaporate the collected upper layer (dichloromethane/n-hexane layer). Formaldehyde, dichloromethane and n-hexane were added to the lower water layer and the mixture was centrifuged again. This extraction step was repeated twice. All the extract was collected in dichloromethane, washed twice with distilled water to remove the formaldehyde, and evaporated to dryness with argon. For HPLC analysis as described below, the extract was dissolved in the eluent comprised of 5% tert-butylmethyl ether, 25% benzene, 0.04% ethanol in n-hexane.

HPLC analysis was carried out with a pump (Hitachi 655, Hitachi Co., Ltd., Tokyo, Japan) at a flow rate of 2 ml/min equipped with a normal phase silica gel column (6x150 mm; YMC-Pack A-012-3 S-3 SIL, Yamamura Chemical Laboratories Co. Ltd., Kyoto, Japan). Two UV-detectors (JASCO 875-UV and JASCO UV-970, Japan Spectroscopic Co. Ltd., Tokyo, Japan) were used to monitor the absorbance at 330 and 360 nm, and the chromatogram was recorded with a two-channel integrator (Labchart 80, System Instruments Co., Ltd., Tokyo, Japan).

In the analysis of extra-ocular tissue, an abundance of unknown substances generally interfered with the detection of peaks of retinal congeners. Therefore, the fraction containing all geometrical isomers of RAL1 and RAL2 from the first HPLC analysis (retention time for 3–6 min) was collected, evaporated, and reduced with a small amount of sodium borohydride (NaBH₄) in ethanol to convert retinal congeners into retinol congeners (Bridges and Alvarez, 1982). The retinol congeners collected with organic solvent were then re-chromatographed under the same conditions described above.

HPLC analysis was first performed for the extract from tail fins without melanophores, second from tail fins with melanophores, and finally from eyes to avoid retinoid contamination. All procedures were carried out under dim red lighting (>610 nm).

Standard 3,4-didehydroretinal and 3,4-didehydroretinol isomers

Following the purification of all-trans RAL2 from crystals using HPLC, an aliquot of all-trans RAL2 in ethanol was reduced to 3,4-didehydroretinol (ROL2) with NaBH₄ (Bridges and Alvarez, 1982). To produce 11-cis RAL2, another aliquot of all-trans RAL2 was added to a membrane preparation of apo-retinochrome in phosphate buffer and exposed to red light exceeding 580 nm for 15 min (Seki et al., 1980). The resulting 11-cis RAL2 was collected using HPLC, according to the conditions mentioned above, following

### Table 1. Ratios of the peak height at 330 nm to that at 360 nm (H330/H360) as detected by successive UV-detectors to monitor the elution profiles of standard samples for HPLC. The eluent is comprised of 5% tert-butylmethyl ether, 25% benzene, 0.04% ethanol in n-hexane.

<table>
<thead>
<tr>
<th></th>
<th>H330/H360</th>
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<tbody>
<tr>
<td><strong>RAL1</strong></td>
<td></td>
</tr>
<tr>
<td>cis</td>
<td>0.75±0.02</td>
</tr>
<tr>
<td>trans</td>
<td>0.62±0.04</td>
</tr>
<tr>
<td><strong>ROL1</strong></td>
<td></td>
</tr>
<tr>
<td>cis</td>
<td>4.37±0.07</td>
</tr>
<tr>
<td>trans</td>
<td>0.93±0.03</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Animals and tissues**

*X. laevis* tadpoles were maintained under an LD cycle at 22–25°C and fed adult *Xenopus* bristle until they grew to 30–50 mm in length (stages 51–56; Nieuwkoop and Faber, 1956). *Xenopus* tadpoles (n=644) were then subjected to darkness for more than 3 hr at 25°C and fed adult *X. laevis* tadpoles were maintained under an LD cycle at 22–25°C until they grew to 30–50 mm in length (stages 51–56; Nieuwkoop and Faber, 1956). *Xenopus* tadpoles (n=644) were then subjected to darkness for more than 3 hr at 25°C and fed adult *X. laevis* tadpoles were maintained under an LD cycle at 22–25°C until they grew to 30–50 mm in length (stages 51–56; Nieuwkoop and Faber, 1956). *Xenopus* tadpoles (n=644) were then subjected to darkness for more than 3 hr at 25°C and fed adult *X. laevis* tadpoles were maintained under an LD cycle at 22–25°C until they grew to 30–50 mm in length (stages 51–56; Nieuwkoop and Faber, 1956).

In the present study, retinal congeners extracted by a formaldehyde method (Suzuki et al., 1986) from the tail fin of *Xenopus* tadpoles were analyzed with high performance liquid chromatography (HPLC), and the presence of 11-cis RAL2 was demonstrated in the tail fin containing photosensitive melanophores.

**ROL1** 4.37 ± 0.07 (n=10)
**ROL2** 0.93 ± 0.03 (n=10)
extraction by the formaldehyde method. An aliquot of purified 11-cis RAL2 was reduced to 11-cis ROL2 with NaBH₄. To produce 9-cis and 13-cis RAL2 isomers, all-trans RAL2 dissolved in ethanol was exposed to light exceeding 440 nm (Tsukida et al., 1980).

The absorption spectrum of each purified RAL2 and ROL2 isomer in ethanol was measured with a spectrophotometer (Hitachi U-3200, Hitachi Co., Ltd., Tokyo, Japan) to determine the concentration based on molar extinction coefficient at the absorption maximum (Tsukida, 1979). For the quantitation of RAL2 and ROL2 isomers using HPLC, different amounts of respective standard samples were applied to HPLC and the peak area for one pmol of each standard sample was obtained from the slope of each regression line with an intercept of 0. For the identification of a peak substance on the chromatograms, a ratio of the peak height at 330 nm to that at 360 nm (H330/H360) was compared with the H330/H360 value for each standard sample (Table 1).

**RESULTS**

Fig. 1 shows HPLC chromatograms of retinoid extracted by the formaldehyde method from the tail fins (A) and eyes (B) of *Xenopus* tadpoles. Based on the elution profile and retention time, peaks 1, 2, and 3 in Fig. 1B were identified as 11-cis, 9-cis and all-trans isomers, respectively, of RAL1 or RAL2. For further identification of the peak substances, the H330/H360 value for peaks 1 (0.57) and 3 (0.43) in Fig. 1B were compared with the H330/H360 values in the text.

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of standard 11-cis and all-trans isomers shown in Table 1. The peak substances were not assigned to 11-cis and all-trans RAL1 (H330/H360 values 0.75 and 0.63, respectively), but instead attributed to 11-cis and all-trans RAL2 (H330/H360 values 0.62 and 0.47, respectively).

In contrast to the obvious RAL2 peaks in the extract from the eyes (Fig. 1B), large peaks interfered with the peak identification of retinal congeners extracted from the tail fins containing melanophores (Fig. 1A). The H330/H360 values of peaks at retention times 3.8 and 5.1 min, shown in Fig. 1A, were 1.74 and 1.04, respectively, indicating a co-elution of substances other than putative RAL1 and/or RAL2 (cf. Table 1). Thus, the substance in the fraction between 3 and 6 min was collected and reduced with NaBH₄ to convert retinal congeners into their corresponding alcohol.

Fig. 2 shows the chromatograms of reduced samples. Geometrical isomers of RAL2 in Fig. 1B (extract from eyes) were reduced to ROL2 isomers (Fig. 2C). The H330/H360 values of peaks 1 (11-cis) and 3 (all-trans) in Fig. 2C were 0.88 and 0.81, respectively; the peak substances were assigned to 11-cis and all-trans ROL2 (H330/H360 values 0.93 and 0.86, respectively) and not to 11-cis and all-trans ROL1 (H330/H360 values 4.37 and 3.96, respectively). In the extract from 402 tail fins containing melanophores (Fig. 2B), two peaks with retention times identical to 11-cis and all-trans ROL2 isomers in Fig. 2C were detected, while no peaks corresponding to ROL2 isomers were detected from 644 tail fins without melanophores (Fig. 2A). The two peaks in Fig. 2B had H330/H360 values of 0.91 and 0.84, confirming the identification of the two peaks as 11-cis and all-trans ROL2, respectively.

**DISCUSSION**

For the HPLC analysis of visual pigment chromatophores in eyes, the oxime method (Groenendijk et al., 1979; Suzuki and Makino-Tasaka, 1983) is an excellent one because of its nearly complete recovery and stability of geometric isomers. However, for the same analysis of retinoids in extraocular tissues, an abundance of unknown substances interfere with the identification of retinoids. This interference is largely attributed to the extremely low concentration of chromophore retinal in extraocular tissues compared to that in ocular tissues.

In this study, the formaldehyde method (Suzuki et al., 1986) was used to extract unmodified retinal isomers and retinal was reduced with NaBH₄ for retinol peak detection by HPLC (Tokioka et al., 1991). The eyes collected from Xenopus tadpoles between stages 51 and 56 were analyzed and the presence of RAL2 was confirmed (see Introduction and Figs. 1B and 2C). With the amounts of 11-cis and all-trans RAL2 obtained from the eye from both the first chromatogram after formaldehyde-extraction (5.12 and 1.38 pmol/eye for 11-cis and all-trans RAL2, respectively; Fig. 1B) and the second chromatogram after NaBH₄ reduction (4.72 and 1.11 pmol/eye for 11-cis and all-trans ROL2, respectively; Fig. 2C), the calculated recovery of ROL2 isomers in the extraction from the second chromatography was 0.92 and 0.90 for 11-cis and all-trans isomers, respectively.

The extract from tail fins containing melanophores had detectable peaks of 11-cis and all-trans ROL2 in the second chromatogram (0.027 and 0.006 pmol/fin, respectively for peaks 1 and 3 in Fig. 2B), but neither ROL1 nor ROL2 peaks were detected in the extract from tail fins without melanophores (Fig. 2A). These results strongly suggest that melanophores in the tail fin contain 11-cis and all-trans RAL2 isomers. The total amount of RAL2 in a fin containing melanophores was 36.9 fmol after correction of recovery from the second chromatography. As a single fin contains about 4,000 melanophores (Miyashita, unpublished observation), about 9 amol (5×10⁵) of RAL2 molecules were estimated to be present in a single melanophore on the assumption that all the RAL2 molecules were equally distributed in melanophores.

Some retinoids have been detected, through HPLC analysis, in photosensitive extraocular tissues such as the pineal organ (Tabata et al., 1985; Tamotsu and Morita, 1990; Provencio and Foster, 1993) and brain (Foster et al., 1993; Masuda et al., 1994) of vertebrates. To our knowledge, however, this report is the first to suggest the presence of 11-cis RAL2 chromophore in the melanophore.

Melanophores in the tail fin of the Xenopus tadpole (stages 51-54) directly respond to light (Bagnara, 1957; van der Lek et al., 1958; Moriya et al., 1996); the melanosomes disperse in darkness and aggregate when exposed to light. While an injection of cGMP into the tail fin of the Xenopus tadpole caused the melanosomes to disperse, that of cAMP exerted only a weak effect (Moriya et al., 1996), implying a cGMP-mediated phototransduction pathway. An opsin-like molecule, similar in sequence to Xenopus opsin, has been detected by the reverse transcription-PCR analysis of the Xenopus tadpole tail fin containing melanophores (Miyashita et al., 2001). These results, along with the present detection of 11-cis RAL2, strongly suggest that porphyropsin-like photosensitive pigment functions as the photoreceptor of the melanophore in the Xenopus tail fin. Alternatively, a less-characterized opsin molecule, melanopsin would be expressed in the melanophore-containing tail fin of Xenopus tadpole at stage 51-54 (Provencio et al., 1998; Miyashita et al., 2001). In this case, both 11-cis and all-trans RAL2 identified in this study (Fig. 2B), might also be bound with melanopisin, because melanopisin is highly related to invertebrate opsin which binds both 11-cis and all-trans retinals tightly. Functional reconstitution of the recombinant melanopsin or rhodopsin expressed in cultured cells with 11-cis RAL2 would enable comparison of the absorption spectrum of the regenerated photosensitive molecule to the action spectrum of melanophore photoresponses at the Xenopus tail fin.

**ACKNOWLEDGMENTS**

We are grateful to Drs. H. Takasaki (Osaka Kyoiku University)
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and K. Yasuda (Nara Women’s University) for providing eggs of *Xenopus laevis*, to Dr. T. Suzuki (Hyogo Medical School) for providing crystals of all-trans 3,4-didehydroretinal, and Drs. K. Yoshihara and K. Tsuchihara (Santory Bio-organic Science) for providing the membrane preparation containing apo-retinochrome. We also thank Mr. T. Soneda (Osaka Kyoiku University) and Miss Y. Haida (Nara Women’s University) for their help with sample preparations. This work was supported in part by a Grant-in-Aid to T. O. from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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(Received July 27, 2001/ Accepted September 26, 2001)