RDH13L, an Enzyme Responsible for the Aldehyde-Alcohol Redox Coupling Reaction (AL-OL Coupling Reaction) to Supply 11-cis Retinal in the Carp Cone Retinoid Cycle*

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Background: In carp cones, 11-cis retinal is formed with the AL-OL coupling reaction.

Results: We identified RDH13L which catalyzes both the AL-OL coupling reaction with tightly-bound NADP+ and the conventional RDH activity with exogenous NADP+. Mouse RDH14 showed similar activity.

Conclusion: Cones have effective mechanisms to regenerate the pigment.

Significance: Molecular bases of effective pigment regeneration in cones are uncovered.

Cone photoreceptors require effective pigment regeneration mechanisms to maintain their sensitivity in the light. Our previous studies in carp cones suggested the presence of an unconventional and very effective mechanism to produce 11-cis retinal, the necessary component in pigment regeneration. In this reaction (aldehyde-alcohol redox coupling reaction, AL-OL coupling reaction), formation of 11-cis retinal, i.e. oxidation of 11-cis retinol is coupled to reduction of an aldehyde at a 1:1 molar ratio without exogenous NADP(H) which is usually required in this kind of reaction. Here, we identified carp retinol dehydrogenase 13-like (RDH13L) as an enzyme catalyzing the AL-OL coupling reaction. RDH13L was partially purified from purified carp cones, identified as a candidate protein, and its AL-OL coupling activity was confirmed using recombinant RDH13L. We further examined the substrate specificity, subcellular localization, and expression level of RDH13L. Based on these results, we concluded that RDH13L contributes to a significant part, but not all, of the AL-OL coupling activity in carp cones. RDH13L contained tightly bound NADP+ which presumably functions as a cofactor in the reaction. Mouse RDH14, a mouse homolog of carp RDH13L, also showed the AL-OL coupling activity. Interestingly, although carp cone membranes, carp RDH13L and mouse RDH14 all showed the coupling activity at 15–37 °C, they also showed a conventional NADP+-dependent 11-cis retinol oxidation activity above 25 °C without addition of aldehydes. This dual mechanism of 11-cis retinal synthesis attained by carp RDH13L and mouse RDH14 probably contribute to effective pigment regeneration in cones that function in the light.

In the vertebrate retina, there are two types of photoreceptors, rods and cones. In both cells, light is absorbed by visual pigment consisting of a chromophore, 11-cis retinal, and a protein, opsin. Upon absorption of a photon, 11-cis retinal is isomerized to all-trans retinal, which leads to generation of electrical responses in these cells (1, 2). Activated pigment is then decomposed into all-trans retinal and opsin. To maintain the level of the visual pigment that can be activated, 11-cis retinal is supplied to opsin to regenerate the pigment. In the vertebrate eyes, there are two metabolic pathways to supply 11-cis retinal to rods and/or cones (3–6).

The first and conventional pathway consists of reactions found in both rods and cones, and in addition, in the cells of the retinal pigment epithelium (RPE). In this pathway (RPE retinoid cycle), all-trans retinal is first reduced in both rods and cones, and the resultant all-trans retinol is sent to the RPE. In the RPE, all-trans retinol is converted to 11-cis retinal that is sent back to rods and cones to regenerate visual pigments (7–9). The second and recently identified pathway consists of the reactions found in cones and in Müller cells. In this pathway (cone-specific retinoid cycle), all-trans retinal is reduced to all-trans retinol in cones and it is sent to Müller cells. In Müller cells, all-trans retinol is isomerized to 11-cis retinol (10, 11) and the product, 11-cis retinol, is sent back to cones where it is oxidized to 11-cis retinal to regenerate visual pigments (12–14). The cone-specific retinoid cycle is suggested to contribute to the effective pigment regeneration in cones (15). However, the enzyme catalyzing 11-cis retinol oxidation in cones has not been identified.

In our previous studies (16, 17), we reported that carp cone inner segment membranes show a very efficient 11-cis retinol oxidation activity that does not require the addition of NADP+ or NAD+ usually required in the oxidation of 11-cis retinol by retinol dehydrogenases (RDHs). Instead, some hydrophobic...
aldehydes possibly produced in the inner segment supported the reaction: 11-cis retinol was oxidized with concomitant reduction of an aldehyde at a 1:1 molar ratio (aldehyde-alcohol redox-coupling reaction, AL-OL coupling reaction; Ref. 17). We estimated that, at its maximum rate, the AL-OL coupling supplies 11-cis retinal at a 240 times higher rate than the RPE retinoid cycle (16). This reaction could also contribute to eliminate toxic aldehydes produced in mitochondrion in the inner segment. In this study, we tried to identify and characterize the enzyme(s) responsible for the AL-OL coupling in carp cones.

EXPERIMENTAL PROCEDURES

Preparation of Cone Membranes—Cones from carp (Cyprinus carpio, ~30 cm in length) were purified as described (18). Experimental animals were cared for in accordance with our institutional guidelines (approval number: FBS-14-005). Cone outer segment membrane-rich (OS-rich) fraction and cone inner segment membrane-rich (IS-rich) fraction were those prepared and reported previously (see Fig. 6, B and C in Ref. 17). Cone visual pigment was enriched in the OS-rich fraction by 36-fold, but detected negligibly in the IS-rich fraction, compared with its content in the starting cone membranes.

Partial Purification and Identification of the Enzyme Catalyzing the AL-OL Coupling—Membranes in the IS-rich fraction was washed once by centrifugation (104,000 × g, 15 min, 4 °C) in 10 mM HEPES buffer (1 mM dithiothreitol and 10 mM HEPES-NaOH, pH 7.5) and stored at −80 °C until use. Stored membranes prepared from 3.7 × 107 purified carp cones were suspended in ~1 ml of 10 mM HEPES buffer containing 9.6 mM dodecyl-β-D-maltoside (DDM) and incubated on a rotator (6 rpm, 30 min, 4 °C). The DDM-solubilized sample was centrifuged (104,000 × g, 20 min, 4 °C) to obtain a soluble supernatant (DDM fraction). This supernatant was loaded on an anion-exchange column (Mono Q PC 1.6/5, GE Healthcare) pre-equilibrated with 10 mM HEPES buffer containing 0.19 mM DDM, and a flow-through fraction (Mono Q fraction) was collected. This fraction was concentrated using a centrifugal concentrator (spin-X UF 500 30k MWCO, Corning).

The concentrated Mono Q fraction was fractionated by high resolution clear native electrophoresis (hrCNE) reported previously (19) with slight modifications using a NATIVEN S preparative electrophoresis system (ATTO). A polyacrylamide gel for hrCNE was prepared, and preparative electrophoresis was performed both according to the manufacturer’s instruction. In this electrophoresis, 0.19 mM DDM was always added to the gel and buffers. To detect the fractions containing the AL-OL coupling activity, the activity was measured in an aliquot of each fraction as reported previously (17).

The fraction containing the activity was concentrated to 30 μl (from ~600 μl) using a centrifugal concentrator. Proteins were separated by SDS-PAGE, and they were stained with silver or a fluorescent probe using a Silver Stain MS kit (Wako) or an Oriole Fluorescent Gel Stain kit (Bio-Rad), respectively. The 37-kDa band in the silver-stained gel was excised, trypsin-digested, and analyzed using tandem mass spectrometry (SYNAPT G2 HDMS PRO, Waters) connected with an HPLC system (nano-Acquity UPLC, Waters) (LC-MS/MS at the Center for Medical Research and Education (Osaka University)).

Measurements of AL-OL Coupling Activity and NADP+-dependent Oxidation of 11-cis Retinol—AL-OL coupling activity was measured basically as described previously (17). Briefly, 100 μl of a membrane suspension or a protein solution was mixed with an aldehyde substrate and a retinol substrate both dissolved in 0.5 μl of ethanol, and incubated for desired time. In most of the present studies, AL-OL coupling activity was determined as the amount of 11-cis retinal formed from 11-cis retinol in the presence of all-trans retinal (Fig. 1E and Fig. 6, and a few measurements in Figs. 3 and 8). In these measurements, we used A1 11-cis retinol and A2 all-trans retinal, and quantified the amount of A1 11-cis retinal or, when appropriate, A2 all-trans retinol, the products formed by the AL-OL coupling activity. In other studies (Fig. 2 and most of the measurements in Fig. 3), we used various kinds of aldehydes. With these aldehydes, we could identify the product, 11-cis retinal, easily. When necessary, kinetic analysis was made to determine the reaction mechanism. In this analysis, all of the data points were globally fitted to an equation formulated for the sequential mechanism or the ping-pong mechanism using Igor Pro software (Wavemetrics). The activity of NADP+-dependent 11-cis retinol oxidation was measured by adding 11-cis retinol (in 0.5 μl of ethanol) together with NADP+ (in 0.5 μl of 10 mM HEPES buffer) to a membrane suspension or a protein solution of 100 μl, and the mixture was incubated for desired time.

In addition to the activity of cone membranes, we measured the activities of three types of recombinant RDHs depending on the studies (see below): non-tagged wild-type (WT RDH), tagged at its C terminus with 6 histidines (RDH-His6) and N terminus glutathione S-transferase fusion (GST-RDH) proteins. In the measurements with WT RDHs, these proteins were expressed in Sf9 cells and their activities were measured using Sf9 cell membranes containing each of WT RDH. In the measurements with purified RDH-His6 proteins, 0.1 mM DDM and 20% (w/v) glycerol were always added to fully solubilize these proteins. In measurements with purified GST-RDH, neither of them was added except in the study shown in Fig. 2B where 20% glycerol was added for the comparison of the reaction mechanisms between mouse GST-RDH14 and carp RDH13L-His6 that was solubilized in 0.1 mM DDM and 20% glycerol. We confirmed that up to 0.2 mM DDM does not affect the coupling activity significantly.

In all of the activity measurements, reactions were terminated by addition of 300 μl of ice-chilled methanol. Samples devoid of one of the two substrates were used for the measurements of controls, and their activities were subtracted for the correction. The reaction products were identified and quantified with HPLC as described previously (17).

Preparation of Retinoids and NADPH(H)—Retinoids used in this study were prepared and purified with HPLC as described previously (17). NADP+ and NADPH were purchased from Kohjin Co. Ltd, dissolved in 10 mM HEPES buffer, quantified spectrophotometrically using a molar extinction coefficient of 18,000 M⁻¹ cm⁻¹ at 260 nm and 6,200 M⁻¹ cm⁻¹ at 340 nm (20), respectively, and stored at −80 °C until use.

Cloning of Carp and Mouse RDH Genes—Carp RDH13L (KM377625) and carp RDH14 (KM377626) were cloned from a carp retinal cDNA library (21) based on partial sequences.
obtained in our previous study (16). Each carp RDH coding sequence was inserted into pGEM-T easy (Promega) and amplified in E. coli (DH5α). Mouse RDH11 (NM_021557.5), mouse RDH12 (NM_030107.3), and mouse RDH14 (NM_023697.2) were cloned from a mouse retinal cDNA library (a kind gift from Prof. T. Furukawa). Mouse RDH13 (NM_175372.4) was cloned from a cDNA clone obtained commercially (FANTOM clone, ID: 4432411109; The FANTOM Consortium, Ref. 22). Each mouse RDH coding sequence was inserted into the EcoRI-Sall sites in a pGEX-5X-1 vector (GE Healthcare) and amplified in Escherichia coli (DH5α).

Expression and Purification of Carp and Mouse RDHs—Carp and mouse RDHs were expressed as WT, RDH-His6, or GST-RDH depending on whether a group of the proteins under study can be expressed in the same type of recombinant. When RDH activities were compared among various RDH subtypes, the same type of recombinant proteins were used.

WT proteins of carp RDH13L, carp RDH8 (AB439579), carp RDH8L2 (AB439580), and carp RDH13 (AB439581) were expressed in Sf9 cells using Bac-to-Bac Baculovirus Expression System (Invitrogen). Sf9 cells (5 x 10⁶ cells) were infected and incubated according to the method described in a manufacturer’s protocol. After the cells were harvested, the cell membranes containing each of the expressed carp WT RDH were obtained according to the method reported previously (23) except that we used potassium gluconate buffer (115 mM potassium gluconate, 2.5 mM KCl, 2 mM MgCl₂, 0.2 mM EGTA, 0.1 mM CaCl₂, 1 mM dithiothreitol, and 10 mM HEPES-KOH, pH 7.5) as the buffer to suspend the membranes and that 1% (v/v) Protease Inhibitor Cocktail (nacalai tesque) was added during preparation. The Sf9 membranes expressing WT RDHs were used for determination of substrate specificity of the AL-OL coupling reaction of carp RDH13L (Fig. 3A), examination of the specificity of anti-RDH13L antiserum (Fig. 4A), and measurements of the activities of carp RDH13L, RDH8, RDH8L2, and RDH13 (Fig. 6A).

Carp RDH13L was also expressed in Sf9 cells as a C terminus His6-tagged protein (RDH13L-His6). In this case, similarly as carp RDH13 (Fig. 6), the expression sequence. Sf9 cell membranes were used for determination of AL-OL coupling activity in carp RDH13L (Fig. 2A), a molar standard in quantification of the amount of endogenous carp RDH13L-His6. For a negative control of the study using this purified antibody, we prepared a solution from normal mouse serum with the procedure used to purify anti-RDH13L antibody. This control solution potentially contains proteins possibly present in both normal serum and purified anti-RDH13L antibody solution, and giving positive signals non-specifically in our immunochemical studies.

Immunofluorescent Staining of Isolated Cones—Immunofluorescence study was performed as reported previously (24) with slight modifications. Briefly, carp photoreceptors were isolated as reported previously (18) in Ringer’s solution (119.9 mM NaCl, 2.6 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 0.5 mM MgSO₄, 1 mM NaHCO₃, 0.5 mM NaH₂PO₄, 16 mM glucose, and 4 mM HEPES-NaOH, pH 7.5). They were mixed with equal volume of Ringer’s solution supplemented with 8% (w/v) paraformaldehyde (PFA), applied onto a stepwise Percoll density gradient consisting of 30%, 45%, and 90% (w/v) Percoll in Ringer’s solution, and centrifuged (33,000 x g, 20 min, 4°C). The layers of 30 and 45% Percoll contained 4% (w/v) PFA. Cones, and rods as a very minor population, were collected at the interface between the 45% and the 90% Percoll layers. The cells were suspended in Ringer’s solution supplemented with 4% (w/v) PFA, kept at 4°C for 12 h in the dark for complete fixation, and mounted on a silane-coated slide glass. Then, they were permeabilized with PBS containing 1% (w/v) Triton X-100 for 15 min, air-dried, blocked with PBS containing 1.5% (v/v) normal goat serum and 0.2% (w/v) Triton X-100, and incubated with an antibody or an antibody solution overnight at 4°C. The antigen or antibody solution used contained purified anti-carp RDH13L antibody (1:5 dilution) in addition to affinity-purified anti-carp red/green-sensitive opsins antibody (1:1000), anti-carp blue-sensitive opsins antibody (40000), or affinity-purified anti-carp UV-sensitive opsins antibody (1:2000). Each anti-opsin antibody or antibody was raised in a rabbit against a partial peptide: STSK-TEVS VIPA for carp red/green-sensitive opsins (no difference in the amino acid sequence in this region between carp red- and
green-sensitive opsins; BAB32496 for red-sensitive opsin, and BAC76806 and BAC76807 for green-sensitive opsin 1 and 2, respectively), KQIFEHEDF for carp blue-sensitive opsin (BAC78822), and GYAEDTNKDYR for carp UV-sensitive opsin (BAC78823). Cells were then immuno-labeled with a mixture of Alexa488-conjugated anti-mouse IgG for RDH13L and Alexa568-conjugated anti-rabbit IgG for carp cone opsins (1:330 dilution each; Molecular Probes).

Quantification of RDH13L in Carp Cones—Purified carp cones (2.0 × 10⁶ cells) were disrupted by freeze-thawing, suspended in 10 mM HEPES buffer, and centrifuged (104,000 g, 20 min, 4°C) to collect a soluble supernatant and a membrane precipitate. The membrane precipitate was resuspended in 10 mM HEPES buffer. RDH13L in the supernatant and that in the precipitate were quantified by immunoblot analysis. Known amounts of purified RDH13L-His6 were used as a molar standard.

Quantification of Protein-bound NADP⁺ and NADPH—The contents of NADP⁺ and NADPH were measured in purified proteins (carp RDH13L-His6 and mouse GST-RDHs including RDH11, RDH12, RDH13, and RDH14), but the detailed quantification was made only for carp RDH13L-His6. NADP⁺ bound to RDH13L-His6 was quantified with the method reported previously (25) with slight modifications. To dissociate the bound NADP⁺, purified RDH13L-His6 in 10 mM HEPES buffer containing 0.19 mM DDM was denatured by adding equal volume of 10 mM urea solution and incubated for 2 min at 85°C. After cooling and centrifugation (17,900 × g, 5 min, 25°C), the supernatant was applied to an HPLC system (SMART system, Pharmacia) equipped with an anion-exchange column (Mono Q PC 1.6/5, GE Healthcare) pre-equilibrated with 10 mM Tris-HCl, pH 8.5. NADP⁺ and NADPH were eluted at a flow rate of 0.1 ml/min with a linear gradient of 0–400 mM KCl, and detected by optical absorption at 260 nm and 340 nm. NADP⁺ and NADPH were identified from their retention times in the chromatogram and quantified by comparing their peak areas with those of standard NADP⁺ and NADPH calibrated spectrophotometrically with their known molar extinction coefficients (see above). The urea and the heat treatments were also made to each of NADPH and NADP⁺ standard samples. Only for carp RDH13L, the amount of the protein used in this quantification was determined with quantitative amino acid analysis (Instrumental Analysis Division, Equipment Management Center Creative Research Institution, Hokkaido University, Japan).

RESULTS

Partial Purification and Identification of RDH13L as an Enzyme Catalyzing the AL-OL Coupling Reaction—We attempted to identify the enzyme catalyzing the AL-OL coupling reaction in carp cones (Fig. 1). Because the AL-OL coupling activity is localized in the inner segment membranes of carp cones (17), first, we prepared an IS-rich fraction from purified carp cones (Fig. 1A, IS-rich). Proteins in the membranes were solubilized with DDM, and solubilized proteins (Fig. 1A, DDM) were loaded to an anion-exchange, Mono Q, column. The AL-OL coupling activity was recovered in the flow-through fraction. This flow-through fraction (Fig. 1A, Mono Q) was concentrated and applied to preparative native-PAGE to obtain a fraction containing the activity. A roughly purified fraction (Fig. 1A, Native-PAGE) showed 1.5% recovery of the activity. Then, the proteins in this fraction were analyzed further in separate native-PAGE: Fig. 1B shows an elution profile of the AL-OL coupling activity (upper) and that of proteins (lower) in each fraction. The elution profile of 37-kDa protein (Fig. 1B, large arrow) was well-correlated with that of the AL-OL coupling activity.
The 37-kDa protein band was analyzed with LC-MS/MS. The mass fingerprint was compared with the proteome database of zebrafish, because carp database was not available. The result showed that the 37-kDa band contained peptide fragments identical to those of zebrafish retinol dehydrogenase-like (RDH-like) protein (NP_001186229.1) and voltage-dependent anion channel 3 (XP_005166801). The number of unique peptides obtained in the LC-MS/MS analysis was larger in RDH-like protein, and the expected molecular mass of RDH-like protein is 37 kDa, which is similar to that of the protein band. In contrast, the expected molecular mass of the anion channel is 34 kDa, which is slightly lower than that of the band in the gel. For these reasons, RDH-like protein seemed to be a good candidate for the enzyme catalyzing the AL-OL coupling reaction. The carp ortholog of RDH-like was partially cloned previously as a homolog of carp retinol dehydrogenase 13 and named as retinol dehydrogenase 13-like (RDH13L; Ref. 16). We cloned the full-length RDH13L-coding sequence from a carp retinal cDNA library and determined its amino acid sequence (Fig. 1C, KM377625). The mass fingerprint of the 37 kDa band obtained with LC-MS/MS was compared with the determined amino acid sequence of RDH13L, and eight peptides covering 26% of the entire RDH13L amino acid sequence were identified (Fig. 1C, underlined). This result strongly suggested that the 37-kDa protein is carp RDH13L.

Peptide fragments having the sequence corresponding to the carp RDH13L protein were also found in the band positioned slightly above the 37-kDa band (Fig. 1B, small arrow). Our attempt to know the reason for this doublet formation was not successful: neither protein phosphatase 2A to remove phosphates nor glycopeptidase F to remove N-acetylglucosamine bound to asparagine residue produced a single band.

**AL-OL Coupling Activity of Recombinant RDH13L-His6**—To ensure that RDH13L has the AL-OL coupling activity, RDH13L-His6 was expressed in Sf9 insect cells, affinity-purified, and its coupling activity was measured. The purity of the obtained RDH13L-His6 was >95% by SDS-PAGE (Fig. 1D). In most of our measurements of AL-OL coupling activity, we used A1 11-cis retinol and A2 all-trans retinol (see “Experimental Procedures”). With incubation of purified RDH13L-His6 and these retinoids, A1 11-cis retinal, the product from A1 11-cis retinol, and A2 all-trans retinol, the product from A2 all-trans retinol, were formed at almost 1:1 ratio (0.86:1 as a mean, Fig. 1E). We did not add exogenous cofactors such as NADP(H), and therefore, the above result strongly suggested that RDH13L catalyzed the reaction with the AL-OL coupling mechanism: 11-cis retinal was formed from 11-cis retinol with oxidation and all-trans retinol was formed from all-trans retinol by reduction, similarly as in cone membranes (Fig. 1E, Ref. 17). The rate of 11-cis retinal formation was 0.13 ± 0.03 11-cis retinol molecules produced/recombiant RDH13L-His6 molecule/s (11-cis retinal/RDH/s, a mean ± the range of variations in two independent measurements).

AL-OL coupling reaction is a two-substrate reaction. There are two possible mechanisms to explain this reaction, the ping-pong mechanism and the sequential mechanism: one product is released from the enzyme each time when one of the substrates is bound and reacted (ping-pong mechanism), or two products are released after both substrates are bound and reacted (sequential mechanism) (26). In our previous study, we showed that the AL-OL coupling reaction has the sequential mechanism in carp cone membranes (17). To confirm that RDH13L shows a similar mechanism, AL-OL coupling activity of RDH13L-His6 was measured by varying the concentrations of 11-cis retinol and benzaldehyde, an aldehyde that supports the AL-OL coupling reaction (26). In our previous study, we showed that the ping-pong reaction (Fig. 2A, red solid lines) is preferred to the sequential reaction (Fig. 2A, black broken lines) (26). In our previous study, we showed that the ping-pong reaction is preferred to the sequential reaction (Fig. 2A, black broken lines) (26).
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FIGURE 3. Comparison of substrate specificity of recombinant WT RDH13L with that in carp cone membranes. A, reactivity of WT RDH13L toward various aldehyde substrates (left) and retinol isomers (right). Each aldehyde (250 μM) was incubated with 11-cis retinol (250 μM) in the presence of 0.1 μM RDH13L and formation of 11-cis retinal was quantified. Bars show 11-cis retinal formation relative to that produced in the presence of all-trans retinal as the aldehyde substrate. Right, each retinol isomer (250 μM) was incubated with benzaldehyde (5 mM) in the presence of 0.2 μM RDH13L and formation of its corresponding retinol isomer was quantified. Bars show the retinol formation relative to that formed in the presence of 11-cis retinol as the alcohol substrate. B, reactivity of cone membranes toward various alde- hydes substrates (left) and retinol isomers (right) measured under similar con- ditions as in A. The data in B are re-plot of the original data published already (Ref. 17; This research was originally published in the Journal of Biological Chemistry. Substrate specificity and subcellular localization of the aldehyde-alcohol redox-coupling reaction in carp cones). All data are indicated as the mean ± S.D. (n = 3) or the range of variations (n = 2).

RDH13L- His6, so that the kinetic values are of RDH13L-His6 in the presence of a detergent. (Similar fitting was made in Fig. 2B for mouse GST-RDH14, which is soluble; see below.)

Substrate Specificity of RDH13L—In our previous study, we examined substrate specificity of the AL-OL coupling reaction in carp cone membranes: many large hydrophobic aldehydes supported the reaction, while only 11-cis retinol and 9-cis retinol were good substrates as alcohols (17). We examined the substrate specificities of recombinant RDH13L toward alde- hydes and alcohols (Fig. 3A) and compared them with those of carp cone membranes reported previously (Fig. 3B, re-drawn from Figs. 3 and 4 in Ref. 17). Because membrane preparations were used in the previous study, S9 cell membranes expressing WT RDH13L were used in this study. Recombinant WT RDH13L showed broad specificity toward large hydrophobic aldehydes and higher reactivity toward 11-cis retinol and 9-cis retinol than other retinol isomers examined. These specificities are similar to those of the AL-OL coupling reaction in cone membranes. However, the activity to each substrate seemed to be slightly different between recombinant WT RDH13L expressed in S9 cell membranes and the endogenous enzyme(s) in cone membranes. It may be the case that recombinant WT RDH13L is slightly different from native RDH13L in their structures, for example, or that enzymes other than RDH13L also contribute to the AL-OL coupling reaction in cone membranes (see below).

Subcellular Localization of RDH13L in Carp Cones—We previously showed that the AL-OL coupling activity is present in the cone inner segment (17). To examine whether RDH13L is expressed in this region of cones, we generated antiserum against RDH13L. The serum specifically reacted to RDH13L among other RDHs (Fig. 4A), but reacted also to other proteins of higher molecular masses in cone membranes in our immu- noblot study. Therefore, we prepared purified antibody against RDH13L (Fig. 4B) and examined subcellular localization of RDH13L.
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Our immunofluorescence study showed that RDH13L is present only in the inner segment of all cone cell-types (red/ green-, blue-, and UV-sensitive cones; Fig. 4, C–E, respectively), but not in rods (Fig. 4F). This localization of RDH13L was also confirmed by immunoblot on carp cone membranes: positive signals were detected in both the total cone membranes (OS-plus-IS) and IS-rich fraction but not in the OS-rich fraction (Fig. 4H).

Expression Level of RDH13L in Carp Cones—Although recombinant RDH13L shows the AL-OL coupling activity as shown above, there might be other enzymes contributing to the coupling activity. To estimate the relative contribution of RDH13L to the AL-OL coupling activity in carp cones, we determined the expression level of RDH13L in purified carp cones using antisera against RDH13L, and estimated the total coupling activity of RDH13L in cones based on the specific activity of recombinant RDH13L-His6 (see Fig. 1E). Using known amounts of RDH13L-His6 as a molar standard, the content of RDH13L was determined in a membrane precipitate and a soluble supernatant fraction of purified carp cones (Fig. 5, A and B). Immunopositive signals were found exclusively in the membrane fraction and the expression level was 2.7 ± 1.5 amol/corne (mean ± S.D., n = 24). With the specific activity of recombinant RDH13L-His6 (0.13 11-cis retinal/RDH/sec, Fig. 1E), contribution of RDH13L to the coupling activity in a carp cone was estimated to be 0.32 ± 0.17 amol of 11-cis retinal formed/cone/sec (amols 11-cis retinal/corne/s, Fig. 5C, RDH13L). In native cone membranes, the AL-OL coupling activity was 1.6 ± 0.4 amol 11-cis retinal/corne/s (Fig. 5C, a cone). Thus, we estimated that RDH13L contributes 21 ± 12% of the total AL-OL coupling activity in carp cones.

AL-OL Coupling Activities of RDH8, RDH8L2, RDH13, and RDH14—In carp cones, in addition to RDH13L, other subtypes of RDHs (RDH8, RDH8L2, and RDH13) are expressed (16). To investigate the contribution of these RDH subtypes to the AL-OL coupling activity in carp cones, we obtained WT recombinants of RDH8, RDH8L2, RDH13, and RDH13L in Sf9 cells. In addition, because we detected the coupling activity in one of the mouse homologs of carp RDH13L (mouse RDH14, see below), we also tried to obtain His6-tagged recombinant carp RDH14. However, we could not express this protein in Sf9 cells, and for this reason, carp RDH14, together with carp RDH13L as a control, was expressed in E. coli. In the present study, depending on whether a group of proteins under study can be expressed as the same type of recombinant, we used WT, His6-tagged, or GST-fusion recombinant RDHs.

We measured the coupling activities of the above WT and His6-tagged RDHs, and found that in addition to RDH13L, RDH13 showed the AL-OL coupling activity, whereas RDH8, RDH8L2 or RDH14 did not (Fig. 6). The activity of WT RDH13 in Sf9 cell membranes was 0.0074 ± 0.0027 (11-cis retinal/RDH/sec), about half of that of WT RDH13L in the Sf9 membranes (0.015 ± 0.009 11-cis retinal/RDH/s) (Fig. 6A). It is evident that, in addition to RDH13L, RDH13 has the coupling activity. However, to determine the contribution of each subtype of RDH to the total AL-OL coupling activity, we need to know the specific coupling activities and the expression levels of all of the RDHs in cones, which we did not examine in this study.

Quantification of NADP⁺ in RDH13L—Analysis with a conserved domain search program (NCBI CD-search) suggests that RDH13L amino acid sequence has an NADP(H)-binding site, but RDH13L-His6 catalyzes the AL-OL coupling reaction without addition of exogenous NADP(H) (Fig. 1). In our previous study, addition of NADP⁺ or NADPH increased the AL-OL coupling activity under some conditions in carp cone membranes (17). From these results, we predicted the presence of a tightly bound NADP(H) in the responsible enzyme(s), and tried to quantify NADP(H) in RDH13L-His6 with HPLC (Fig. 7). We detected NADP⁺, not NADPH, in RDH13L-His6 (Fig. 7D), and the content was 0.93 ± 0.15 moles of NADP⁺ per mol of this enzyme (Fig. 7F). This result strongly suggests that one RDH13L molecule binds one NADP⁺ molecule tightly.

AL-OL Coupling Activities of Mouse RDH11, RDH12, RDH13, and RDH14—To examine whether the AL-OL coupling reaction is present in the mammalian retina, we searched for mouse orthologs of carp RDH13L using NCBI BLAST against NCBI Protein Reference Sequences of mouse. Although the mouse ortholog was not found, amino acid sequences of mouse RDH11, RDH12, RDH13, and RDH14 showed significant similarity to that of carp RDH13L (55–66% similarity). To test
RDH13L as an Enzyme Catalyzing the AL-OL Coupling Reaction

![Diagram](image)

**FIGURE 7. Quantification of NADP⁺ and NADPH bound to RDH13L-His6.**

A–D, representative HPLC chromatograms of control buffer (A); standard NADP⁺ (B); standard NADPH (C); an extract obtained from urea-denatured RDH13L-His6 (D). NADP⁺ and NADPH were monitored by the absorbance at both 260 nm (red traces) and 340 nm (blue traces). Gray traces indicate the conductance increase due to the increase in the KCl concentration. E, a calibration curve for the quantification of NADP⁺. Peak areas obtained in the HPLC chromatograms of standard NADP⁺ were plotted against the amount of NADP⁺ (open circle) to obtain a calibration curve (solid line). The amount of NADP⁺ detected in the extract obtained from urea-denatured RDH13L-His6 was determined (filled red circle). F, molar ratio of detected NADP⁺ to RDH13L-His6. Bars indicate the mean ± S.D. (n = 3).

whether these proteins have the AL-OL coupling activity, N terminus GST-fusion protein of each RDH (mouse GST-RDH) and corresponding protein of carp RDH13L (carp GST-RDH13L) as a control, were expressed in *E. coli*. They were affinity purified, and their coupling activities were measured at room temperature (25°C). As a result, carp GST-RDH13L and mouse GST-RDH14 showed the AL-OL coupling activity of the rate of 0.068 ± 0.002 and 0.025 ± 0.004 11-cis retinal/RDH/sec, respectively (Fig. 8A). To test whether mouse RDH14 catalyzes the AL-OL coupling with the sequential mechanism as carp RDH13L, kinetic analysis was performed (Fig. 2B). In this analysis, coupling activities were measured at 37°C. The result showed that mouse GST-RDH14 also catalyzes the reaction with the sequential mechanism. Although detailed quantitative measurement was not made, we found detectable amount of NADP⁺, not NADPH, only in mouse GST-RDH14, not in other mouse GST-RDHs.

**Dual Mechanism of Oxidation of 11-cis Retinol in Cones: Aldehyde-dependent and NADP⁺-dependent Mechanisms—** Mouse GST-RDH14 showed the AL-OL coupling activity without addition of NADP(H) (Fig. 8A). However, it was previously reported that human RDH14 has NADPH-dependent 11-cis retinal reductase activity (27). From this result, we thought that mouse RDH14 may also have an activity to produce 11-cis retinal from its retinol in the absence of aldehydes but in the presence of added NADP⁺. To test this possibility, we measured NADP⁺-dependent 11-cis retinol oxidation activity of mouse GST-RDH14 at 37°C. Interestingly, our measurement showed that mouse GST-RDH14 oxidized 11-cis retinol to 11-cis retinal in an NADP⁺-dependent manner at the rate comparable with that attained by the AL-OL coupling reaction. Furthermore, carp GST-RDH13L, an enzyme not showing the NADP⁺-dependent oxidation of 11-cis retinol in our earlier measurement made at 25°C, showed similar NADP⁺-dependent oxidation of 11-cis retinol at 37°C. These results led us to examine the temperature-dependence of AL-OL coupling reaction and that of NADP⁺-dependent oxidation of 11-cis retinol.

The AL-OL coupling activity and the NADP⁺-dependent oxidation were measured with mouse GST-RDH14 (Fig. 8B), carp GST-RDH13L (Fig. 8C) and in carp cone membranes (Fig. 8D) at 15, 25, and 37°C. As seen in Fig. 8, in all of the samples, both reactions were temperature-dependent, but their dependence profiles were clearly different: at 15 and 25°C, the AL-OL coupling activity was significantly higher than the NADP⁺-dependent activity, while, at 37°C, these two reaction activities were almost comparable.

**DISCUSSION**

In this study, we identified carp RDH13L as a candidate of an enzyme catalyzing the AL-OL coupling reaction in carp cones (Fig. 1B). In fact, recombinant RDH13L shows the AL-OL coupling activity (Fig. 1, D and E), and in addition, its reaction mechanism (Fig. 2), substrate specificity (Fig. 3) and subcellular localization (Fig. 4) are all similar to those observed in cone membranes. However, the expression level of RDH13L in cone membranes does not seem to explain all of the coupling activity in cones (Fig. 5). Other enzyme(s) such as RDH13 (Fig. 6A) in cones could contribute to the coupling. Although carp RDH13L and carp RDH13 do not require exogenous NADP⁺ for the
 oxidation of 11-cis retinol, carp RDH13L contains tightly-bound NADP\(^+\) (Fig. 7). Mouse RDH14, which probably contains tightly-bound NADP\(^+\), could be a functional homolog of carp RDH13L (Fig. 8A). Carp RDH13L and mouse RDH14 together with carp cone membranes oxidize 11-cis retinol with the AL-OL coupling mechanism at 15, 25, and 37 °C but all of them oxidize 11-cis retinol also in an NADP\(^+\)-dependent manner without addition of aldehydes at 25 and 37 °C (Fig. 8, B–D).

**Contribution of RDH13L in the AL-OL Coupling Activity in Carp Cones**—As shown in the present study, based on the maximum specific activity of a recombinant protein (purified RDH13L-His6 expressed in Sf9 cells) and the expression level of recombinant WT RDH13 expressed in Sf9 cells (0.0074 11-cis retinal/RDH/sec) was about a half of that of WT RDH13L (0.015 11-cis retinal/RDH/sec) (Fig. 6A). If the expression level of each of these proteins is proportional to that of mRNA and if RDH13 is exclusively expressed in the inner segment in cones, the rest of 80% of the coupling activity could be mostly explained by the activity of RDH13. Unfortunately, our attempt to obtain purified carp RDH13-His6 and specific antibody against carp RDH13 was not successful, so that further study was not possible. Alternatively, it could be the case that RDH13 is present in cells other than cones and that our recombinant RDH13L-His6 showed only a partial activity, namely 20%, of endogenous RDH13L.

As shown in Fig. 8A, mouse RDH14 showed the coupling activity. Expression level of mouse RDH14 mRNA is significant in a culture cell line (661W) established from mouse cones, and the level is higher in the all-cone Nrl knock-out retina compared with the wild-type retina (28), which suggests its expression in wild-type mouse cones. In our study, carp RDH14 did not show the coupling activity appreciably. The expression level of RDH14 mRNA in carp retina is about a half of that of RDH13L (16). We do not know the expression level of RDH14 protein in carp cones, but the contribution of RDH14 to the coupling activity in carp cones could be small.

**Catalytic Mechanism of the AL-OL Coupling Reaction in Carp RDH13L**—As shown in Fig. 7, recombinant RDH13L-His6 contains tightly bound NADP\(^+\), not NADPH, at a molar ratio of almost 1:1 to RDH13L-His6. Other members of RDH family enzymes require exogenous NAD(H) or NADP(H) as a cofactor of the retinoid reduction/oxidation (27). Based on these findings, we propose that RDH13L catalyzes the AL-OL coupling using tightly bound NADP\(^+\). In this study, we showed that the AL-OL coupling catalyzed by RDH13L-His6 proceeds through the sequential mechanism (Fig. 2A) as in cone membranes: both aldehyde and retinol substrates must bind to the enzyme before the products are formed and released. We previously proposed that there are two substrate binding sites in a postulated enzyme in cone membranes, one for a retinol and the other for an aldehyde (17). Taken together, the reaction mechanism of the AL-OL coupling could be as follows. The tightly bound NADP\(^+\) is situated at the very vicinity of, probably between, the two substrate binding sites, and a ternary complex of retinol-NADP\(^+\)-aldehyde is formed to couple oxidation of a retinol and reduction of an aldehyde. One proton and two electrons are transferred from retinol to aldehyde via NADP\(^+\) to form corresponding retinal and alcohol products. Formed retinal dissociates from the retinol binding site and formed alcohol also dissociates from the aldehyde binding site. However, bound NADP\(^+\) does not dissociate from its binding site under the condition that allows AL-OL coupling (see below). This mechanism explains the sequential reaction mechanism, 1:1 product formation of a retinal and an alcohol, and no needs of addition of exogenous cofactors. It is interesting that this coupling reaction rather preferably takes place below 25 °C (Fig. 8, see below).

Although carp RDH13 and RDH13L showed the AL-OL coupling activity (Fig. 6A), mouse GST-RDH13 did not show the activity significantly (Fig. 8A). Our previous molecular phylogenetic tree analysis suggested that carp RDH13 and RDH13L are grouped together with mouse RDH13 rather than with mouse RDH14 (16). Because carp RDH14 did not show the coupling activity significantly, functional classification of RDH13 and RDH14 in carp and mice is not so simple.

**Dual Mechanism of Oxidation of 11-cis Retinol in Carp RDH13L, Mouse RDH14, and Carp Cone Membranes**—At 15–25 °C, oxidation of 11-cis retinol is mainly attained by the AL-OL coupling reaction, while at 37 °C, the oxidation is catalyzed by both the coupling reaction and the conventional NADP\(^+\)-dependent reaction (Fig. 8). This finding raises an interesting question how the reactions proceed in the enzyme(s). We suggest that NADP\(^+\) binds firmly to the responsible enzyme regardless of the temperature we employed, and that NADPH dissociates from the enzyme easily at 37 °C.

RDH13L-His6 retains NADP\(^+\), not NADPH (Fig. 7), and catalyzes the AL-OL coupling reaction at 15–37 °C. In the case of the AL-OL coupling reaction, we assume that a retinol-NADP\(^+\)-aldehyde ternary complex is formed in the enzyme, and predict that the bound NADP\(^+\) would be transiently reduced to NADPH by a retinol substrate in the course of the reaction. However, this NADPH would not be released from the enzyme, possibly because formed NADPH is oxidized quickly to NADP\(^+\) by an aldehyde in the ternary complex. As a result, NADP\(^+\) stays at its binding site at 15–37 °C throughout the AL-OL coupling reaction period. In the absence of an aldehyde, the ternary complex is not formed. In this case, a retinol substrate first reacts to the bound NADP\(^+\), and NADP\(^+\) is reduced to NADPH to produce a retinal product. NADPH thus produced could be released from the enzyme at 37 °C. If it is the case, exogenous NADP\(^+\) is required to continue the reaction. NADP\(^+\)-dependent oxidation of 11-cis retinol does not take place at 15 °C, possibly because either formed NADPH is not released from the enzyme or the reaction of a retinol substrate to NADP\(^+\) does not take place at this temperature. Obviously, further study is required to fully understand the mechanisms of these reactions.

**Physiological Relevance of the Dual Mechanism of the Synthesis of 11-cis Retinol in Cones**—Dual mechanism of 11-cis retinol formation provides biological advantages for carp and mice.
RDH13L as an Enzyme Catalyzing the AL-OL Coupling Reaction

AL-OL coupling activity is localized in the cone inner segment where mitochondria are densely packed. Some aldehydes having a long carbon chain are produced in mitochondria under oxidative stress (29) and these are good aldehyde substrates in the AL-OL coupling reaction (17). It is possible that the coupling reaction acts as a scavenger of those aldehydes and produces 11-cis retinal from 11-cis retinol transported from Müller cells. The product, 11-cis retinal, could be sent to the outer segment through the binding to opsin (17). This mechanism can be utilized at a temperature range of 15–37 °C (Fig. 8). The NADP⁺-dependent mechanism can be utilized at the temperature above 25 °C as shown. Thanks to the presence of the dual mechanisms depending on the availability of the substrates, aldehydes or NADP⁺, the mechanisms preferentially at lower temperatures and either of the reactions at higher temperatures. Mice can use either of reactions at their body temperature (38 °C). Because cones function in the light where the rate of bleaching of the pigment should be very high, the effective mechanisms to produce 11-cis retinal would help cones function in the light.

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