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Enzymatic characterization of recombinant mouse retinal dehydrogenase type 1

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Abstract

Retinal dehydrogenases (RALDHs) convert retinal into retinoic acids (RAs), which are important signaling molecules in embryogenesis and tissue differentiation. We expressed mouse RALDH type 1 (mRALDH1) in *Escherichia coli* and studied the kinetic properties of the recombinant enzyme for retinal substrates. Purified recombinant mRALDH1 catalyzed the oxidation of all-*trans* and 9-*cis* retinal but not 13-*cis* retinal, and exhibited two pH optimums, 7.8 and 9.4, for all-*trans* and 9-*cis* retinal substrates, respectively. The K_m for all-*trans* retinal (11.6 μM) was 3-fold higher than for 9-*cis* retinal (3.59 μM). However, the conversion efficiencies of either all-*trans* or 9-*cis* retinal to the respective RAs were similar. $MgCl_2$ inhibited the oxidation of both all-*trans* and 9-*cis* retinal. Chloral hydrate and acetaldehyde competitively suppressed all-*trans* retinal oxidation with inhibition constants (K_i) of 4.99 and 49.4 μM, respectively. Retinol, on the other hand, blocked the reaction uncompetitively. These data extend the kinetic characterization of mRALDH1, provide insight into the possible role of this enzyme in the biogenesis of RAs, and should give useful information on the determination of amino acid residues that play crucial roles in the catalysis of all-*trans* and 9-*cis* retinal.

Keywords: Retinal dehydrogenase; Recombinant enzyme; Retinal; Retinoic acid

1. Introduction

The retinol metabolites all-*trans* RA and 9-*cis* RA regulate numerous biological functions by altering gene expression during embryogenesis and adulthood [1,2]. RAs function as specific ligands for nuclear receptors (retinoic acid receptors and retinoid X receptors) [3]. All-*trans* RA is the ligand for retinoic acid receptors, whereas its 9-*cis* isomer binds and activates both retinoic acid receptors and retinoid X receptors [4]. One aspect of retinoid signaling that is under intense investigation in our laboratory is the metabolic regulation of the formation of RA isomers from precursor retinoids *in vivo*. RA is biosynthesized *in vivo* from retinol by two-step oxidation that involves specific retinol and retinal dehydrogenases [5]. RALDHs that convert retinal to RA belong to the ALDH superfamily [6]. Several ALDHs purified or cloned from human, bovine, or murine tissues (RALDH1, RALDH2, and RALDH3, also known as ALDH1A1, ALDH1A2, and ALDH1A3[1]) have been shown to catalyze the oxidation of retinal to RA [7–11]. However, detailed kinetic studies on the retinal isomer substrates of these enzymes have been limited. Rat and human RALDH (rRALDH and hRALDH, respectively) and, recently, mouse RALDH2 (mRALDH2) have been found to catalyze the oxidation of all-*trans* and 9-*cis* retinal with different efficiencies [12–14]. On the other hand, mRALDH1, which is highly expressed in the mouse liver but not in the kidneys [15,16], remains largely uncharacterized as to its role in the synthesis of 9-*cis* RA.

The retinal-oxidizing activity of purified RALDH1 from the mouse liver was first demonstrated by Lee *et al.* [8], and

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Abbreviations: ALDH, aldehyde dehydrogenase; CHES, 2-*N*-cyclohexylaminoethanesulfonic acid; GST, glutathione-*S*-transferase; MES, 2-*N*-morpholinoethanesulfonic acid; RA, retinoic acid; and RALDH, retinal dehydrogenase.

[1] Unified aldehyde dehydrogenase nomenclature (http://www.uchsc.edu/sp/alcdbase/aldhcov.html).

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its cDNA was cloned [17]. mRALDH1 is detected in the embryonal stage, E9.0-E10.5, primarily in the dorsal retina, and expressed prominently in the developing lung [16,18,19]. High levels of mRALDH1 expression are seen in several adult mouse organs [16,20], suggesting that it may function in providing RA for the maintenance of adulthood.

The purpose of this work was to conduct detailed kinetic studies of mRALDH1 for retinal isomer substrates, to further characterize the recombinant enzyme with various ALDH inhibitors, and to compare its kinetic properties with those of rat and human RALDH1.

2. Materials and methods

2.1. Expression and isolation of mRALDH1 enzyme

Cloning of the coding region of mRALDH1 and its expression in *Escherichia coli* as N-terminal fusions to *Schistosoma* GST have been described previously [14]. Briefly, 1.6 kb full-length cDNA was cloned into the *Eco*RI site of pGEX-4T-2, and the resulting recombinant plasmid was transformed into *E. coli* BL-21DE3 cells. The cell pellet, obtained after induction of transfected *E. coli* with 0.5 mM isopropyl-β-D-thiogalactopyranoside, was sonicated in 20 mM Tris–HCl (pH 7.4) containing 1 mM EDTA, 100 mM NaCl, and a mixture of protease inhibitors. The lysate was centrifuged (1000 *g* at 4° for 10 min), and expressed protein was purified with a GST-affinity column, followed by excision with thrombin, as described by the manufacturer (Pharmacia Biotech). The purity of the recombinant enzyme was verified by gel electrophoresis.

2.2. Enzyme assays

RALDH assays were performed by quantitating RAs formed in the incubation mixture by HPLC, as described previously [21]. Recombinant RALDH1 was incubated at 25° for 1 hr in 100 mM phosphate buffer (pH 7.5) containing 0.02% Tween-80, 161 mM dithiothreitol, and 603 μM NAD (final volume 250 μL). Protein concentrations for the assays used were 0.3 to 0.6 μg. Reactions were initiated by the addition of retinal substrates (2–20 μM of all-*trans*, 9-*cis*, and 13-*cis* retinal in 2.5 μL dimethyl sulfoxide). After incubation, the retinoids were analyzed by HPLC. Kinetic constants were determined in hyperbolic and double-reciprocal plots under initial velocity conditions linear with protein and time.

3. Results and discussion

Gel electrophoresis of purified recombinant RALDH1 showed an expected molecular mass of 55 kDa (Fig. 1). The oxidation of all-*trans*, 9-*cis*, and 13-*cis* retinal to respective RAs by various amounts of recombinant mRALDH1 was examined at two substrate concentrations. At 10 μM retinal substrates, all-*trans* retinal showed higher activity than 9-*cis* retinal, and the reaction rates were proportional to the enzyme concentrations (Fig. 2A). However, at a lower retinal substrate concentration (2 μM), 9-*cis* retinal had higher activity than all-*trans* retinal, and the rates of RA production took a hyperbolic curve because of substrate limitation (Fig. 2B). Interestingly, recombinant RALDH1 did not show any activity towards 13-*cis* retinal oxidation (Fig. 2A and B). RALDH1 exhibited two pH optimums, 7.8 and 9.4, for all-*trans* and 9-*cis* retinal oxidation, respectively (Fig. 3A and B).

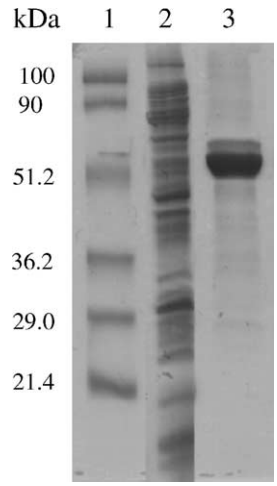

Fig. 1. Electrophoresis of recombinant mRALDH1 protein under denaturing conditions. Lane 1, molecular weight markers; lane 2, bacterial cell extracts expressing GST-mRALDH1; and lane 3, mRALDH1 purified from a GST-affinity column. Five micrograms of each protein was loaded on the gel.

To determine the kinetic constants of recombinant mRALDH1 for retinal isomers, saturation curves were charted with retinal substrates (Fig. 4). As expected, at higher substrate concentrations (7–20 μM, Fig. 4A) all-*trans* retinal exhibited the highest activity, while 9-*cis* retinal showed high activity at lower substrate concentrations (1–4 μM, Fig. 4B). At these substrate concentrations, mRALDH1 did not catalyze 13-*cis* retinal oxidation (Fig. 4A). The K_m for 9-*cis* retinal (3.59 μM) was 3-fold lower than for all-*trans* retinal (11.6 μM) (Table 1). However, no significant difference was observed in the catalytic

Table 1
Kinetic constants of mRALDH1 for retinal isomers

Retinal isomers	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m
All-*trans*	11.6	85.6	7.37
13-*cis*			
9-*cis*	3.59	38.1	10.6

K_m and V_{max} values represent the average of two independent determinations where each point on the curve of each experiment is an average of three replicates.

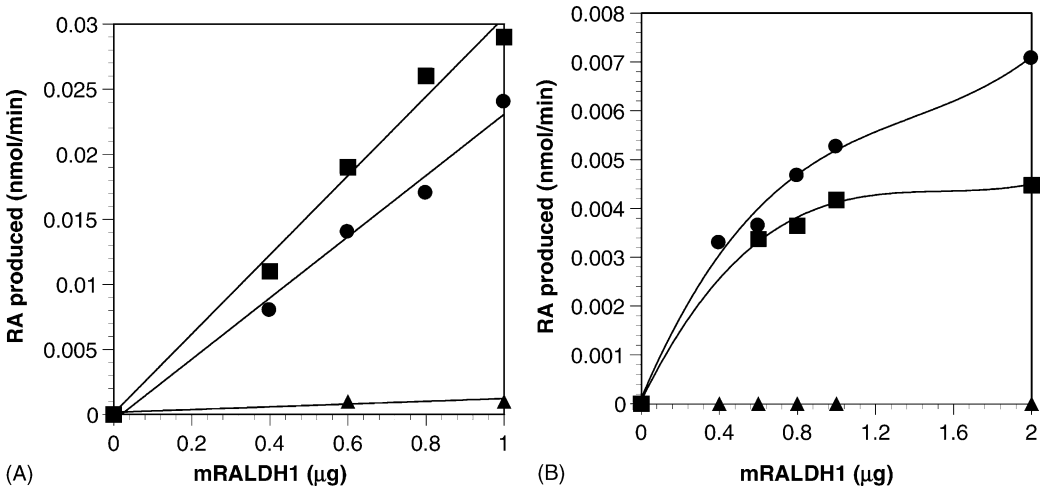

Fig. 2. Activity of purified, bacterially expressed mRALDH1 for all-*trans* (■), 9-*cis* (●), and 13-*cis* (▲) retinal oxidation. Assays were performed at 10 μM (A) and 2 μM (B) retinal substrate concentrations under standard incubation conditions. Points represent the average of triplicate experiments.

efficiency (V_{max}/K_m) of the conversion of all-*trans* and 9-*cis* retinal to the respective RAs (Table 1).

Several investigators [22–24] have reported that magnesium influences class 1 and class 2 ALDH activity. Its effect on mRALDH1 activity for retinal oxidation is unknown. Therefore, we examined the impact of $MgCl_2$ on recombinant mRALDH1 activity for all-*trans* and 9-*cis* retinal oxidation. $MgCl_2$ suppressed the oxidation of both all-*trans* and 9-*cis* retinal (Fig. 5). Inhibition was strongest at $MgCl_2$ concentrations >1 mM.

We examined mRALDH1 inhibition by chloral hydrate, acetaldehyde, and retinol, which have been demonstrated previously to block class 1 ALDHs [12,25]. Chloral hydrate was a potent inhibitor of mRALDH1 activity (Fig. 6A) and depressed all-*trans* retinal oxidation by 80% at a 100 μM concentration. Acetaldehyde and retinol suppressed the reactions by 60 and 40% at concentrations of 400 and 12 μM, respectively (Fig. 6B and C). Next, we investigated the nature of inhibition by these compounds on all-*trans* retinal oxidation. Chloral hydrate and acetaldehyde competitively suppressed all-*trans* retinal oxidation by mRALDH1 with inhibition constants of 4.5 and 49.4 μM, respectively (Fig. 7A and B). On the other hand, all-*trans* retinol inhibited the reaction uncompetitively (Fig. 7C).

We have shown previously that in spite of close amino acid sequence identity (87%), rat and human RALDH1 differ significantly in their abilities to catalyze retinal isomer substrates [12,13]. rRALDH1 has 2-fold higher catalytic efficiency for 9-*cis* retinal oxidation compared to all-*trans* retinal oxidation, and is inactive towards 13-*cis* retinal [12]. On the other hand, hRALDH1 catalyzes all

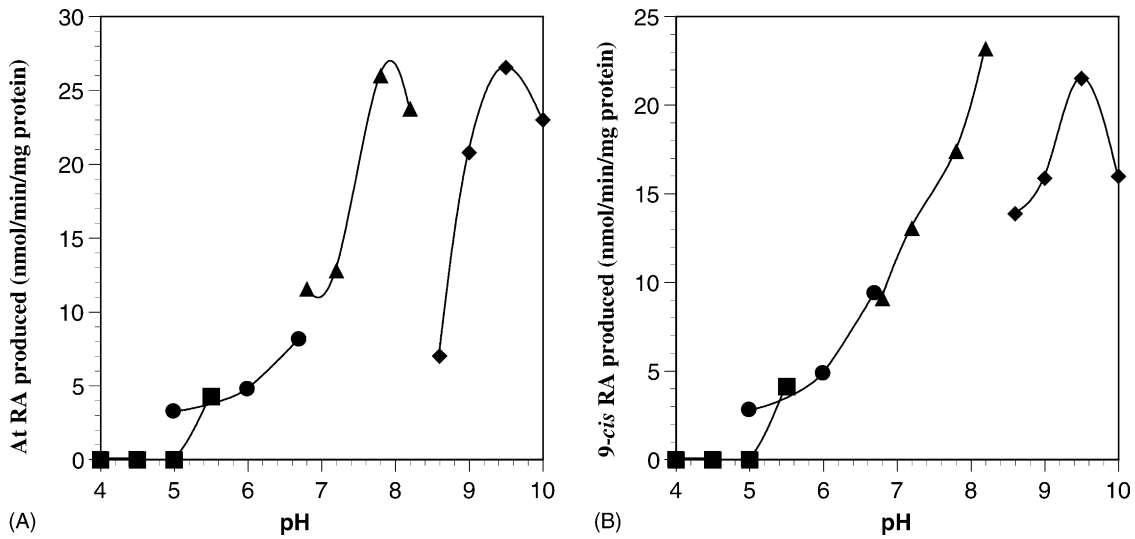

Fig. 3. Effects of pH on mRALDH1 activity. Formation of RAs was measured using 0.5 μg protein and 10 μM each of all-*trans* (A) and 9-*cis* (B) retinal. Key: (■) potassium acetate buffer, pH 4 to 5.5; (●) MES buffer, pH 5 to 6.7; (▲) HEPES buffer, pH 6.8 to 8.2; and (◆) CHES buffer, pH 8.6 to 10. All buffers were used at a 50 mM concentration. Each point is the average of triplicate experiments.

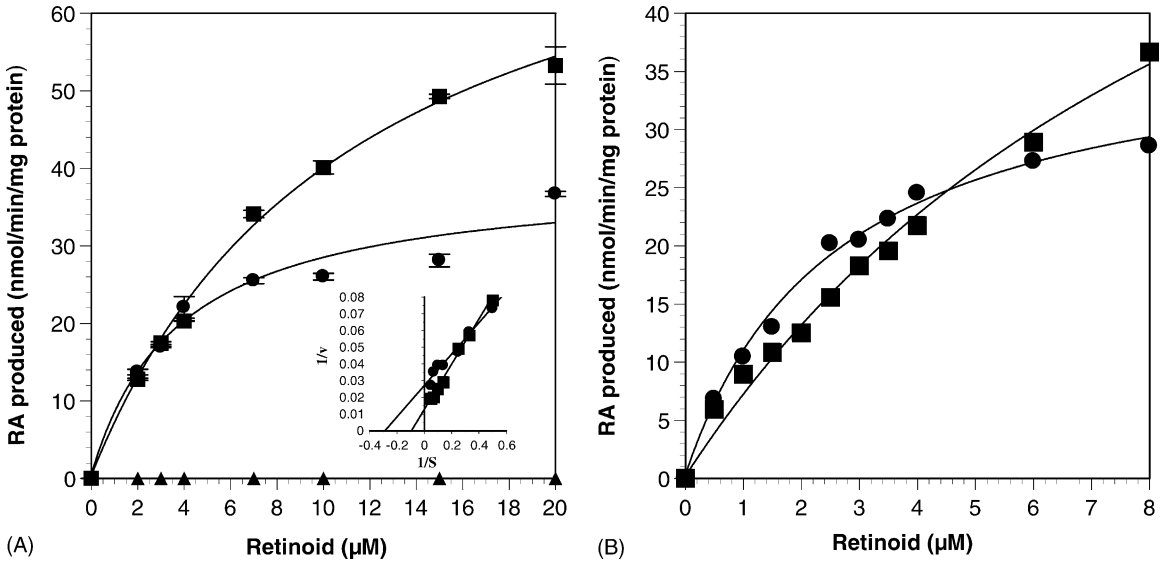

Fig. 4. Kinetics of recombinant mRALDH1 for retinal isomer substrates. Rates of reaction at 2–20 μM (A) and 0.5–8 μM (B) retinal concentrations. Key: all-*trans* (■), 9-*cis* (●), and 13-*cis* (▲) retinal. The reactions were performed with 0.3 μg protein. Each experiment was conducted twice with three replicates per point (less than 5% variation between each replicate). The inset depicts double-reciprocal Lineweaver–Burk plots.

three retinal isomers with equal efficiency [13]. Interestingly, another closely related cytosolic ALDH, namely, phenobarbital-induced ALDH, which has 89% amino acid sequence identity with rRALDH1, does not exhibit any activity for all-*trans* or 13-*cis* retinal oxidation, and has very little activity for 9-*cis* retinal [26]. Using point mutants and chimeric enzymes, we have demonstrated that the amino acids of rRALDH1, which are necessary and sufficient for the catalysis of all-*trans* retinal, differ from those required for the oxidation of 9-*cis* retinal [26].

mRALDH1 shares 96% amino acid sequence identity with rRALDH1, and is considered as the rat and human homologue. However, the patterns of tissue- and stage-specific

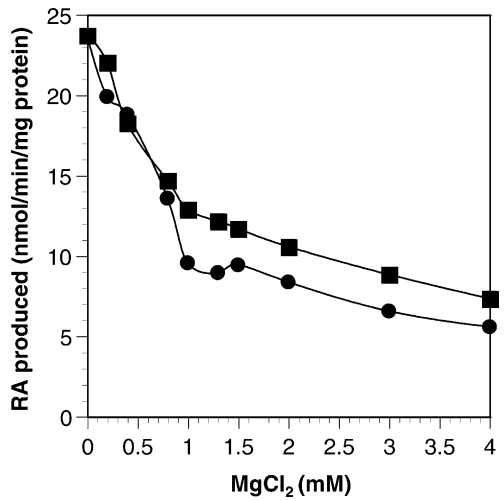

Fig. 5. Inhibitory effect of $MgCl_2$ on all-*trans* (■) and 9-*cis* (●) retinal oxidation activity of mRALDH1. The assays consisted of 0.5 μg mRALDH1 protein with 10 μM substrates under standard incubation conditions. Points are the average of duplicate experiments, each carried out in triplicate.

expression of mouse, rat, and human RALDH1 are different, suggesting a defined physiological role of these RALDHs in providing all-*trans*, 9-*cis*, and 13-*cis* RAs. We undertook a detailed kinetic study to explore whether the 4% difference in amino acid sequence of mRALDH1 results in any variation in the specificity for all-*trans* retinal and its isomers. The kinetic behavior of mRALDH1 for retinal isomers was similar to that of rRALDH1. Both mouse and rat RALDH1 catalyze all-*trans* and 9-*cis* retinal efficiently and have no activity for 13-*cis* retinal. One notable difference is that at low substrate concentrations, mRALDH1 shows higher activity for 9-*cis* retinal (Fig. 4B), and such a difference is not seen for rat and human RALDH1 [12,13]. The difference in activity for 9-*cis* and all-*trans* retinal at low and high substrate concentrations could be due to change in the configuration of the enzyme–substrate complex. It appears from our saturation kinetics experiments that a high concentration of all-*trans* retinal activates the enzyme (Fig. 4A), which results in higher V_{max} (Table 1).

The crystal structure of sheep liver ALDH1 indicates a substrate entrance tunnel that fits the structure of all-*trans* retinal [27]. The main structural elements in the substrate entrance tunnel comprise helix αC (amino acids 113–135), helix αD (amino acids 170–185), helix αH (amino acids 282–296), and a loop (amino acids 455–461). We have demonstrated that N-terminal amino acids 1–131 of rRALDH1 that contain helix αC are essential for all-*trans* retinal catalysis, whereas amino acids 131–343 that contain helices αD and αH are important for 9-*cis* retinal oxidation [26]. Comparison of amino acid sequences between rRALDH1 and mRALDH1 in these regions showed one amino acid substitution at positions 125 (helix αC) and 172 (helix αD). These two amino acid substitutions in the region of the substrate entrance tunnel may be

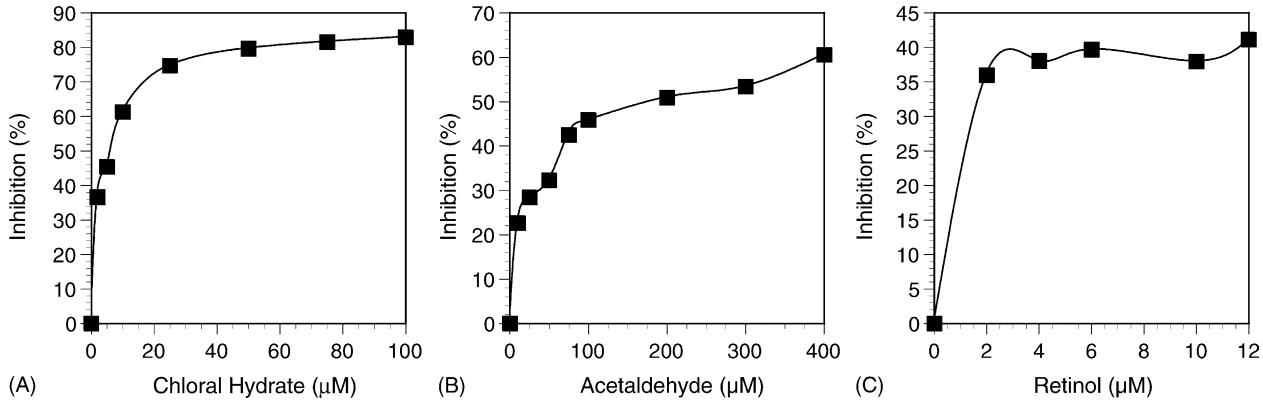

Fig. 6. Inhibition of mRALDH1 activity by chloral hydrate (A), acetaldehyde (B), and all-*trans* retinol (C). The substrate all-*trans* retinal and protein concentrations used in the incubations were 10 μM and 0.5 μg, respectively. Each point on the curves represents the average of two experiments.

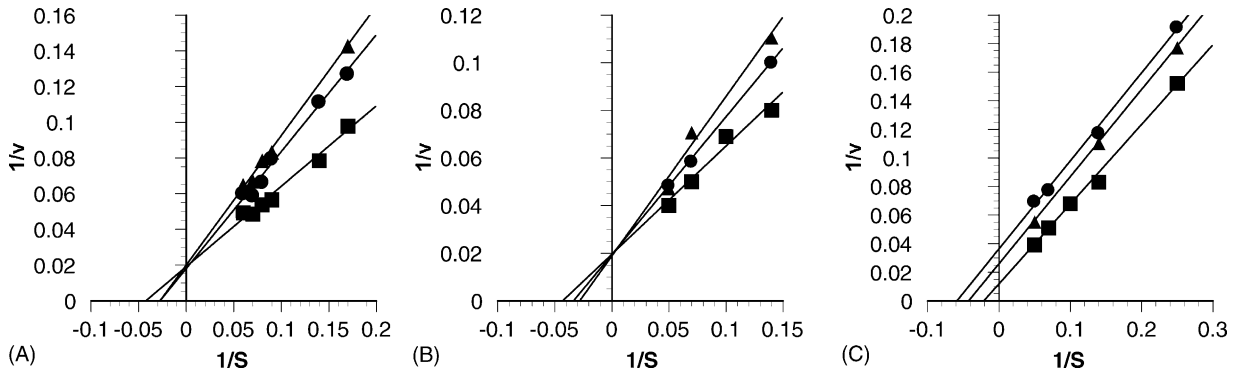

Fig. 7. Double-reciprocal plot of inhibition of mRALDH1 activity by chloral hydrate (A), acetaldehyde (B), and all-*trans* retinol (C). All-*trans* retinal was used as the substrate in the enzyme reaction. (A) Chloral hydrate concentrations: none (■), 2 μM (●), and 3 μM (▲); (B) acetaldehyde concentrations: none (■), 10 μM (●), and 20 μM (▲); (C) all-*trans* retinol concentrations: none (■), 3 μM (●), and 4 μM (▲). Each point on the curve represents the average of three replicates (less than 8% variation between each replicate).

responsible for the slight differences in the K_m values observed between these two RALDHs for two retinal isomers. It is noteworthy that retinol inhibits mRALDH1 (Fig. 7C) and hRALDH1 activity uncompetitively [13]. On the other hand, retinol suppresses rRALDH1 activity competitively [12]. At present, it is not clear whether amino acid differences at helices αC and αD play a role in the nature of inhibition of RALDHs by retinol.

Another important difference in the structural components of the substrate entrance tunnel is the two amino acid substitutions between rRALDH1 and mRALDH1 in the loop (amino acids 457 and 458). It will be interesting in future studies to examine whether these two amino acid differences in mRALDH1 contribute to a structural change that results in differences in activities for all-*trans* and 9-*cis* retinal catalysis at two different substrate concentrations.

Acknowledgments

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