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Retinoic acid biosynthesis catalyzed by retinal dehydrogenases relies on a rate-limiting conformational transition associated with substrate recognition

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Abstract

Retinoic acid (RA), a metabolite of vitamin A, exerts pleiotropic effects throughout life in vertebrate organisms. Thus, RA action must be tightly regulated through the coordinated action of biosynthetic and degrading enzymes. The last step of retinoic acid biosynthesis is irreversibly catalyzed by the NAD-dependent retinal dehydrogenases (RALDH), which are members of the aldehyde dehydrogenase (ALDH) superfamily. Low intracellular retinal concentrations imply efficient substrate molecular recognition to ensure high affinity and specificity of RALDHs for retinal. This study addresses the molecular basis of retinal recognition in human ALDH1A1 (or RALDH1) and rat ALDH1A2 (or RALDH2), through the comparison of the catalytic behavior of retinal analogs and use of the fluorescence properties of retinol. We show that, in contrast to long chain unsaturated substrates, the rate-limiting step of retinal oxidation by RALDHs is associated with acylation. Use of the fluorescence resonance energy transfer upon retinol interaction with RALDHs provides evidence that retinal recognition occurs in two steps: binding into the substrate access channel, and a slower structural reorganization with a rate constant of the same magnitude as the k_{cat} for retinal oxidation: 0.18 vs. 0.07 s⁻¹ and 0.25 vs. 0.1 s⁻¹ for ALDH1A1 and ALDH1A2, respectively. This suggests that the conformational transition of the RALDH-retinal complex significantly contributes to the rate-limiting step that controls the kinetics of retinal oxidation, as a prerequisite for the formation of a catalytically competent Michaelis complex. This conclusion is consistent with the general notion that structural flexibility within the active site of ALDH enzymes has been shown to be an integral component of catalysis.

Keywords

Aldehyde dehydrogenase; retinal; molecular recognition; conformational flexibility

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1. Introduction

All *trans*-retinoic acid (RA), the most potent metabolite of vitamin A (all *trans*-retinol, hereafter referred to as retinol), is a diffusible factor which acts as a paracrine signaling molecule throughout life in vertebrate organisms [1]. The major mode of RA action consists in regulating the expression of responsive genes, which is mediated by interaction with specific nuclear receptors [2]. RA thus regulates numerous processes in embryonic development and morphogenesis, and in adult tissue homeostasis, cell proliferation, differentiation, and apoptosis [3–6]. RA has been used in the treatment and chemoprevention of certain cancers [7, 8]. Because of its pleiotropic effects, RA action must be tightly regulated at the spatial and temporal levels, through the coordinated action of biosynthetic and degrading enzymes [9].

RA biosynthesis from Vitamin A occurs in two oxidative steps. The first is the retinol to retinal (referring to all *trans*-retinal) reaction that is catalyzed by members of the medium-chain or short-chain dehydrogenase/reductase enzyme superfamilies. In the second step, retinal is irreversibly oxidized to RA by members of the aldehyde dehydrogenase (ALDH) superfamily: ALDH1A1, ALDH1A2 and ALDH1A3, also known as retinal dehydrogenases RALDH1, RALDH2 and RALDH3, respectively [9–11]. In mammalian embryonic development, all three enzymes contribute to RA synthesis in a specific spatiotemporal pattern. ALDH1A2, which is expressed earliest, has been shown to be essential based on mouse gene knockout studies and responsible for 80% RA synthesis in the embryo [12–14]. In the adult, ALDH1A1 is responsible for the major RALDH activity in rodent kidney and liver and is expressed in several epithelial tissues. ALDH1A1 has been involved in the catabolism of excess retinol, and has been linked to obesity [10, 12, 15]. ALDH1A2 is highly expressed in the adult testis [16] and has been proposed as a candidate tumor suppressor in prostate cancer [17]. Interestingly, ALDH1A2 expression is highly induced in regenerating tissues in zebrafish [18].

Tight control of retinoid metabolism is reflected in low *in vivo* concentrations of retinoids and particularly retinal, which production is rate-limiting *in vivo* [11, 15, 19, 20]. Furthermore, retinol, but also retinal to a lesser extent, are bound by the cellular retinol binding protein which thus limits the availability of free molecules for the metabolizing enzymes [21]. Therefore, this suggests the importance of efficient substrate molecular recognition of RALDH enzymes to ensure high affinity and specificity for retinal. The three-dimensional structures of sheep ALDH1A1 [22] and rat ALDH1A2 [23] have been reported as a complex with the NAD cofactor, but in the absence of substrate. In the X-ray structure of ALDH1A1, the substrate binding site is shaped as a tunnel with a large entrance that can accommodate retinal [22]. Although the same secondary structure elements contribute to the substrate binding site, the loop that makes up the bottom wall of the tunnel is disordered in the X-ray structure of ALDH1A2 [23]. A disorder to order transition has been proposed to be linked to catalysis and to act as a mechanism conferring substrate specificity for retinal *vs.* short chain aldehydes [23, 24]. This hypothesis is consistent with the general notion that in ALDH enzymes, structural flexibility within the active site induced by ligand binding or associated with cofactor dynamics is an integral component of catalysis [25–32].

This study addresses the molecular basis of retinal recognition in human ALDH1A1 and rat ALDH1A2, through the comparison of the catalytic behavior of retinal analogs and use of the fluorescence properties of retinol. Our results provide for the first time direct evidence that retinal recognition involves at least two steps: first, the binding into the substrate access channel, and second, a structural reorganization that leads to the formation of a catalytically competent ternary complex, which would be rate-limiting for the overall reaction of retinal oxidation.

2. Material and Methods

2.1 Materials

NAD was purchased from Roche (Mannheim, Germany). Retinal, retinol, decanal, hexanal, citral, 2,4-decadienal (*trans, trans* isomer) and Hepes were sourced from Sigma-Aldrich (St. Louis, MO, U.S.A.). 2-methyl-2,4-pentanediol (MPD) was from Fluka (Sigma-Aldrich).

Retinoids are lipophilic compounds with low solubility in aqueous buffers [33]. Stock solutions of retinal and retinol were prepared in acetonitrile. Prior to enzymatic analysis, conditions were set up to increase the solubility of retinal and retinol, avoiding the use of micellar detergents such as Tween 80 that could affect enzyme kinetics by sequestering the substrate. MPD used as a solubilization agent allowed preparation of retinal solutions up to 100 μ M. The effect of increasing concentrations of the solubilizing agent MPD was tested on ALDH1A1 and ALDH1A2 steady-state activity with decanal and retinal. No inhibitory effect was observed up to 20 % MPD. To assess that the presence of 20 % MPD did not change the rate-limiting step of the reaction, we verified that the presteady-state kinetics of decanal oxidation in 20 % MPD and in 0.05% Tween 80 were the same. Indeed, for decanal which is more soluble than retinal, conditions using Tween 80 or 20% MPD allowed us to observe the acylation burst of NADH, thus showing that the overall acylation step is not rate-limiting.

2.2 Site-directed mutagenesis, production and purification of wild type and mutated RALDHs

Mutant enzymes were generated by standard PCR site-directed mutagenesis.

Plasmid pET/RALDH(III) expressing a long form of *Rattus norvegicus* testis ALDH1A2 including 11 N-terminal additional residues, was a generous gift from J.L. Napoli [16]. Recombinant rat ALDH1A2 was produced from a pET14b-derived expression vector prepared by amplification with the *Pfu* polymerase and subcloning of the ALDH1A2 ORF encoding a 499-residue protein, between the *NdeI* and *BamHI* restriction sites, under the control of the T7 promoter. The construction was sequenced in order to confirm that no mutations had been introduced in the amplification reaction. This plasmid allowed production of N-terminal His-tagged ALDH1A2, hereafter referred to as ALDH1A2.

Escherichia coli C41(DE3) transformants were grown at 37 °C for 16 h in the ZYM5052 or N5052 autoinducible medium [34] supplemented with ampicillin (200 mg/L). For purification of wild-type and mutated ALDH1A2, cells were harvested by centrifugation, resuspended in 20 mM potassium phosphate buffer, pH 7.5, 200 mM KCl, 2 mM DTT and disrupted by sonication. The extract was clarified by centrifugation at 17000 g for 1 h and dialyzed twice successively against 20 mM potassium phosphate buffer, pH 7.5 containing 200 mM KCl and a 20 mM potassium phosphate buffer pH 7.5 containing 1 M NaCl to remove small compounds that copurify with the protein. The supernatant was applied to a high performance nickel-Sepharose column previously equilibrated with the same buffer containing 50 mM imidazole, connected to an FPLC system (Amersham Biosciences). ALDH1A2 was eluted using a 0.5 M imidazole step. Enzyme concentration was determined spectrophotometrically by using a molar absorption coefficient of $2.11 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm.

Recombinant human ALDH1A1 was produced from a pET20b-derived vector prepared by subcloning of the ALDH1A1 ORF amplified with the *Pfu* polymerase from the IRAUp969H0146D clone obtained from the IMAGE consortium, between the *NdeI* and *EcoRI* restriction sites under the control of the T7 promoter. The resulting plasmid was sequenced in order to confirm that no mutations had been introduced in the amplification reaction. The human ALDH1A1 protein was expressed in *Escherichia coli* C41(DE3) cells

in the same conditions as described above. For purification of wild-type and mutated ALDH1A1, cells were harvested by centrifugation, resuspended in 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT and disrupted by sonication. The extract was clarified by centrifugation at 17000 g for 1 h, and solid ammonium sulfate was added to 50% saturation. The precipitate was applied to an Ultrogel® AcA34 gel (Biosepra, Pall Corporation) filtration column, the fractions containing ALDH1A1 were then applied to anion exchange Q-Sepharose column connected to an FPLC system equilibrated with the same buffer, and ALDH1A1 was eluted using a 0 to 1 M KCl gradient. Final purification was achieved by hydrophobic chromatography on a phenyl-Sepharose column connected to a FPLC system equilibrated with 50 mM potassium phosphate buffer, pH 7.5 containing 1 M ammonium sulfate, with elution by a decreasing 1 to 0 M ammonium sulfate gradient. Enzyme concentration was determined spectrophotometrically using a molar absorption coefficient of $2.5 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm. In the text, both ALDH1A1 and ALDH1A2 concentrations are expressed per monomer (normality, N).

2.3 Kinetic parameters for wild type and mutated RALDHs under steady-state conditions

For non-retinal substrates, initial rate measurements were carried out at 25 °C on a SAFAS UV mc2 spectrophotometer, by following the appearance of NADH at 340 nm in 20 mM Hepes/K⁺ pH 8.5 buffer containing 150 mM KCl, 1 mM EDTA, 20% MPD (standard buffer). The initial rate data were fit to the Michaelis–Menten equation using non linear least-squares regression analysis to determine the k_{cat} and K_{M} values. For ALDH1A1 in the presence of retinal, the reaction was followed at 340 nm as the overall combination of increasing absorbance of produced NADH and RA and decreasing contribution of retinal absorbance. A global extinction coefficient of $31000 \text{ M}^{-1} \cdot \text{s}^{-1}$ was experimentally determined by measuring the absorbance increase upon oxidation of a given concentration of retinal by an excess of ALDH1A1 in these conditions. All K_{M} values were determined at saturating concentrations of NAD (2 mM). For ALDH1A2 with retinal, due to its low K_{M} value, the reaction was monitored by NADH fluorescence, more sensitive than absorbance, using a rapid-mixing SX18MV-R stopped-flow apparatus.

2.4 Presteady-state kinetic measurements

Presteady-state kinetic analyses were carried out on a SX18MV-R stopped-flow apparatus (Applied PhotoPhysics) and collected data were analyzed using the SX18MV-R software package. To study the acylation step, progress curves of NADH production were recorded at 25 °C in standard buffer by following fluorescence emission using a 395 nm cutoff filter after excitation at 350 nm. One syringe was filled with 2 μN enzyme and 2 mM NAD⁺ and the other contained 2 mM NAD and the substrate (final concentrations after mixing). To study retinol interaction with RALDHs, fluorescence emission filtered above 455 nm was monitored after excitation at 295 nm at 25 °C in standard buffer. One syringe was filled with 2 μN enzyme and the other contained 30 μM retinol in the presence or absence of 2 mM NAD⁺ (final concentrations after mixing). Progress curves were fit according to a biphasic expression.

2.5 Fluorescence and fluorescence anisotropy binding assay

Fluorescence spectra and anisotropy measurements were carried out on a spectrofluorimeter SAFAS Flx-Xenius equipped with dual monochromators. ALDH1A1 dissociation constants for retinol were deduced from anisotropy titrations of 10 μM retinol in standard buffer at 25 °C with increasing ALDH1A1 concentrations recorded at 325 nm excitation and 482 nm emission wavelengths using 10 nm slits. The instrument computed anisotropy from samples illuminated with vertically polarized light $A = (I_{\text{VV}} - G \cdot I_{\text{VH}}) / (I_{\text{VV}} + 2G \cdot I_{\text{VH}})$ where I_{VV} is the fluorescence intensity recorded with excitation and emission polarization in vertical position, and I_{VH} is the fluorescence intensity recorded with the emission polarization

aligned in horizontal position. The G-factor of the spectrofluorometer represents the ratio of the sensitivities of the detection system for vertically and horizontally polarized light and was calculated by the instrument for each individual sample. Data obtained were expressed as the fraction of complex formed $(A - A_0)/(A_{\max} - A_0)$, where A_0 and $A_{\max}$ represent the anisotropy of free and bound retinol, vs. the concentration of ALDH1A1. Data sets were fit to a single-site binding model.

3. Results and discussion

3.1 The chemical structure of long-chain aldehyde substrates determines the rate-limiting step of ALDH1A1 and ALDH1A2

Previous structural and enzymatic studies on rat ALDH1A2 using model substrates with saturated linear aliphatic chain suggested a role of a disorder to order transition within the substrate access channel for long-chain aldehyde selectivity [24]. Assuming this hypothesis, we surmised that the conformational flexibility of the substrate should impact catalysis. Therefore, the steady-state and presteady-state kinetics of rat ALDH1A2 were analyzed using retinal and its unsaturated analogs citral and 2,4-decadienal, and compared to those obtained with the saturated analogs decanal and hexanal (Fig. 1, Table 1). For all substrates, ALDH1A2 followed classical Michaelis-Menten kinetics with Michaelis constants values ranging from 2 to 6.4 μM for all substrates but hexanal, characterized by a 23 μM K_M , all of which were consistent with values previously reported in studies from other groups on rat or mouse ALDH1A2 (sharing 99% sequence identity) [16, 24, 35]. Regarding the catalytic constant however, significantly higher values were measured compared to most older reports, which could be related to the enzyme purification protocol or to experimental conditions of enzyme activity determinations. In these conditions, oxidation of citral was found to be catalyzed by ALDH1A2, unlike previous results obtained on the rat enzyme [16]. Citral is a mixture of *cis* and *trans* isomers of 3,7-dimethyl 2,6-octadienal (neral and geranial, respectively), of which geranial bears closer structural similarity to retinal and is a better substrate than neral [36].

The characteristic feature revealed by this kinetic profile was the distinct behavior of ALDH1A2 with decanal or hexanal, which were oxidized with k_{cat} of $\sim 2 \text{ s}^{-1}$, 13 to 22-times higher as compared to the corresponding values for retinal, 2,4-decadienal or citral. Similarly, ALDH1A1 oxidized decanal and retinal with 1.0 and 0.07 s^{-1} k_{cat} values, respectively (Table 1). Such differences could be correlated with a distinct rate-limiting step in the catalytic cycle (scheme 1). This hypothesis was thus tested by following the kinetics of the reaction in presteady-state conditions using the fluorescence signal of the NADH produced, which are shown in Fig. 2 for ALDH1A2 with decanal, retinal, citral and 2,4-decadienal. When decanal was used as substrate, an exponential burst of NADH production was observed, corresponding to the first cycle of decanal oxidation and characterized by a rate constant of 36 s^{-1} , 16-times higher than the k_{cat} value, followed by a slower, linear phase corresponding to steady-state NADH release. Such behavior shows that the rate-limiting step occurs after the hydride transfer. In contrast, no significant burst was observed compared to the control recorded in the absence of substrate when the unsaturated substrates retinal, citral and 2,4-decadienal were used, revealing that in these cases, the rate-limiting step corresponded to a process occurring within the acylation step. Likewise, the rate-limiting step of retinal and decanal oxidation by ALDH1A1 was observed to correspond to acylation and deacylation, respectively. Indeed, the rate constant of the burst of NADH production for ALDH1A1 with decanal was of 37 s^{-1} , 37-fold higher than the k_{cat} value (1 s^{-1}) (data not shown). Therefore, these results suggest a correlation between the saturated/unsaturated chemical structure of the substrate, including the associated physicochemical characteristics such as conformational flexibility, and the rate-limiting event of the catalytic cycle.

3.2 Probing the molecular mechanism of substrate recognition by FRET using the fluorescent analog retinol

Within acylation, the rate-limiting process can be associated with molecular recognition events including binding or conformational reorganization, to the nucleophilic attack of the catalytic Cys on the carbonyl group of the substrate, or to the hydride transfer (scheme 1). To probe substrate recognition by the RALDHs, we searched for the existence of a FRET signal between the Trp residues of the RALDHs and retinol, a fluorescent analog close to retinal that is likely to bind to RALDHs in a similar manner. Indeed, the fluorescence emission maximum wavelength of Trp at ~330 nm matches the absorption maximum wavelength of retinol at 325 nm, which makes possible fluorescence energy transfer from Trp to a retinol molecule bound to the enzyme. In addition, retinol is not a substrate for RALDHs, which allows characterizing a ternary complex with NAD⁺ (see below). As shown in Fig. 3, addition of excess retinol resulted in significant quenching of Trp emission on the spectrum of ALDH1A1 and ALDH1A2 after excitation at 295 nm, while a new emission band showed up at wavelengths above 400 nm. Due to the existence of a shoulder in this region of the spectrum of ALDH1A1 in the absence of retinol, this band was better analyzed from the difference spectra recorded after and before addition of retinol, as shown in the inset of Fig. 3. In the case of ALDH1A2, the difference spectrum reveals an envelope very similar to the retinol emission spectrum with a maximum intensity at 483 nm, thereby indicating Trp to retinol FRET. In the case of ALDH1A1, the signal was characterized by a lower intensity and a blue-shift of 15 nm, suggesting that retinol is bound in a physicochemical environment different from ALDH1A2. ALDH1A2 contains six Trp residues per monomer as also found in ALDH1A1, among which the conserved Trp177 is of particular interest due to its location within the substrate access channel defined in the X-ray structures of rat ALDH1A2 and sheep ALDH1A1 (sharing 92% sequence identity with the human enzyme) (Fig. 4) [22, 23]. Therefore, the contribution of Trp177 to the FRET signal was tested by comparing the effect of retinol on the Trp fluorescence spectrum of wild-type and W177F ALDH1A2. In the mutant, the intensity of the FRET signal at 383 nm was depressed by at least 80%, showing the major contribution of Trp177 to the fluorescence transfer. This suggests that retinol binds in the close environment of Trp177, thus validating the specificity of this probe for the study of retinol binding in these enzymes.

The mechanism of retinol recognition was analyzed by using the FRET property of retinol within the complex formed with RALDHs as a probe to monitor the kinetics of retinol binding interactions in a rapid kinetics apparatus. The progress curve for the FRET signal, recorded after rapid mixing of 2 μ M ALDH1A2 monomers and 30 μ M retinol (Fig. 5, trace 1), is best described by a combination of two exponential phases characterized by rate constants of 6.2 and 0.35 s⁻¹, the amplitude of the slower phase representing 82 % of the total amplitude. Similar behavior was found for ALDH1A1, although the rapid and slow phases were characterized by rate constants of 0.82 and 0.16 s⁻¹, respectively, and the amplitude of the slower phase represented 22 % of the total amplitude (Fig. 5, trace 2). As expected from the intensity of the FRET band in the spectra (Fig. 3), the total amplitude of the progress curves was lower for ALDH1A1 than for ALDH1A2. Because the competent Michaelis species for the catalysis of retinal oxidation is a ternary complex with NAD⁺, the interaction of retinol with the RALDHs was also recorded in the presence of NAD⁺ (Fig. 5, traces 2 and 4). Apart from a significant effect on the total amplitude due to the screening effect due to NAD⁺ absorption at the excitation wavelength 295 nm, very similar results were obtained in the presence of 2 mM NAD⁺, with e.g. for the slower phase, rate constants of 0.25 and 0.18 s⁻¹, and amplitudes of 85 and 28 % of the total, for ALDH1A2 and ALDH1A1, respectively. Therefore, although the kinetic mechanism of several non-CoA dependent ALDHs was described to be ordered sequential [37, 38], retinol is able to bind the

apo-RALDHs and the RALDH/NAD binary complex with similar kinetics when probed by the retinol FRET signal.

The biphasic nature of the kinetics of interaction of retinol, suggests that after entrance into the substrate access channel, which could correspond to the fast phase, a conformational transition of the complex occurs that results in a modification of the distance and/or orientation between the retinol and Trp177 fluorophores and to the signal observed in the second, slower phase. This interpretation is supported by the fact that the rate of the fast phase increases with retinol concentration (data not shown), whereas the rate of the slower phase remains unchanged. Because retinol and retinal have very similar structures, the same mechanism could likely also hold for retinal recognition. Such results could be directly correlated to the disorder to order transition of the region comprising residues 462–478 proposed to provide substrate specificity to ALDH1A2, based on the X-ray structure of the enzyme/NAD complex [23]. In the case of ALDH1A1 however, the X-ray structure of the sheep enzyme revealed an ordered substrate access channel. This does not preclude the occurrence of a conformational transition, although it could in part explain the lower relative magnitude of the corresponding kinetic step in the progress curve recorded by the FRET signal (28% vs. 85% for ALDH1A2).

Furthermore, when compared to the kinetics of retinal oxidation, the rate constants of the slower phase of retinol interaction with the enzymes appear to be of the same magnitude as the k_{cat} (0.18 vs. 0.07 s⁻¹ and 0.25 vs. 0.1 s⁻¹ for ALDH1A1 and ALDH1A2, respectively). This suggests that the conformational transition of the enzyme-retinal complex proposed above significantly contributes to the rate-limiting step that controls the kinetics of retinal oxidation. By extension, the rate-limiting process of citral and 2,4-decadienal oxidation, that is shown to be associated with acylation, would also be controlled by a structural reorganization concomitant to substrate molecular recognition. Retinal, citral and 2,4-decadienal share a common unsaturated structure of the carbon chain which imparts lower flexibility for these molecules than for the saturated substrates such as decanal or hexanal (Fig. 1). This therefore suggests that the formation of an enzyme/NAD/substrate Michaelis complex competent for the subsequent chemical steps of acylation requires a conformational adaptation of the substrate access channel in the case of the retinal/citral/2,4-decadienal substrates. Noticeably, the β -ionone ring of retinal seems not to play a critical role for efficient recognition of substrate, in agreement with the results of retinal docking on the sheep ALDH1A1 structure, showing that the ring sticks out from the tunnel entrance and is partially exposed to solvent in this model [22]. By contrast, the efficient binding of saturated substrates would be promoted by their intrinsic flexibility, making this process not rate-limiting for the overall reaction.

3.3 Mutations in ALDH1A1 substrate binding site affect the conformational transition that conveys substrate recognition in ALDH1A1

In the known X-ray structures of sheep ALDH1A1 and rat ALDH1A2, the substrate access channel is built up by residues from three helices and a loop (455–461). As mentioned above, this loop appears disordered in the structure of ALDH1A2, while it is well-defined in the structure of ALDH1A1 [22, 23]. In an attempt to identify structural determinants of retinal recognition, we selected residue positions in ALDH1A1, also defined in the ALDH1A2 structure, that line the entrance of the tunnel and could thus participate in the motions occurring with retinal recognition when the competent catalytic complex forms (Fig. 4). In ALDH1A1, residues Asn120 (Ile/Val in ALDH1A2), Lys127 (conserved in ALDH1A2) and Tyr296 (Phe in ALDH1A2), were substituted by Ala, Ala and Ala/Val, respectively, and the binding affinity of the mutant enzymes for retinol was determined by fluorescence anisotropy and compared with the catalytic parameters for retinal oxidation (Table 2). Fluorescence anisotropy allows the monitoring of complex formation between

retinol and the protein, irrespective of the conformational state of the complex. Determination of the affinity constants K_D by this method shows that the mutations had little effect on the overall affinity of ALDH1A1 for retinol, which likely also applies for retinal. However, although only a slight effect is observed on the steady state kinetic parameters for the N120A and K127A mutants, the k_{cat} for retinal oxidation catalyzed by the Y296A and Y296V ALDH1A1s is lowered 4 and 9-times compared to the wild type. If the rate-limiting step of retinal oxidation by ALDH1A1 is associated with the structural reorganization occurring upon the formation of the competent catalytic complex, this result suggests that position 296 participates in this process either directly or indirectly.

3.4 Conclusion

Structural dynamics within the active site are essential components of the catalytic cycle of ALDH enzymes. In this study, direct evidence supports the role of a conformational transition in the molecular recognition and specificity of ALDH1A1 and ALDH1A2 for retinal. This step would be a prerequisite for the formation of a catalytically competent complex and could thus control the process of RA synthesis from low *in vivo* levels of retinal. The approaches developed herein, including use of the fluorescent analog retinol, will allow in the future to probe for this critical step in RA biosynthesis and understand the role of structural elements within the active site of RALDH enzymes in retinal recognition.

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Abbreviations

ALDH	aldehyde dehydrogenase
FRET	fluorescence resonance energy transfer
Hepes	hydroxyethyl piperazine ethanesulfonic acid
MPD	2-methyl-2,4-pentanediol
retinal	all <i>trans</i> -retinaldehyde
retinol	all <i>trans</i> -retinol
RALDH	retinal dehydrogenase
RA	all <i>trans</i> -retinoic acid

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Highlights

Retinal dehydrogenase catalytic mechanism is explored by presteady-state kinetics

The rate-limiting step of retinal dehydrogenases depends on the nature of substrate

Retinol fluorescence properties reveal retinoid binding and recognition mechanism

Substrate specificity of retinal dehydrogenases relies on conformational transition

Figure 1.
Chemical structures of retinal, retinol and the aldehyde analogs used in this study.

Figure 2.

Presteady state kinetics of NADH production for the reaction catalyzed by ALDH1A2 with retinal and aldehyde analogs: 100 μ M decanal (1), 5 μ M retinal (2), 100 μ M citral (3), 100 μ M 2,4-decadienal (4), control in absence of substrate (5). A solution of 1 μ N ALDH1A2, 2 mM NAD is rapidly mixed at 25 $^{\circ}$ C with an equal volume of 2 mM NAD and substrate in standard buffer. The excitation wavelength is set at 350 nm, and the fluorescence signal of NADH is monitored using a 395 nm cutoff filter.

Figure 3.

Fluorescence emission spectra of RALDHs in the absence and presence of retinol. The excitation wavelength is set at 295 nm corresponding to Trp fluorescence excitation and the spectra recorded for 10 μ M enzyme in the absence (black traces) and presence (gray traces) of 10 μ M retinol in standard buffer. For each enzyme spectra in absence and presence of retinol are recorded in the same conditions. Spectra for ALDH1A1 (solid), ALDH1A2 (dash) and ALDH1A2 W177F (dots) are normalized according to the maximum fluorescence intensity of the apo enzyme. Inset: difference spectra of the effect of retinol addition in the 400–550 nm region, compared with the emission spectrum of retinol (solid).

Figure 4.

View of the substrate access channel of sheep liver ALDH1A1 (coordinates from the pdb entry 1BXS). The large entrance is lined with Tyr-296 (blue), Met-120 (green)(Asn-120 in human ALDH1A1), Lys-127 (cyan) and a loop makes up the bottom wall of the channel. The positions of Trp-177 (orange), of the catalytic Cys-302 (yellow) and of the nicotinamide moiety of the NAD cofactor are shown deeper in the tunnel. The figure was prepared using PyMOL 0.99 (www.pymol.org).

Figure 5.

Kinetics of interaction of retinol with RALDHs monitored by FRET. Times courses are recorded after mixing of 2 μ N ALDH1A2 (1,2) or ALDH1A1 (3,4) with 30 μ M retinol in the absence (1,3) or presence of 2 mM NAD (2,4), in standard buffer at 25°C. The excitation wavelength is set at 295 nm, and the fluorescence signal is monitored using a 455 nm cutoff filter. The collected data were fit to according to a biphasic expression.

Scheme 1.

The general catalytic mechanism of hydrolytic ALDHs showing the global steps of acylation and deacylation. Within acylation, the rate-limiting process may be associated with the formation of the Michaelis complex including binding or conformational reorganization events, the nucleophilic attack of the catalytic Cys on the carbonyl group of the substrate, or the hydride transfer.

Table 1

Steady-state kinetics parameters of wild-1 type RALDHs with retinal and analogs.

Substrate	ALDH1A2 ^a		ALDH1A1 ^a	
	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})
Retinal	2.0 ^b	0.10	8.1	0.07
2,4 decadienal	6.3	0.15	ND	ND
Citral	2.9	0.10	ND	ND
Decanal	6.4	2.2	10.0	1.0
Hexanal	23.0	2.0	ND	ND

^aKinetic parameters were deduced from non-linear regression of experimental data sets, according to the Michaelis-Menten equation. All K_M values were determined at saturating concentrations of the NAD cofactor. The steady-state initial rates of the reaction of both RALDHs were measured at 25°C in standard buffer, by following NADH absorbance or the resulting absorbance of retinal, RA and NADH. The standard deviation is below 20%. ND, not determined.

^bMeasured by monitoring NADH fluorescence on a stopped flow apparatus.

Table 2

Steady-state kinetics parameters of wild-type and mutant ALDH1A1 with retinal compared with dissociation constants for retinol

ALDH1A1	Retinal ^a		Retinol ^b
	K_M (μM)	k_{cat} (s^{-1})	K_D (μM)
WT	8.1	0.07	1.8
N120A	11	0.03	3.1
K127A	8.6	0.03	3.7
Y296A	8.9	0.017	1.7
Y296V	15	0.008	2.6

^a Kinetic parameters were determined as in Table 1.

^b Determined by monitoring retinol fluorescence anisotropy recorded at 325 nm excitation and 482 nm emission wavelengths. Data sets obtained at 10 μM retinol by varying ALDH1A1 concentration in standard buffer at 25°C, were fit to a single-site, saturable binding model. The standard deviation is below 20%.