Comparative Biochemistry and Physiology, Part B 157 (2010) 113–118

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Retinoid storage in the egg of reptiles and birds

Toshiaki Irie [a,*], Tamiko Sugimoto [b], Nobuo Ueki [b], Haruki Senoo [c], Takaharu Seki [d]

[a] Laboratory of Biology, Department of General Education, Hakodate National College of Technology, Tokura-cho, Hakodate, Hokkaido 042-8501, Japan
[b] Hitec Co. Ltd., Sonezakishinchi, Kita-ku, Osaka 530-0002, Japan
[c] Department of Cell Biology and Morphology, Akita University Graduate School of Medicine, Hondo, Akita 010-8543, Japan
[d] Research and Development Center for Teacher Education, Osaka Kyoiku University, Kashiwara, Osaka 582-8582, Japan

ARTICLE INFO

Article history:
Received 15 February 2010
Received in revised form 20 May 2010
Accepted 21 May 2010
Available online 27 May 2010

Keywords:
Bird
Egg
Reptile
Retinal
Retinoid
Retinol
Vitamin A
Yolk protein

ABSTRACT

Storage of retinal has been confirmed in eggs from a range of anamniotic vertebrates (teleosts and amphibians) and an ascidian, but the retinoid-storage state in eggs of oviparous amniotic vertebrates (reptiles and birds) has yet to be clarified in detail. We studied four reptilian and five avian species and found that retinal was commonly stored in their egg yolk. Furthermore, retinal was the major retinoid in reptilian eggs, with only low levels of retinol, whereas significant amounts of retinol as well as retinal were stored in avian eggs. In both reptilian and avian eggs, retinal was commonly bound to proteins, which were assumed to be homologous to the proteins that bind retinal in the eggs of anamniotic vertebrates. Despite the common storage state of retinal, retinol would be bound to different proteins. In the reptilian eggs, retinol was found in the yolk-granule fraction, which also contained retinal. However, retinol in avian eggs was found largely in the yolk-plasma fraction, separate from retinal. These results suggest that retinol storage in avian eggs acquired after the divergence of birds from the reptiles, while retinal storage was acquired before the appearance of the vertebrates, and has subsequently been conserved during evolution of oviparous vertebrates.

1. Introduction

Retinoids, derivatives of vitamin A, play two distinct roles, involving unrelated mechanisms, and derived from different evolutionary backgrounds. The function of 11-*cis* retinal (RAL) in vision is widely distributed in the animal kingdom. In contrast, the function of retinoic acid (RA) in regulating genes is commonly present in chordates, but RA function in non-chordate animals has been controversial (Shimeld, 1996; Duester, 2000; Marlétaz et al., 2006; Campo-Paysaa et al., 2008; Simões-Costa et al., 2008). Because vitamin A homeostasis is essential for the maintenance of normal physiological conditions, animals have evolved retinoid-storing mechanisms to prevent hypovitaminosis A.

In adult vertebrates, the esterified product of retinol (ROL), retinylester (RE), is stored in the stellate cells, which are mostly localized in the liver (Blomhoff et al., 1990), although extrahepatic stellate cells also play an important role in RE storage in lower vertebrates (Wold et al., 2004; Yoshikawa et al., 2006). ROL and fatty acids are produced by hydrolysis of hepatic RE, and the resulting ROL then combines with serum ROL-binding protein (RBP4, formerly RBP), which is synthesized in the hepatocytes. In mammals and birds, the RBP4 associated with ROL (holo-RBP4) subsequently combines with transthyretin (TTR), a carrier protein of thyroid hormones. The holo-RBP4–TTR complex is secreted into the blood, and then delivered to the cells *via* the circulation (Vogel et al., 1999).

Retinoids in vertebrate eggs, however, are stored differently from those in adults. Plack et al. (1959) and Plack and Kon (1961) described the distribution of RAL in the eggs or ovaries in a wide range of oviparous vertebrates, but the biological significance of RAL in eggs has not been investigated. In our previous studies, we demonstrated that RAL was the major form of retinoid stored in amphibian and teleostean eggs (anamniotic vertebrates) (Seki et al., 1987; Irie et al., 1991, 2002; Irie and Seki, 2002), and was also the major form of retinoid stored in the eggs of the ascidian, *Halocynthia roretzi* (Urochordata) (Irie et al., 2003). We determined that RAL in the eggs of anamniotic chordates was commonly bound to lipovitellin 1 or its homologous proteins, by Schiff-base linkages (Irie et al., 1991, 2002, 2003; Irie and Seki, 2002). The common storage state of RAL in these diverse species suggests that the utilization of RAL for vitamin A storage in eggs was acquired before the appearance of vertebrates in chordate evolution (cf. Irie et al., 2004).

In contrast to the eggs of anamniotic chordates, considerable amounts of ROL are known to be present in the yolks of chicken and quail eggs, though RAL has also been demonstrated in the eggs of poultry (Al-Hasani and Parrish, 1972; Joshi et al., 1973; Sivell et al.,

Abbreviations: RAL, retinal (vitamin A aldehyde); ROL, retinol (vitamin A alcohol); RE, retinylester (vitamin A ester); RBP4, serum retinol-binding protein; TTR, transthyretin.

* Corresponding author. Tel./fax: +81 138 59 6391.
E-mail addresses: irie@hakodate-ct.ac.jp (T. Irie), senoo@ipc.akita-u.ac.jp (H. Senoo), seki@cc.osaka-kyoiku.ac.jp (T. Seki).

1096-4959/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2010.05.009

1982; Dong and Zile, 1995). However, the content of RAL in avian eggs remains to be confirmed, as simple extractions with organic solvents are unable to dissociate RAL adequately from its binding protein because of the Schiff-base linkage. The retinoid-storage state in eggs of reptiles, which belong to the amniotic taxon together with birds and mammals, also remains to be determined.

In the present study, we compared the retinoid storage strategies in the egg yolks of reptiles and birds. The results clearly showed that RAL was the major form of retinoid stored in the reptilian eggs, whereas ROL as well as RAL was stored in the avian eggs. Based on the results, we discuss the retinoid-storage state in eggs of oviparous amniotic vertebrates in comparison with that in anamniotic vertebrates, and in relation to vertebrate phylogeny.

2. Materials and methods

2.1. Eggs

Chicken (*Gallus gallus*, order Galliformes) and Japanese quail (*Coturnix japonica*, order Galliformes) eggs were purchased from a supermarket in Osaka prefecture, Japan. An ostrich (*Struthio camelus*, supraorder Palaeognathae) egg was supplied from Akita Prefectural College of Agriculture (Akita, Japan). Ostrich and duck (*Anas platyrhynchos domestica*, order Anseriformes) eggs were purchased from Jonan Green System Co. Ltd. (Ibaraki, Japan). Common kestrel (*Falco tinnunculus*, order Falconiformes) eggs were supplied by Akita City Omoriyama Zoo (Akita, Japan). Red-eared slider (*Trachemys scripta elegans*, order Testudines) eggs were from Himeji City Aquarium (Hyogo, Japan), soft-shell turtle (*Pelodiscus sinensis*, order Testudines) eggs were from Ashida Kikaku Ltd. (Ishikawa, Japan), and Siamese crocodile (*Crocodylus siamensis*, order Crocodylia) eggs were from Koike Wani Souhonpo Co. Ltd. (Shizuoka, Japan). Loggerhead turtle (*Caretta caretta*, order Testudines) eggs were supplied by Yakushima Umigame-kan, NPO (Kagoshima, Japan), mediated by the Sea Turtle Association of Japan, NPO (Osaka, Japan). Because the loggerhead turtle is listed as endangered in the Red List, the turtle eggs were obtained with formal permission based on the regulations for sea turtle preservation of Kagoshima Prefecture, Japan.

For all species, the eggshell was broken and the intact egg yolk was isolated from the albumen, after which the appropriate amount of yolk was collected. The yolk was suspended immediately in buffer (see below) for retinoid analysis, or stored in a deep freeze (−80 °C) until use.

2.2. Fractionation of the yolk and analysis of the yolk proteins

All the reptilian egg yolks consisted of an aqueous part, which accounted for the majority of the yolk, and a small amount of floating lipids. No floating lipids were visible in the avian egg yolks. The floating lipids were obtained by layering an appropriate amount of *n*-hexane onto the yolk mass of the reptilian eggs and shaking gently. The lipids dissolved in the *n*-hexane layer were then collected using a Pasteur pipette.

The aqueous part of the yolk was diluted 10-fold with 20 mM Tris-HCl buffer (pH 7.4), suspended using a manually operated Teflon-pestle homogenizer, and centrifuged at 17,000×*g* for 20 min to separate the yolk-granule (precipitate) and yolk-plasma (supernatant) fractions. The precipitate was resuspended in the same buffer, and centrifuged again under the same conditions to remove the remaining yolk-plasma proteins from the yolk-granule fraction.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed largely according to the procedure described by Laemmli (1970), using 1-mm thick 12.5% slab gels. The protein bands were visualized after staining with Coomassie Brilliant blue R-250. Precision Protein Standards (Bio-Rad Lab., CA, USA) were used as a molecular weight marker for SDS–PAGE.

Protein content was measured using the method of Lowry et al. (1951), with bovine serum albumin as the standard protein.

2.3. Retinoid extraction and analysis

The samples (whole egg yolk or yolk fractions) were extracted with organic solvents using the oxime method (Suzuki and Makino-Tasaka, 1983), following routine procedures (Irie et al., 1991). Briefly, each sample was treated with hydroxylamine hydrochloride (freshly neutralized NH_2OH-HCl) and then extracted using dichloromethane and hexane. The oxime method results in good dissociation of the Schiff-base linkage between RAL and its binding protein. To confirm the Schiff-base linkage of RAL to proteins, the yolk suspension was treated with sodium borohydride ($NaBH_4$) (Bownds and Wald, 1965) before extraction using the oxime method.

High-performance liquid chromatography (HPLC) was performed as described previously (Irie and Seki, 2002). The HPLC system (PU-2080, Jasco Corp., Tokyo, Japan or L-2130, Hitachi Ltd., Tokyo, Japan) was equipped with a 6×150-mm column of 3-μm silica gel (YMC-Pack SIL, YMC Co. Ltd., Kyoto, Japan), and was used at a flow rate of 2 mL/min. Absorbances were monitored using UV/visible detectors (dual wavelength, 875-UV and UV-970; Jasco Corp.) or a diode array detector (spectral surveillance, 300–400 nm, L-2450 Hitachi Ltd.). The eluent was 5% *tert*-butylmethyl ether, 0.04% ethanol and 25% benzene in *n*-hexane. Because RE isomers elute out in the void fraction under these eluent conditions, the fraction (retention time for 1.5–2.2 min) was collected, saponified to ROL with ethanolic KOH (Bridges and Alvarez, 1982), and rechromatographed under the same conditions as described above. RE isomers are detected as ROL isomers on the second round of HPLC. Extraction and analyses of retinoids were performed under dim red light to avoid photo-isomerization of retinoids during the experiments.

2.4. Gel chromatography of yolk-granule proteins

Analysis of the protein-binding RAL was performed using the previously described method (Irie and Seki, 2002). The isolated yolk-granule proteins were solubilized in buffer containing 0.4 M NaCl. The solution was then centrifuged at 17,000×*g* for 20 min to remove any insoluble matter. The supernatant was treated with $NaBH_4$, followed immediately by the addition of SDS (final concentration 2%) to form a retinyl–protein complex. The complex was stood on ice for 30 min and then dialyzed against 20 mM Tris-HCl buffer (pH 7.4) containing 0.1% SDS to avoid the formation of hydrogen gas bubbles due to any remaining $NaBH_4$, during the chromatography. Mercaptoethanol (final concentration 5%) was then added and the sample was chromatographed using a Sephacryl S-300 HR column (2.5×86 cm) equilibrated with buffer containing 0.1% SDS and 0.02% dithiothreitol. The eluent was collected in 5-mL fractions and the absorbances of each fraction at 280 nm (proteins) and 330 nm (retinyl–protein complex) were measured.

3. Results

3.1. Amounts and composition of retinoids

Fig. 1A_1 and B_1 show the HPLC chromatograms of extracts from the whole egg yolks of loggerhead turtles and Japanese quail. High levels of RAL were detected in the eggs of both reptilian and avian species. RAL was the major retinoid component in the turtle eggs, with a small amount of ROL, although considerable amounts of both ROL and RAL were present in the quail eggs.

Table 1 shows the contents (μg/g yolk) and concentrations (ng/mg protein) of retinoids in the egg yolks. RAL, ROL and RE were present in varying amounts in the different species. Fig. 2 summarizes the retinoid compositions of the eggs examined in the present and