Characterization of subunit interactions in the hetero-oligomeric retinoid oxidoreductase complex.

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Running title: subunit interactions in the retinoid oxidoreductase complex

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ABSTRACT

The hetero-oligomeric retinoid oxidoreductase complex (ROC) catalyzes the interconversion of all-trans-retinol and all-trans-retinaldehyde to maintain the steady-state output of retinaldehyde, the precursor of all-trans-retinoic acid that regulates the transcription of numerous genes. The interconversion is catalyzed by two distinct components of the ROC: the NAD(H)-dependent retinol dehydrogenase 10 (RDH10) and the NADP(H)-dependent dehydrogenase reductase 3 (DHRS3). The binding between RDH10 and DHRS3 subunits in the ROC results in mutual activation of the subunits. The molecular basis for their activation is currently unknown. Here, we applied site-directed mutagenesis to investigate the roles of amino acid residues previously implied in subunit interactions in other SDRs to obtain the first insight into the subunit interactions in the ROC. The results of these studies suggest that the cofactor binding to RDH10 subunit is critical for the activation of DHRS3 subunit and vice versa. The C-terminal residues 317-331 of RDH10 are critical for the activity of RDH10 homo-oligomers but not for the binding to DHRS3. The C-terminal residues 291-295 are required for DHRS3 subunit activity of the ROC. The highly conserved C-terminal cysteines appear to be involved in inter-subunit communications, affecting the affinity of the cofactor binding site in RDH10 homo-oligomers as well as in the ROC. Modeling of the ROC quaternary structure based on other known structures of SDRs suggests that its integral membrane-associated subunits may be inserted in adjacent membranes of the endoplasmic reticulum (ER), making the formation and function of the ROC dependent on the dynamic nature of the tubular ER network.

Keywords: retinol, short-chain dehydrogenase/reductase, retinaldehyde, retinoic acid, vitamin A

Introduction
As we have reported previously, the hetero-oligomeric retinoid oxidoreductase complex (ROC) is composed of at least two subunits of each: human retinol dehydrogenase 10 (RDH10) and human dehydrogenase reductase 3 (DHRS3) [1]. RDH10 and DHRS3 share 50% sequence similarity and belong to the 16C family of the short-chain dehydrogenase/reductase (SDR) superfamily of proteins [2, 3]. RDH10 and DHRS3 also exist as homo-oligomers. Both proteins are associated with the membranes of endoplasmic reticulum, mitochondria-associated membranes, and lipid droplet membranes [4]. In vitro, RDH10 homo-oligomers catalyze interconversion of all-trans-retinol and all-trans-retinaldehyde with NAD(H) as the preferred cofactor, whereas DHRS3 homo-oligomers display negligible catalytic activity [2]. However, upon binding to RDH10, DHRS3 acquires a robust catalytic activity, catalyzing interconversion of all-trans-retinol and all-trans-retinaldehyde with NADP(H) as the preferred cofactor. Thus, the RDH10 activity in the ROC can be assessed by utilizing NAD(H) as cofactor, whereas the activity of DHRS3 subunit towards retinol or retinaldehyde can be measured in vitro by using NADP(H).

Gene knockout studies demonstrated that inactivation of either RDH10 or DHRS3 disrupts the normal process of all-trans-retinoic acid (RA) biosynthesis and results in mid- to late-gestation embryonic lethality [2, 5-7]. RA regulates gene expression through binding to nuclear transcription factors, retinoic acid receptors, and its concentration in tissues and cells is tightly controlled as either too much or too little of RA is equally harmful [reviewed in ref. 8]. RA is produced from the cellular stores of retinol in two steps: first, retinol is oxidized reversibly to retinaldehyde, and then retinaldehyde is oxidized irreversibly to RA [reviewed in ref. 3]. Thus, the flux from retinol to retinaldehyde, the immediate precursor of RA, has to be controlled in order to maintain the RA homeostasis. As we have reported previously, the ROC fulfills this function because of the differential cofactor preferences of its subunits, RDH10 and DHRS3 [1, 2].

In most types of cells, NAD⁺ and NADPH are the predominant forms of each cofactor [9]. Consequently, the NAD⁺-preferring RDH10 functions in the oxidative direction [10], oxidizing retinol to retinaldehyde, whereas the NADPH-dependent DHRS3, activated through binding to RDH10 in the ROC, functions in the reductive direction [2], reducing retinaldehyde back to retinol. In intact living cells, this creates a biological circuit that controls and stabilizes the flux from retinol to retinaldehyde to maintain RA homeostasis. When the circuit is disrupted by substituting wild-type DHRS3 for its catalytically inactive Y188A mutant, the RA output in the cells increases several-fold for two reasons: 1) DHRS3 can no longer convert retinaldehyde to retinol; 2) the activation of subunits in the ROC is mutual, and the inactive DHRS3 continues to activate RDH10, thereby raising the flux from retinol to retinaldehyde and hence to RA [2]. Importantly, the mutual activation of the two antagonistic components of the ROC makes the output of retinaldehyde largely independent of the concentration of individual ROC subunits [1], which might vary among individuals. This further ensures the robustness of RA homeostasis.
The majority of SDRs have either homo-dimeric or homo-tetrameric quaternary structures [11]. The ROC is a rare example of a hetero-oligomeric SDR complex. To our knowledge, the only other example of an SDR hetero-oligomer known to date is the complex formed by 17β-hydroxysteroid dehydrogenase type 8 (HSD17B8) and carbonyl reductase type 4 (CBR4) [12]. This complex was termed human 3-ketoacyl-ACP reductase/3R-hydroxyacyl-CoA dehydrogenase (HsKAR). The recently solved X-ray structure of HsKAR showed that it is a dimer of HSD17B8 and CBR4 dimers. Interestingly, one set of homodimers exhibits preference for NAD\(^+\) as cofactor (HSD17B8), and the other – for NADPH (CBR4), suggesting possible similarities in the structure-function relationships between the hetero-oligomeric HsKAR and the hetero-oligomeric ROC.

To date, very little is known about the structure-function relationships in the SDR hetero-oligomeric complexes. Considering that the function of the ROC is essential for the maintenance of RA homeostasis, we undertook a series of mutagenesis studies to obtain the first insight into the properties of the human ROC subunits and the molecular basis for their mutually activating interaction.

Materials and Methods

Expression constructs

The constructs encoding human DHRS3 with the C-terminal FLAG tag (DHRS3-FLAG-pCMV-Tag-4a) and human RDH10 with the C-terminal 3x HA tag (RDH10-3xHA-pIREShrGFP-2a) for expression in HEK 293 cells and Sf9 cells have been described previously [2]. The cofactor binding site mutants and deletion mutants were generated using primers listed in Table S1, and constructs carrying wild type DHRS3 and RDH10 cDNA sequences as templates.

To generate N-linked glycosylation consensus motifs NXT or NXS, amino acid substitutions (DHRS3-H169T, DHRS3-F238T and RDH10-G190S) were introduced via site-directed mutagenesis using wild type untagged constructs in pIRES-neo vector for DHRS3 and in pCMV-Tag-4a for RDH10 as templates, and Pfx-polymerase (Invitrogen) and primers listed in Table S1.

Determination of Kinetic Constants

RDH10 and/or DHRS3 variants were expressed in Sf9 cells and microsomal fractions were isolated as previously described [13]. To examine enzyme activities, microsomes were resuspended in Reaction Buffer (90 mM potassium phosphate, pH 7.4, 40 mM potassium chloride). The \(K_m\) values for oxidation of retinol were determined at a fixed concentration of NAD\(^+\) as indicated and varied concentrations of retinol (0.032-8 µM); for reduction of retinaldehyde, values were determined at a fixed NADH concentration as indicated and varied concentrations of retinaldehyde (0.0625-8 µM). Each \(K_m\) determination was repeated at least three times. A control without added cofactor was included with each experiment. The apparent \(K_m\) values for cofactors were determined with six concentrations between 0.5–20 mM for NAD\(^+\).
and 0.1–4 mM for NADH. Initial velocities (nmol of product formed/mg of protein) were obtained by linear regression. The amount of product formed was less than 15% within the 15-min reaction time and was linearly proportional to the amount of microsomes added. The reactions were carried out in the dark at 37 °C for 15 min. Substrates and reaction products were extracted with hexane and quantified via normal phase HPLC as previously described [10].

Expression and activity assays in HEK 293 cells

HEK 293 cells (American Type Culture Collection, Manassas, VA) were cultured and maintained as monocultures at 37 °C and 5% CO₂ in complete medium (minimal essential medium containing 10% horse serum and penicillin/streptomycin). Expression constructs encoding DHRS3 and/or RDH10 variants were transfected into HEK 293 cells using Invitrogen Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) as previously described [10]. For control transfections, pCMV-Tag-4a, pIREShrGFP-2a, or both vectors in combination were transfected into cells. Approximately 24 h after transfections, growth medium was supplemented with 2 µM all-trans-retinol or 5 µM all-trans-retinaldehyde and the cells were cultured for an additional 7–8 h or 4 h, respectively. Retinoids were extracted from cell medium and quantified by normal phase HPLC as described previously [10].

Western blots and antibodies

Proteins from HEK 293 cells extracts or Sf9 microsomes were separated by SDS-PAGE and transferred to Amersham Hybond P PVDF membranes (GE Healthcare, Little Chalfont, UK). Membranes were probed with rabbit polyclonal DHRS3 antibodies (Proteintech Group, Inc., Rosemont, IL); rabbit polyclonal RDH10 antibodies (Proteintech Group, Inc.); and monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich, St. Louis, MO) at dilutions described in figure legends. Antibodies were diluted in TBST supplemented with 4% BSA. Amounts of protein loaded on gels varied as described in figure legends. The amounts of mutant variants were compared using UN‑SCAN‑IT gel Version 7.1 (Gel Analysis Software, Silk Scientific, Inc., Orem, UT).

Immunoprecipitations

Co-immunoprecipitation of ROC complex was performed as previously described [1] with minor modifications. Recombinant RDH10-HA was isolated by incubating 30 µg of Sf9 cell microsomes with 15 µl of Pierce Anti-HA Agarose (Thermo Fisher Scientific) overnight at 4 °C. Agarose was washed with wash buffer (RIPA buffer supplemented with 1 M NaCl) for 30 min at 27 °C. Washes were repeated for a total of 120 bead volumes of wash buffer. Agarose was resuspended in SDS-PAGE loading buffer and proteins were eluted by heating agarose to 90 °C for 20 min. Proteins were separated via SDS-PAGE and examined by Western blotting.
Alkaline and detergent extractions

Alkaline extractions were performed as previously described [14]. Briefly, 10 µg of microsomes from Sf9 cells expressing RDH10-HA3, DHRS3-FLAG, or RDH10-HA3 + DHRS3-FLAG were re-suspended in 100 µL of either PBS, 1% Triton X-100 in PBS, or extraction buffer (100 mM sodium carbonate, 25 mM potassium acetate, pH 11.5) and incubated on ice for 30 min. Samples were then loaded onto 100 µL-cushions of 0.5 M sucrose prepared in extraction buffer, PBS, or 1% Triton/PBS and centrifuged at 200,000 x g for 1 h at 4 °C. Pellets were resuspended in 20 µL of SDS-PAGE loading buffer. The supernatants were incubated with 50% trichloroacetic acid on ice for 30 min and centrifuged for 5 min at 12,000 x g at 4 °C. Pellets were washed two times with ethyl ether, dried, and resuspended in 20 µL of SDS-PAGE loading buffer for western blot analysis.

Results

Intact cofactor binding domains are required for the mutual activation of the ROC subunits.

Our previous studies showed that substitution of the catalytic tyrosine residues with alanines in either RDH10 or DHRS3 does not affect their ability to activate each other in the ROC: the inactive Y188A mutant of DHRS3 increased the activity of WT RDH10 and vice versa, the inactive Y210A mutant of RDH10 activated WT DHRS3 [2]. Here, we investigated whether mutations in the cofactor binding sites affect the mutual activation of the ROC subunits. All three of the cofactor binding glycines in RDH10 (G43, G47, and G49) and two in DHRS3 (G49 and G51) were substituted for alanine residues. The resulting constructs encoded HA-tagged RDH10-G43/47/49A and FLAG-tagged DHRS3-G49/51A. The mutant and WT RDH10 and DHRS3 were co-expressed in different combinations in Sf9 cells using the baculovirus expression system.

The mutant variants of both proteins were expressed at the levels comparable to WT proteins (Fig. 1 A). To assess the subunit activities in the ROC, the Sf9 microsomes were incubated either with all-trans-retinaldehyde (RAL) and NADPH (DHRS3 activity) or with all-trans-retinol (ROL) and NAD+ (RDH10 activity). Remarkably, while DHRS3 complexed with WT RDH10 displayed a robust activity, the DHRS3 subunit activity in the ROC containing RDH10-G43/47/49A was undetectable (Fig. 1 B). This was not due to the lack of binding between the WT DHRS3 and RDH10-G43/47/49A, because as we have reported previously, the introduced mutations of the glycine residues did not disrupt the ROC formation [15]. Likewise, the activity of RDH10 subunit in the ROC was not enhanced by its binding to DHRS3-G49/51A variant with a mutated cofactor binding site (Fig. 1 C and Table 1). Thus, unlike the WT RDH10 and DHRS3 or their respective catalytically impaired mutants, i.e., Y210A and Y188A, respectively, the mutants defective in cofactor binding were unable to activate the WT partners in the ROC, strongly
suggesting that intact cofactor binding sites were required for the mutual activation of the ROC subunits.

**The C-terminal residues 317-331 are required for the activity of RDH10 homo-oligomers but not for binding to DHRS3.**

The X-ray structure of the *Hs*KAR showed that binding between HSD17B8 and CBR4 occurs via the corresponding β7 β-strands from each subunit (Fig. S1), which associate in an anti-parallel manner. Two helices (α7) from both subunits are also aligned with each other. The β7 β-strands and α7 helices are located at the end of the C-termini of HSD17B8 and CBR4 polypeptides. If, as suggested by the structural alignments of RDH10 with HSD17B8 and DHRS3 with CBR4 (Figs. S2 and S3), RDH10 and DHRS3 are organized in a similar manner in the ROC, then the binding and interactions within RDH10-DHRS3 heterodimer would occur through their respective C-termini.

To test this hypothesis, we generated C-terminally truncated mutants of each protein and investigated their mutual activation, resulting from the subunit interactions, by co-transfecting different combinations of variants into HEK 293 cells (Figs. 2 and 3). For RDH10, expression construct RDH10∆1 encoded a 331-amino acid polypeptide that lacked the C-terminal 10 residues, whereas RDH10∆2 encoded a shorter 316-amino acid variant lacking 25 amino acids (Fig. 2 A). For DHRS3, expression construct DHRS3∆1 encoded residues 1-295 while the shorter DHRS3∆2 construct encoded residues 1-290 (Fig. 3 A).

Expression of RDH10 variants was confirmed by western blot analysis (Fig. 2 B). Since RDH10 homo-oligomers are catalytically active, we first investigated the impact of the C-terminal truncations on the activity of RDH10 homo-oligomers. The transfected cells were incubated with all-trans-retinol, and the cells and culture medium were collected for analysis of retinoid metabolites. The cells expressing the longer variant, RDH10Δ1, produced significantly more of all-trans-retinaldehyde and RA compared to mock transfected cells (Fig. 2, C and D). This was similar to the full-length RDH10 [10] and suggested that residues 332-341 were not required for the enzymatic function of RDH10 homo-oligomers. In comparison, the cells expressing RDH10Δ2 produced retinaldehyde and RA at levels only slightly above the levels measured in empty vector-transfected cells (Fig. 2, C and D). In part, the lower retinol dehydrogenase activity in cells expressing RDH10Δ2 could be due to the 4.4-fold lower levels of RDH10Δ2 as determined by densitometry (Fig. 2 B). This strongly suggests that residues 317-331 might be essential for the stability of RDH10 homo-oligomers and that RDH10Δ2 homo-oligomers have a very low catalytic activity due to increased protein turnover.

To investigate the role of the RDH10 C-terminus in the function of hetero-oligomeric complex, RDH10 deletion mutants were co-expressed with WT DHRS3 to allow the formation of the ROC (Fig. 2 B). Co-expression of the longer variant RDH10Δ1 with WT DHRS3 showed that the conversion of ROL to RAL and RA was reduced compared to cells expressing only RDH10Δ1
(Fig. 2, C and D). This suggested that RDH10Δ1 stimulated DHRS3 activity, which in turn, catalyzed the conversion of RAL back to ROL, reducing the flux of ROL to RAL and RA. Interestingly, co-expression of the less active RDH10Δ2 variant with DHRS3 raised the flux from ROL to RAL (Fig. 2 C), indicating that the shorter RDH10Δ2 retained its ability to bind and become activated by DHRS3. In addition, co-expression with DHRS3 appeared to stabilize RDH10Δ2 protein, increasing its levels in the cells (Fig. 2 B). In agreement with the binding of RDH10Δ2 to DHRS3, the flux from ROL to RAL and RA was similar to that observed in the cells co-expressing DHRS3 with RDH10Δ1 (Fig. 2, C and D). Thus, while the homo-oligomers of RDH10Δ2 were catalytically inactive, RDH10Δ2 appeared to retain its capacity for binding to DHRS3 and reciprocal activation similarly to the longer RDH10Δ1 variant.

To directly measure whether DHRS3 is activated by RDH10 truncation mutants, cells expressing DHRS3 alone or in combination with RDH10Δ1 or RDH10Δ2 (Fig. 2 E) were incubated with RAL as substrate for DHRS3. Consistent with our previous report, DHRS3 expression alone did not result in increased ROL production (Fig. 2 F). However, co-expression of DHRS3 with either RDH10Δ2 or RDH10Δ1 resulted in increased conversion of RAL to ROL compared to control cells (Fig. 2 F), although the corresponding decrease in RA production was insignificant (Fig. 2 G). Taken together, these results indicated that RDH10 residues 317-331 were required for the full catalytic potential of RDH10 homo-oligomers, but were not required for the binding to DHRS3 and stimulation of its retinaldehyde reductive activity in the ROC.

**The C-terminal residues 291-295 are required for DHRS3 subunit activity of the ROC.**

The structural alignment of DHRS3 with CBR4 suggested that DHRS3 residues 287 and above might be involved in the binding to RDH10 (Fig. S3). To investigate the significance of the DHRS3 C-terminal residues, we prepared two constructs: DHRS3Δ1, which encoded residues 1-295, and DHRS3Δ2, which encoded residues 1-290 (Fig. 3 A).

DHRS3 homo-oligomers are inactive; therefore, to evaluate the impact of truncations on the function of DHRS3, DHRS3Δ1 or DHRS3Δ2 were co-expressed with RDH10 in HEK 293 cells (Fig. 3 B). The cells were cultured in medium supplemented with ROL. As expected, expression of WT RDH10 alone resulted in increased conversion of ROL to RAL and RA compared to mock-transfected control cells (Fig. 3, C and D). Co-expression of RDH10 with the longer DHRS3Δ1 resulted in decreased RA production compared with RDH10-expressing cells, indicating that like WT DHRS3, truncated DHRS3Δ1 was activated by RDH10, and it reduced the flux of retinol to RA by converting RAL back to ROL. Unexpectedly, co-expression of RDH10 with DHRS3Δ2, which is shorter than DHRS3Δ1 by 5 residues, resulted in an increased rather than decreased flux of ROL to RA through the ROC (Fig. 3, C and D). This observation is consistent with interpretation that DHRS3Δ2 was unable to reduce RAL to ROL, but it was still able to bind and activate RDH10 thus increasing the flux from ROL to RAL through the ROC. This finding is similar to the stimulation of RDH10 activity by a catalytically inactive Y188A form of DHRS3 [2].
Indeed, when the cells co-expressing DHRS3Δ2 variant with WT RDH10 (Fig. 3 E) were incubated with RAL, the substrate for DHRS3, there was no increase in production of ROL from RAL, confirming that DHRS3Δ2 was not catalytically active. In contrast, the cells co-expressing DHRS3Δ1 variant with WT RDH10 produced ~2-fold more ROL from RAL and ~ 3-fold less RA (Fig. 3 F and G), indicating that DHRS3Δ1 was active as a retinaldehyde reductase. Thus, the C-terminal residues 291-295 were essential for the catalytic activity of DHRS3 subunits in the ROC, but not for their binding to RDH10.

**Conserved C-terminal cysteines are essential for maintaining the integrity of the cofactor binding sites.**

Experiments described above identified the C-terminal segments in RDH10 and DHRS3 that were essential for the activity of RDH10 homo-oligomers and the activity of DHRS3 subunits in the ROC. Alignment of RDH10 and DHRS3 sequences revealed that these segments contain a highly conserved Cys residue, Cys^{320} in RDH10 and Cys^{294} in DHRS3 (Fig. S4). To investigate the roles of these residues, the corresponding cysteines in RDH10 and DHRS3 were substituted for serines by site-directed mutagenesis to generate HA-tagged RDH10-C^{320}S and FLAG-tagged DHRS3-C^{294}S. The constructs were expressed in Sf9 cells, and microsomes containing the recombinant proteins were isolated for assays. Stability of mutant forms of RDH10 and DHRS3 expressed in Sf9 microsomes was confirmed via Western blotting (Fig. 4 A).

Since only RDH10 homo-oligomers but not DHRS3 homo-oligomers display catalytic activity, we examined the activity of RDH10-C^{320}S-containing Sf9 microsomes. Surprisingly, the microsomes containing RDH10-C^{320}S homo-oligomers displayed no appreciable activity when incubated with 2 µM retinol and 1 mM NAD^+, our standard assay conditions for WT RDH10 (Fig. 4 B). However, when co-expressed with either WT DHRS3 or DHRS3-C^{294}S, RDH10-C^{320}S subunit was activated by DHRS3 nearly to the same extent as WT RDH10 (Fig. 4 B), indicating that binding to DHRS3 partially restored RDH10-C^{320}S activity in the ROC.

The effect of RDH10-C^{320}S on DHRS3 subunit activity in the ROC was examined using 2 µM retinaldehyde and 1 mM NADPH as co-substrates. RDH10-C^{320}S activated WT DHRS3 similarly to WT RDH10 (Fig. 4 C). Interestingly, DHRS3-C^{294}S subunit appeared to display a higher activity in the ROC with either WT RDH10 or RDH10-C^{320}S as partner proteins. Thus, both RDH10 and DHRS3 Cys mutants were reciprocally activated upon forming the ROC, similar to their respective WT variants.

To determine whether the lack of activity of RDH10-C^{320}S homo-oligomers under normal assay conditions was due to altered kinetic parameters, we gradually increased the concentrations of retinol or NAD^+ in the reaction mixture (Table S2). These assays demonstrated that increasing the concentration of retinol from 2.5 µM to 10 µM had no effect on the rate of the reaction. However, increasing the concentration of NAD^+ from 1 mM to 10 mM resulted in a 3-fold increase in the reaction rate. This observation suggested that the $K_m$ value of RDH10-C^{320}S for
all-trans-retinol did not change, but the $K_m$ value for NAD$^+$ might have increased. To determine whether this was the case, we performed kinetic analysis of RDH10-C$_{320}$S with variable concentrations of NAD$^+$ in the presence of fixed 10 µM retinol. Indeed, the $K_m$ value of RDH10-C$_{320}$S for NAD$^+$ (2.78 mM) increased ~28-fold compared to the $K_m$ value of WT RDH10 (0.1 mM) (Table 1). The $K_m$ value of RDH10-C$_{320}$S for NADH (0.13 mM) also increased ~12-fold compared to WT RDH10 (0.011 mM) [2]; whereas the $K_m$ value for all-trans-retinaldehyde (0.24 µM) remained essentially unchanged (0.46 µM for WT RDH10). These assays demonstrated that Cys-320 mutation indirectly affected the cofactor binding by RDH10, likely through altered inter-subunit communications.

Interestingly, the $K_m$ value of RDH10-C$_{320}$S subunit in the ROC for NAD(H) was lower than that of RDH10-C$_{320}$S homo-oligomers (~3-fold down to 0.97 mM for NAD$^+$ and ~1.4-fold down to 0.09 mM for NADH), suggesting that binding to DHRS3 restored the $K_m$ values for cofactors to some extent, but was insufficient to fully restore the WT values. These observations suggested that the C-terminal Cys-320 was essential for maintaining the integrity of the cofactor binding site in RDH10 homo-oligomers as well as in the ROC.

Prior studies suggested that Cys residues are important for subunit interactions in SDR homo-oligomers [16]. To assess the roles of DHRS3 Cys$^{294}$ and RDH10 Cys$^{320}$ in the ROC formation, microsomes containing the recombinant proteins were subjected to HA immunoprecipitation to enrich HA-tagged RDH10. Immunoprecipitation of HA-tagged RDH10 also enriched FLAG-tagged WT DHRS3 as well as DHRS3-C$_{294}$S (Fig. 4 D). Thus, substitution of DHRS3 Cys$^{294}$ for Ser did not disrupt protein-protein interactions between RDH10 and DHRS3. Likewise, immunoprecipitation of HA-tagged RDH10-C$_{320}$S pulled down both FLAG-tagged WT DHRS3 as well as DHRS3-C$_{294}$S (Fig. 4 D). These assays demonstrated that, in agreement with mutual activation of RDH10-C$_{320}$S and DHRS3 Cys$^{294}$ subunits in the ROC, the mutation of the Cys residues did not prevent the binding of the two partner proteins. Thus, the conserved cysteines in RDH10 and DHRS3 appeared to be essential for inter-subunit communications that affect the affinity of the cofactor binding sites, but not for the binding of the subunits in the ROC.

The subunits of the ROC are integral membrane proteins with their active sites facing the cytoplasm.

A major difference between the hetero-oligomeric HsKAR and the ROC is that the subunits of HsKAR are soluble proteins whereas RDH10 and DHRS3 are associated with membranes. To understand how RDH10 and DHRS3 can form a complex, we investigated the type of membrane association for each protein. Alkaline and detergent extractions of Sf9 microsomes containing RDH10 or DHRS3 were performed to determine whether RDH10 and DHRS3 are integral or peripheral membrane proteins. The two proteins displayed similar properties, resisting extraction with sodium carbonate and being only partially solubilized by treatment with 1% Triton X-100 (Fig. 5 A). Thus, both RDH10 and DHRS3 behaved as integral membrane proteins spanning the
lipid bilayer. Indeed, this result is in a good agreement with transmembrane prediction plots for RDH10 and DHRS3 (Fig. S5). RDH10 protein is predicted to have two transmembrane helices composed of amino acids 5-27 and 34-53. Amino acids 1-4 and 54-341 are predicted to face the cytoplasm, with residues 28-33 located in the ER lumen. DHRS3 protein is predicted to have one transmembrane helix spanning amino acids 7-29, with residues 1-6 located inside the ER lumen and residues 30-302 located in the cytoplasm. To determine the orientation of RDH10 and DHRS3 in the membrane, we applied an N-glycosylation scanning mutagenesis approach. N-Glycosylation of eukaryotic membrane proteins occurs cotranslationally in the lumen of the endoplasmic reticulum and is manifested by the slower electrophoretic mobility of glycosylated polypeptide. Neither RDH10 nor DHRS3 polypeptides contain native N-glycosylation consensus motifs (NxS/T); therefore, we introduced novel acceptor sites by site directed mutagenesis at DHRS3 amino acid residue 238 (F238T), and at RDH10 amino acid residue 190 (G190S). The resulting cDNAs encoded N-glycosylation consensus motif N236LT in DHRS3 and N188HS in RDH10. The constructs were expressed in HEK293 cells, and the wild type (WT) and mutant proteins were analyzed by western blotting. DHRS3-F238T and RDH10-G190S variants were both expressed and their electrophoretic mobility was similar to that of the WT proteins (fig. 5 B). This result indicated that the polypeptide segments located within 49 amino acid residues from the active site Tyr in DHRS3 (Y188CTS) and 19 amino acid residues from the active site Tyr in RDH10 (Y190CASK) (Fig. S4) are located on the cytoplasmic side of the membrane, suggesting that both the cofactor binding sites and the substrate binding sites of RDH10 and DHRS3 are facing the cytoplasm. Analysis of the three-dimensional structure of HsKAR (Fig. 6) shows that in hetero-tetramer, the N-termini of HSD17B8 and CBR4 are located on the opposite sides of the structure (shown in red in Fig. 6). In the ROC, these would be the sites attached to the corresponding trans-membrane segments. Thus, assuming that the quaternary structure of the ROC is similar to that of HsKAR, these observations suggest that the transmembrane segments of RDH10 and DHRS3 have to be inserted into adjacent membranes to form the ROC.

Discussion

This study provides the first insight into the interactions between subunits of the heterooligomeric ROC. The ROC represents a molecular mechanism for maintaining the homeostasis of a biologically active RA [1]. This mechanism operates due to the circuit created by its antagonistically bifunctional components, RDH10 and DHRS3, which simultaneously have two opposing effects on the same biological process, i.e. interconversion of retinol and retinaldehyde. An increase in the concentration of either component increases both the oxidation of retinol and the reduction of retinaldehyde, making the steady-state output of RA largely independent of the concentration of either RDH10 or DHRS3. The mutual activation of the ROC subunits is critical for enabling this mechanism to work. However, what triggers the activation of the subunits has not yet been determined. Unlike HsKAR, which is soluble, the ROC is formed by the membrane bound proteins and has not yet been isolated in a purified catalytically active form to allow
solving its X-ray structure. Therefore, we applied a mutagenesis approach to identify the residues required for the mutual activation of the ROC subunits.

As shown in this study, mutations within the cofactor binding sites in RDH10 or DHRS3 completely abolish their mutual activation. This finding is consistent with the reaction mechanism described for SDRs [11]. Typical SDR enzymes follow sequential order bi–bi reaction mechanism in which the cofactor, NAD(P)H, first binds to the cofactor-binding pocket of the enzyme followed by binding of the substrate in the C-terminal region. Upon the completion of the reaction, the product is released first followed by the release of the cofactor. The release of the cofactor was shown to be the rate-limiting step for typical SDRs.

SDR proteins have been shown to undergo structural rearrangements upon binding of the cofactor. It has been reported that the presence of nicotinamide adenine dinucleotides is critical for stabilization of the conformation of the substrate-binding loop and this region is disordered in many SDRs without a ligand [18, 19]. Binding of cofactor usually induces this region to form an ordered structure [18]. Interestingly, the crystals of HsKAR could only be obtained when cocrystallized with 2 mM NAD⁺. HsKAR tetramer was reported to have two binding sites for NAD(H) and NADP(H) with differing affinities. The stronger binding sites exhibit $K_d$ values in the micromolar range, whereas the weaker (second) binding sites display $K_d$ values in the millimolar range [12]. The authors speculated that the binding of the cofactor to one of the subunits reduces the affinity of the second binding site to millimolar levels, which would be a case of negative cooperativity.

Interestingly, kinetic characterization of the ROC subunits revealed two sets of constants for NAD(H), NADP(H), retinol and retinaldehyde. The ROC displayed low and high $K_m$ values for NADH (12 µM and 470 µM), NADPH (2.6 µM and 280 µM), all-trans-retinol (0.029 µM and 3.9 µM) and all-trans-retinaldehyde (0.15 µM and 6.8 µM) [2]. At the time, we interpreted this dichotomy as evidence for the simultaneous presence of homo-oligomers and hetero-oligomers in SF9 microsomes. However, DHRS3 homo-oligomers are inactive; thus, the cofactor-binding sites and substrate-binding sites with different $K_m$ values had to be present in the ROC.

Negative cooperativity of cofactor binding has been observed in _E. coli_ and _Plasmodium falciparum_ β-ketoacyl-[acyl carrier protein] reductase (FabG) in particular in the presence of acyl carrier protein (ACP). FabG is an allosteric enzyme whereby the binding of NADPH to one site increases the affinity at that site for the ACP-bound substrate and decreases the affinity for the cofactor at other sites [19]. It has been proposed that the movements associated with NADPH and ACP binding could be transmitted within the tetramer to promote the open conformation of the adjacent active site, thus reducing the substrate and cofactor affinities for a site where the reaction is complete. This could provide a mechanism by which product is expelled and the active site cleared to allow the binding of a NADPH molecule and β-keto-substrate for the next catalytic cycle.
In the case of the ROC, it appears that the binding of the cofactor triggers a conformational change that translates into a greater activity of the partner subunit. Binding of the cofactor is critical for starting the cycle of retinol to retinaldehyde interconversion. Normally, the concentration of retinol in the cells greatly exceeds that of retinaldehyde [20, 21]; therefore, the cycle is likely to be initiated by the binding of retinol to the ROC complexed with NAD$^+$.  

The structure of the HsKAR suggested that the hetero-oligomer is formed through the interactions between the C-termini of its subunits (Fig. S1). Therefore, we explored the roles of RDH10 and DHRS3 C-termini in the formation of the ROC. These studies demonstrated that deletion of the C-terminal 10 residues in RDH10 (RDH10Δ1) or 7 residues in DHRS3 (DHRS3Δ1) had no effect on either the binding or activity of the ROC subunits. However, deletion of 25 amino acids in RDH10 significantly reduced the activity of RDH10Δ2 homo-oligomers. At the same time, this variant retained the ability to activate DHRS3, indicating that binding was not affected. Similarly, deletion of 12 amino acids in DHRS3 abolished the activity of DHRS3Δ2 subunits in the ROC, but this variant bound and activated RDH10. The common feature between RDH10Δ2 and DHRS3Δ2 was the absence of the highly conserved C-terminal cysteine residues. Site-directed mutagenesis of these residues in RDH10 and DHRS3 showed that RDH10-C$^{320}$S displayed significantly increased $K_m$ values for NAD$^+$ and NADH, whereas DHRS3-C$^{294}$S subunit appeared to be more active than WT DHRS3 in the ROC. The binding of the cysteine mutants was not disrupted and, furthermore, appeared to normalize the RDH10-C$^{320}$S $K_m$ values for NAD(H) in the ROC to some extent compared to RDH10-C$^{320}$S homo-oligomers. This observation highlighted the mutually stabilizing effect of RDH10 and DHRS3 subunits of the ROC. Importantly, the impact of altered Cys residue on RDH10 $K_m$ values for cofactors suggests that this residue is involved in involved in inter-subunit communications in RDH10 homo-oligomers.  

Previous studies indicated that RDH10 and DHRS3 are inserted into the endoplasmic reticulum (ER) membranes via their N-termini [22-24]. Here, we have established that both RDH10 and DHRS3 are integral membrane proteins with cytoplasmic orientation of the majority of their polypeptide chains. Based on the orientation of subunits in HsKAR, this finding suggests that the dimers of RDH10 and DHRS3 would likely be inserted into apposing ER membranes through their N-termini, potentially crosslinking the membranes (Fig. 6). The ER is spread throughout the cytoplasm of eukaryotic cells and includes the nuclear envelope, peripheral ER cisternae, and an interconnected tubular network [25]. The morphology of the tubular peripheral ER has been shown to be regulated by the Reticulon family of integral membrane proteins and DP1/Yop1, which are thought to stabilize the tubular network and other regions of high membrane curvature through their unique topology and by forming higher-order oligomers [26, 27]. It is possible that the hetero-oligomers of the ROC are located between the adjacent membranes of peripheral ER tubules. The tubular ER network is very dynamic and constantly rearranges its structure along the microtubule cytoskeleton. The changes in the structure of ER tubules caused by ER stress
could in turn affect the binding between the RDH10 and DHRS3 homo-oligomers and the function of the ROC, potentially disrupting the RA homeostasis.

Recently, we reported that hepatic RDH10 and DHRS3 appear to translocate to lipid droplets during fasting [4]. We have also observed that in spite of being enriched in RDH10, the specific NAD+-dependent retinol dehydrogenase activity of lipid droplets is lower than that of the microsomal membranes. Whether this difference is due to conformational changes in RDH10 or ROC quaternary structure or changes in RDH10 interaction with DHRS3 in the single-layer round lipid droplet membrane versus double-layer membranes of tubular ER network remains to be established.

**Abbreviations:** SDR, short-chain dehydrogenase/reductase; RDH10, retinol dehydrogenase 10; DHRS3, dehydrogenase/reductase 3; ROC, retinoid oxidoreductase complex.

**Author Contributions**

M.K.A, O.V.B., L.W., K.D.R., and I.F.C. performed experiments; M.K.A, O.V.B., and N.Y.K. planned experiments; M.K.A, O.V.B., N.Y.K., and K.M.P. wrote the manuscript.

**Data availability statement**

All the data are presented in the main manuscript and supplementary information.

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**Additional information**

Supplementary Information accompanies this manuscript.

**Competing financial interests**

The authors declare no competing financial interests.

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Legends to Figures

Figure 1. Impact of abolished cofactor binding sites on mutual activation by RDH10 and DHRS3. A. Western blot analysis of microsomes (1 µg) containing wild type (WT) or cofactor binding site mutant RDH10 and DHRS3 expressed individually or in combination in Sf9 cells. Membranes were probed with anti-FLAG antibodies (1:3,000) followed by anti-RDH10 antibodies (1:3,000). B. Analysis of DHRS3 activity towards retinaldehyde with NADPH as cofactor in complex with WT or RDH10 G43/47/49A mutant. Note the lack of stimulation of DHRS3 activity by RDH10 mutant. C. Analysis of RDH10 activity towards retinol with NAD+ as cofactor in complex with WT or mutant DHRS3. Note that DHRS3 G49/51A mutant does not stimulate RDH10 activity above the WT RDH10 activity level.

Figure 2. Characterization of C-terminally truncated RDH10 variants. A. Diagram of truncation mutants. B. Western blot analysis of RDH10 and DHRS3 in total lysates (25 µg) of cells incubated with 2 µM retinol (ROL) for 8 h. RDH10 truncated mutants were expressed in HEK293 cells individually or co-transfected with WT DHRS3. A separate gel was run for detection of DHRS3 in samples co-transfected with RDH10 truncated mutants as the shorter RDH10 polypeptides overlapped with DHRS3 proteins bands and could not be visualized on the same blot. C and D. Normal phase HPLC analysis of RAL and RA levels, respectively, produced in cells incubated with ROL. E. Western blot analysis of DHRS3 and RDH10 in total lysates of cells incubated with 5 µM retinaldehyde (RAL) for 4 h. F and G. Normal phase HPLC analysis of ROL and RA, respectively, in cells incubated with RAL. **, p≤0.01; ***, p≤0.001; mean ± S.D. The predicted N-terminal transmembrane segments are indicated in blue.

Figure 3. Characterization of C-terminally truncated DHRS3 variants. A. Diagram of truncation mutants. B. Western blot analysis of RDH10 and DHRS3 in total lysates (25 µg) of cells incubated with 2 µM retinol (ROL) for 7 h. DHRS3 truncated mutants were expressed in HEK293 cells individually or co-transfected with WT RDH10. Retinoids were extracted and the amounts of produced RAL, ROL, and RA were determined by normal phase HPLC. C and D. Normal phase HPLC analysis of RAL and RA levels, respectively, produced in cells incubated with ROL. E. Western blot analysis of DHRS3 and RDH10 in total lysates of cells incubated with 5 µM retinaldehyde (RAL) for 4 h. F and G. Normal phase HPLC analysis of ROL and RA, respectively, in cells incubated with RAL. **, p≤0.01; ***, p≤0.001; mean ± S.D. The predicted N-terminal transmembrane segments are indicated in blue.

Figure 4. Analysis of the role of conserved cysteine residues in ROC formation and activity. A. Western blot analysis of wild type or mutant RDH10 and DHRS3 expressed individually or in combination in Sf9 microsomes. B and C. Activity assays of wild type and mutant RDH10 and DHRS3. B. Microsomes (2 µg) containing individual or co-expressed RDH10 and DHRS3 variants were incubated with 2 µM retinol and 1 mM NAD+. Retinaldehyde produced by RDH10 was quantified by HPLC; mean ± S.D., n=3. C. Microsomes (2 µg) containing individual or co-expressed RDH10 and DHRS3 variants were incubated with 2 µM retinaldehyde and 1 mM NADPH. Retinol produced by DHRS3 was quantified by HPLC; mean ± S.D., n=3. D. Pull down assays of interaction between wild type versus Cys mutant forms of RDH10 and DHRS3. Co-immunoprecipitations were performed on microsomes containing HA-tagged RDH10 or RDH10-C320S using HA-agarose beads and the bound DHRS3-FLAG was detected by FLAG antibodies (1:3,000). 1/20th fraction by volume of the input (I) and unbound supernatant (U), and
all of HA-beads’ eluate (E) were loaded per lane on an SDS-PAGE gel for Western blotting; star * denotes a non-specific band.

**Figure 5. Alkaline extraction (A) and glycosylation screening mutagenesis (B) of RDH10 and DHRS3.** A. Microsomal membranes were extracted with sodium carbonate buffer, pH 11.5 (Alk Extr), phosphate-buffered saline, pH 7.4 (PBS), or 1% Triton X-100 in PBS (1% Tri). Solubilized proteins (S) were separated from integral membrane proteins (P) by centrifugation. Distribution of proteins between the soluble and the membrane-bound fractions was analyzed by Western blotting as described under “Materials and Methods.” B. HEK293 cells were transfected with wild type (WT) or mutant constructs using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer’s protocol and harvested 24 h after transfection. After separation in SDS-PAGE and transfer to PVDF membranes, DHRS3 protein was detected using DHRS3 antibodies at a 1:2,000 dilution. RDH10 protein was detected using RDH10 antibodies at a 1:3,000 dilution.

**Figure 6. Space-fill representation of the tetrameric organization of HsKAR based on 4CQM coordinates.** Shown are front and side views of HsKAR heterotetramer (panels A and B, respectively). Two HSD17B8 subunits are shown in yellow (chain A) and lime green (chain D). Two CBR4 subunits are represented in teal (chain B) and aquamarine (chain C). NAD⁺ bound to HSD17B8 (chain A) is shown in blue, red, orange, and white stick model. NADP⁺ molecule bound to CBR4 (chain B) is represented in blue, white, orange, and red stick model. N-Terminal ends of chains A, B, C, and D, where corresponding transmembrane-spanning domains of RDH10 and DHRS3 would be attached, are highlighted in red. Model was created using PyMOL software (DeLano Scientific).
Table 1. Kinetic Properties of RDH10 and DHRS3 Homo-oligomeric and Hetero-oligomeric Variants

| Enzyme                  | Substrate          | Cofactor | $K_m$ for cofactor (mM) | $K_m$ for substrate (µM) | $V_{max}$ (nmol·min$^{-1}$·mg$^{-1}$) |
|-------------------------|--------------------|----------|-------------------------|--------------------------|----------------------------------------|
| RDH10                   | at-retinol         | NAD$^+$  | 0.1*                    | 0.07 ± 0.02              | 0.84 ± 0.05                            |
| RDH10                   | at-retinaldehyde   | NADH     | 0.011*                  | 0.46*                    | 1.1*                                   |
| RDH10 + DHRS3           | at-retinol         | NAD$^+$  | N.M.                    | 0.66 ± 0.08              | 6.81 ± 0.23                            |
| RDH10 + DHRS3-G$^{49,51}$A | at-retinol       | NAD$^+$  | N.M.                    | 0.27 ± 0.04              | 1.39 ± 0.05                            |
| RDH10-C$^{320}$S        | at-retinol         | NAD$^+$  | 2.78 ± 0.63             | N.D.                     | 0.47 ± 0.03                            |
| RDH10-C$^{320}$S        | at-retinaldehyde   | NADH     | 0.13 ± 0.01             | 0.24 ± 0.03              | 0.72 ± 0.02                            |
| RDH10-C$^{320}$S + DHRS3| at-retinol         | NAD$^+$  | 0.96 ± 0.03             | 0.43 ± 0.06              | 1.05 ± 0.04                            |
| RDH10-C$^{320}$S + DHRS3| at-retinaldehyde   | NADH     | 0.093±0.013             | 1.49 ± 0.25              | 10.07 ± 0.61                           |

RDH10 was expressed individually or in combination with DHRS3 to obtain ROC. Kinetic curves for RDH10 alone, RDH10+DHRS3, and RDH10+DHRS3-G$^{49,51}$A are shown in Figure 1C. The $K_m$ value of RDH10-C$^{320}$S for NAD$^+$ was measured with fixed 10 µM all-trans-retinol (at-retinol); for NADH – with 5 µM all-trans-retinaldehyde (at-retinaldehyde); and for all-trans-retinaldehyde – with 2 mM NADH. The $K_m$ value of RDH10-C$^{320}$S +DHRS3 for all-trans-retinol was measured with saturating 3 mM NAD$^+$; and for all-trans-retinaldehyde – with saturating 2 mM NADH. N.M.: not measured. All kinetic measurements were repeated at least 3 times. *, Data are from ref. [10]; *data are from ref. [2].
