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Identification of 3,4-Didehydroretinal Isomers in the *Xenopus* Tadpole Tail Fin Containing Photosensitive Melanophores

Keiko Okano[1], Tadashi Oishi[1], Yoko Miyashita[2], Tsuneo Moriya[2], Motoyuki Tsuda[3], Toshiaki Irie[4], Nobuo Ueki[5], and Takaharu Seki[6]*

[1]*Department of Life Environment, Graduate School of Human Culture, Nara Women's University, Kitauoyanishi-machi, Nara 630-8506, Japan*
[2]*Department of Biology, School of Medicine,Sapporo Medical University, Sapporo, Hokkaido 060-8556, Japan*
[3]*Department of Life Science,Himeji Institute of Technology, Harima Science Garden City, Akoh-gun, Hyogo 678-1297, Japan*
[4]*Osaka Meijo Women's College, Kumatori, Sennan-gun, Osaka 590-0493, Japan*
[5]*Hitec Co. Ltd., Sonezakishinchi, Kita-ku, Osaka 530-0002, Japan*
[6]*Division of Health Sciences, Osaka Kyoiku University, Asahigaoka, Kashiwara, Osaka 582-8582, Japan*

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 5×10^6 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 porphyropsin-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-

* Corresponding author: Tel: +81-729-78-3611;
Fax: +81-729-78-3554.
E-mail: seki@cc.osaka-kyoiku.ac.jp

iolos *et al.,* 1990). Rollag (1996) has also reported that in cultured *Xenopus* melanophores, melanosomes dispersed with exposure to light after treatment with all-*trans* retinal (abbreviated to RAL1). Although the switch mechanism of the photo-response during development has yet to be clarified, melanophores have been suggested to absorb photons with visual pigment-like photoreceptor(s).

Provencio *et al.* (1998) isolated a cDNA that encodes a putative opsin-like molecule, melanopsin, which is close to invertebrate opsins, from cultured melanophores derived from the *Xenopus* embryo. Recently, Miyashita *et al.* (2001) demonstrated, using *Xenopus* opsin specific primers, rhodopsin mRNA expression in the *Xenopus* tail fin containing melanophores. These results further support the possibility that visual pigment-like photoreceptive molecules are involved in the photo-response of the melanophore, although the endogenous chromophore retinal has yet to be reported.

Whether RAL1 or 3,4-didehydroretinal (abbreviated to RAL2) is used as the chromophore of putative visual pigment-like photoreceptors in the melanophore also remains elusive. The visual pigment contained within vertebrate rod photoreceptor cells consists of an opsin and a chromophore, 11-*cis* RAL1 (chromophore of rhodopsin) and/or 11-*cis* RAL2 (chromophore of porphyropsin). In some frogs, the rod visual pigment switches from porphyropsin in tadpoles to rhodopsin during metamorphosis (Bridges, 1972); however *X. laevis* is an exception. In an egg of *X. laevis*, nearly equal amounts of all-*trans* RAL1 and all-*trans* RAL2 are present (Seki *et al.*, 1987; Irie *et al.*, 1991). In the eyes of the early-stage tadpole (stages 43–50), a mixture of RAL1- and RAL2-derived visual pigments was detected (Crescitelli, 1973; Azuma *et al.*, 1988), and the RAL2/RAL1 ratio increased from 1 to 20 between stages 42 and 46 (Azuma *et al.*, 1988). In the later-stage tadpole (stages 58–59), as well as the adults after metamorphosis, porphyropsin has been exclusively detected in the retina (Crescitelli, 1973; Bridges *et al.*, 1977).

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.

MATERIALS AND METHODS

Animals and tissues

X. laevis tadpoles were maintained under an LD cycle at 22–25°C and fed adult *Xenopus* brittle 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 and the following operations were done under dim red lighting (>610 nm). Ventral parts of tail fins were dissected, cut into two parts, posterior with melanophores and anterior without melanophores, and stored at –80°C. The eyes were also dissected from forty-nine tadpoles, about half of which were excised under natural light condition.

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 (Physcotron, Nichion Irikakikai, 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*-butylmethylether, 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 ($6\phi \times 150$ 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 ($NaBH_4$) 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 $NaBH_4$ (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 sucsessive UV-detectors to monitor the elution profiles of standard samples for HPLC. The eluent is comprised of 5% *tert*-butylmethylether, 25% benzene, 0.04% ethanol in *n*-hexane.

	H330/H360	
	11-*cis*	all-*trans*
RAL1	0.75±0.02 (n=24)	0.63±0.01 (n= 4)
RAL2	0.62±0.04 (n= 5)	0.47±0.02 (n=16)
ROL1	4.37±0.07 (n=10)	3.96±0.07 (n= 3)
ROL2	0.93±0.03 (n= 5)	0.86±0.04 (n=12)

extraction by the formaldehyde method. An aliquot of purified 11-*cis* RAL2 was reduced to 11-*cis* ROL2 with $NaBH_4$. 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).

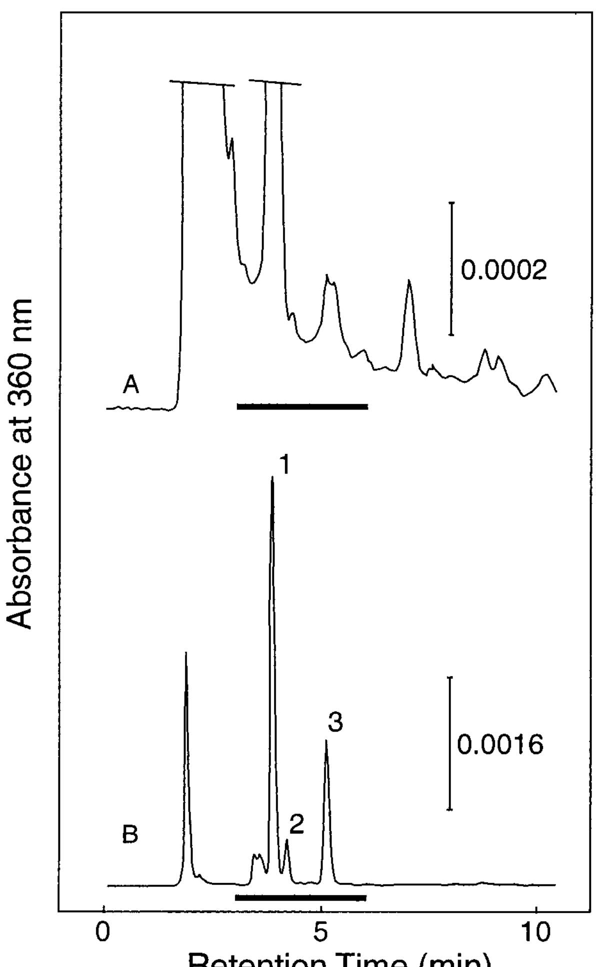

Fig. 1. Chromatograms of lipid soluble substances extracted from *Xenopus* tadpoles by the formaldehyde method. The extracts obtained from 402 tail fins,which contained melanophores (A; wet weight 560 mg), and 98 eyes (B; wet weight 91 mg) were analyzed using a normal-phase column and HPLC (detection at 360 nm). Two peaks (1 and 3) on chromatogram B were identified as 11-*cis* and all-*trans* RAL2, respectively, as mentioned in the text; and peak 2 was identified as 9-*cis* RAL2 on the basis of the elution profile. In the extracts from tail fins containing melanophores (A), large peaks interfered with the detection of retinal congeners. Thus, substances eluted between 3 and 6 min (filled bar) were collected and treated with $NaBH_4$ to convert retinal congeners into corresponding alcohols (ROL1 or ROL2) for the second HPLC analysis (Fig. 2). Scale bars indicate the absorbance at 360 nm.

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

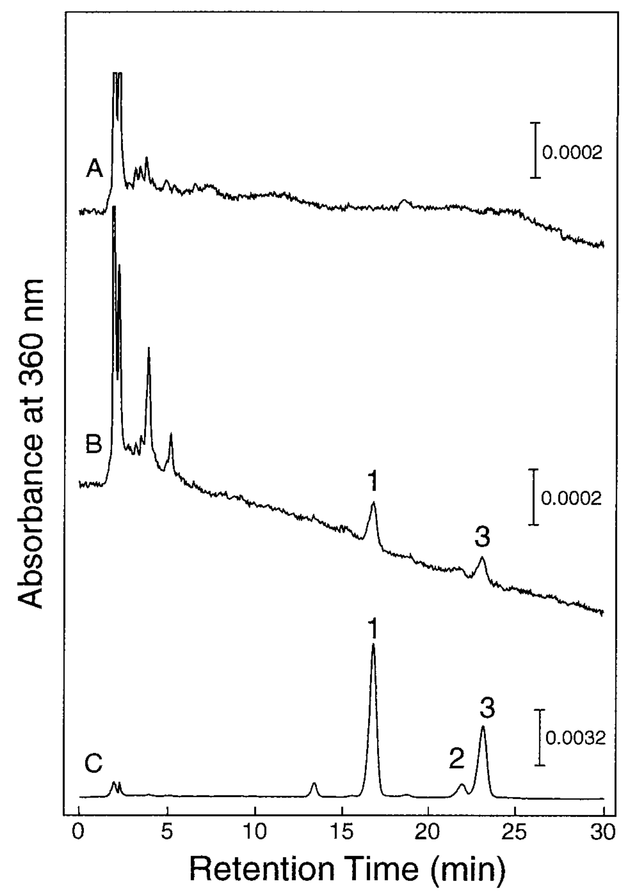

Fig. 2. Elution profiles on the second chromatogram of 3–6 min fractions obtained from the first chromatography (cf. Fig.1) after the reduction with $NaBH_4$. The reduced substances obtained from 644 tail fins without melanophores (A; wet weight 519 mg), from 402 tail fins containing melanophores (B), and from 98 eyes (C) of *Xenopus* tadpoles were analyzed by HPLC under the same conditions as those of the first chromatography. In the extract from eyes (C), two peaks, 1 and 3, were identified as 11-*cis* and all-*trans* ROL2, respectively, as mentioned in the text, and peak 2 as 9-*cis* ROL2 on the basis of the elution profile of authentic ROL2 isomers. In the extract from tail fins containing melanophores (B), 11-*cis* (peak 1) and all-*trans* (peak 3) ROL2 isomers were detected as mentioned in the text, while no peaks corresponding to ROL1 and ROL2 isomers were detected in the extract from tail fins without melanophores (A). Scale bars indicate the absorbance at 360 nm.

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 $NaBH_4$ 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. 2 B), 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 chromophores 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 extra-ocular 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 extra-ocular 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 $NaBH_4$ 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 $NaBH_4$ 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 (5×10^6) 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 extra-ocular 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 melanopsin, because melanopsin 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.

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