RALDH3 (retinal dehydrogenase 3) was characterized by kinetic and binding studies, protein engineering, homology modelling, ligand docking and electrostatic-potential calculations. The major recognition determinant of an RALDH3 substrate was shown to be an eight-carbon chain bonded to the aldehyde group whose kinetic influence \( k_{cat}/K_m \) at pH 8.5 decreases when shortened or lengthened. Surprisingly, the \( \beta \)-ionone ring of all-trans-retinal is not a major recognition site. The dissociation constants \( K_d \) of the complexes of RALDH3 with octanal, NAD\(^+\) and NADH were determined by intrinsic tryptophan fluorescence. The similarity of the \( K_d \) values for the complexes with NAD\(^+\) and with octanal suggests a random kinetic mechanism for RALDH3, in contrast with the ordered sequential mechanism often associated with aldehyde dehydrogenase enzymes. Inhibition of RALDH3 by tri-iodothyronine binding in competition with NAD\(^+\), predicted by the modelling, was established kinetically and by immunoprecipitation. Mechanistic implications of the kinetically influential ionizations with macroscopic pK\(_a\) values of 5.0 and 7.5 revealed by the pH-dependence of \( k_{cat} \) are discussed. Analogies with data for non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus mutans*, together with the present modelled structure of the thioacyl RALDH3, suggest (a) that \( k_{cat} \) characterizes deacylation of this intermediate for specific substrates and (b) the assignment of the pK\(_a\) of the major ionization (approximating to 7.5) to the perturbed carboxy group of Glu\(^{280}\) whose conjugate base is envisaged as supplying general base catalysis to attack of a water molecule. The macroscopic pK\(_a\) of the minor ionization (5.0) is considered to approximate to that of the carboxy group of Glu\(^{488}\).

Key words: enzymology, mouse retinaldehyde dehydrogenase type 3 (RALDH3, ALDH1A3), pH-dependent kinetics, retina, retinoic acid, 3,3′,5-tri-iodothyronine (T\(_3\)).

INTRODUCTION

Retinoic acid biosynthesis in the retina involves the generation of retinal from retinol (vitamin A) in reactions catalysed initially by retinol dehydrogenases and subsequently by further oxidation catalysed by RALDHs (retinal dehydrogenases). All are part of the superfamily of ALDHs (aldehyde dehydrogenases). The RALDHs are a group of cytosolic ALDHs that exert high specificity for the NAD\(^+\) oxidation of all-trans- or 9-cis-retinal to all-trans- or 9-cis-retinoic acid respectively [1–4]. The recently identified *raldh4* gene is mainly expressed in embryonic and adult liver and kidney and has not been identified in the developing retina [4].

Differences in their locations and the domains defined by the expression of each *raldh* gene and of cyp26, the gene encoding the retinoic acid-catabolizing enzyme CYP26 (cytochrome P450RA1), result in the formation of defined zones containing high concentrations of retinoic acid. During embryogenesis, *raldh2* is the first dehydrogenase mRNA expressed in the eye region at E8 (embryonic day 8). It appears transiently in the ventral region and, shortly after, it can be detected in the ectoderm, where *raldh3* is also expressed. The dorsal tip of the eye vesicle then expresses *cyp26*, followed by expression of *raldh1* in the dorsal neural retina. By E11.5, distinct spatiotemporal expression patterns are made up of the expression of *raldh1*, *raldh3* and *cyp26*, but *raldh2* is no longer present. CYP26 forms a horizontal boundary between the dorsal RALDH1 region and the ventral RALDH3 region [5,6].

Both RALDH1 and RALDH2 exert substantial substrate specificity for retinals relative to non-physiological aldehydes. Also RALDH2 appears to discriminate markedly in favour of all-trans-retinal, as demonstrated by the reported values of maximal specific activity (incorrectly given the symbol \( V_{max} \)) divided by \( K_m \) [7], whereas RALDH1 appears not to so discriminate [1]. These conclusions depend on the assumption that initial rates were measured by the HPLC point assay after 1 h of reaction. The structures of RALDH1 [8] and RALDH2 [9] have been solved by X-ray crystallography and reveal three domains: a novel NAD\(^+\)-binding domain containing a five-stranded parallel \( \beta \)-sheet, a catalytic domain comprising a six-stranded parallel \( \beta \)-sheet, and a three-stranded anti-parallel \( \beta \)-sheet oligomerization domain. The substrate-binding tunnel of ovine RALDH1 [8] is the largest of any of the ALDH structures solved so far. The entrance is wide and contains amino acid residues whose side chains adopt conformations that maintain the tunnel open and allow access for the retinal substrates [8]. The active site of RALDH1 contains the conserved cysteine residue (Cys\(^{280}\)) of the ALDHs, which is transiently acylated during catalysis [10]. The kinetic mechanism [11] involves the sequential binding of NAD\(^+\), followed by the aldehyde, which reacts with Cys\(^{280}\) to form a thiohemiacetal intermediate. Hydride transfer to the nicotinamide ring of NAD\(^+\) then results in formation of a thiol ester intermediate. This undergoes base-catalysed hydrolysis by Glu\(^{288}\) [12] and, finally, the carboxylic acid product and NADH are released sequentially. The structure of rat RALDH2 in the presence of NAD\(^+\) [9] suggests mobility in the catalytic machinery. The substrate-access tunnel is considerably smaller than that in RALDH1 and slightly larger than that in the class 2 ethanal-metabolizing enzyme ALDH2. There is a lack of inter-monomer contacts and a disordered loop that might contribute to the mobility of the catalytic
enzymes are similar (studied, being limited to showing that the recently, chicken RALDH3 (ALDH6) was identified and cloned human RALDH3 [14] (originally designated ALDH6 [15]). More identity with that of mouse RALDH2 and 94 number AF28044) has 70% identity with that of mouse RALDH2 and 94% identity with human RALDH3 [14] (originally designated ALDH6 [15]). More recently, chicken RALDH3 (ALDH6) was identified and cloned [3]. The catalytic characteristics of RALDH3 have been little studied, being limited to showing that the K_m values for the oxidation of all-trans-retinal catalysed by the chicken and mouse enzymes are similar (≈0.3 μM) and the K_m for the oxidation of the short-chain aldehyde, ethanal, by the chicken enzyme is much larger (≈3 mM). Also, by contrast with RALDH1 and RALDH2, the three-dimensional structure of RALDH3 has not been reported. Differential regulation of RALDH3s in the developing retina results in the formation of a steep gradient of retinoic acid, which defines the dorsal and ventral domains. Differences in the kinetic characteristics of the various RALDHs would be expected to contribute to the mechanisms available for the control of retinoic acid synthesis. In view of the lack of structural and kinetic information available for RALDH3, the work reported in the present paper was undertaken to remedy this deficit as a contribution to the eventual understanding of retina development. We here report a modelled three-dimensional structure for RALDH3 and key kinetic and binding characteristics relevant to specificity and catalytic mechanism, including the involvement of ionizing groups. In addition, a study of the possible involvement of the thyroid hormone T3 (3,3′,5′-triiodothyronine) as a physiological regulator of RALDH3 was initiated.

MATERIALS AND METHODS

Chemicals and reagents
Most chemicals were purchased from Sigma, Poole, Dorset, U.K. Other materials were provided by the following suppliers: restriction enzymes and modification enzymes were from Promega, Chilworth, Southampton, U.K.; Chelating Sepharose Fast Flow resin and gel-filtration columns were from Amersham Biosciences, Little Chalfont, Bucks., U.K.; pET14b was from Novagen, Madison, WI, U.S.A.; tryptone and yeast extract were from Oxoïd, Basingstoke, U.K.; sodium dithione was from Roche, Poole, U.K.; and primers were from Invitrogen, Paisley, Renfrewshire, Scotland, U.K.

Cloning and site-directed mutagenesis
Mouse Raldh3 was amplified from Raldh3-pcDNA (kindly donated by Dr P. McCaffery, Eunice Kennedy Shriver Center at the University of Massachusetts Medical School, Waltham, MA, U.S.A.) with primers specific for the 5′ and 3′ of each cDNA incorporating NdeI or BamHI restriction sites respectively. Following PCR, the product was ligated into the cloning vector pGEM-T Easy (Promega) and subcloned into the expression vector pET14b (Novagen). Sequence analysis confirmed the correct clone of mouse Raldh3-pET14b. Site-directed mutagenesis of mouse Raldh3-pET14b was performed using the GeneEditorTM in vitro Site-Directed Mutagenesis System (Promega). The mutagenic oligonucleotide (TACAATGCACTTTATGCAAC) was used in conjunction with the sense-strand selection oligonucleotide, Top Select Oligo, following the manufacturer’s instructions. To confirm the T1411C mutation, raldh3_F471L-pET14b was digested with NsiI and NdeI, and the absence of a 1.4 kb fragment indicated the incorporation of the base change at position 1411. The positive clone was sequenced using the DNA sequencing service at Qiagen (Hilden, Germany).

Recombinant-protein production and purification
Raldh3-pET14b was transformed into BL21(DE3) pLysS cells and grown to an attenuation (D_600) of 0.6, and protein production was induced with 0.4 mM IPTG (isopropyl β-D-galactopyranoside) at 16°C overnight. Cells were collected by centrifugation (8000 g, 4°C, 10 min) and resuspended in lysis buffer (0.5 M NaCl/20 mM Tris/HCl, pH 7.9) containing 5 mM imidazole and frozen at −20°C. Cells were defrosted and lysed by sonication in an ice bath in the presence of 1 M PMSF. The cell suspension was centrifuged (27000 g, 25 min, 4°C) and the cell cytosol loaded on to a pre-charged nickel-chelating Sepharose 4B column. The column was washed with 10 vol. of lysis buffer containing 5 mM imidazole, followed by 10 vol. containing 80 mM imidazole. The protein was eluted with 5 vol. of lysis buffer containing 400 mM imidazole. The eluted protein was separated on a SDS 5% gel-filtration column (26/60; Amersham Biosciences), equilibrated in 25 mM Tris/HCl pH 8.0/50 mM NaCl/5 mM DTT (dithiothreitol). Proteins were analysed by both denaturing and non-denaturing PAGE and concentrated in Vivaspin 10K 20 ml concentrators (Vivascience AG, Göttingen, Germany). Protein concentration was calculated by measuring the A_280 under denaturing conditions on a Hitachi Europe Ltd (Maidenhead, Berks., U.K.) 2010 UV–visible spectrophotometer. An ε value of 46 380 M^-1·cm^-1 was used.

Immunoprecipitation
The binding of T3 to human and mouse RALDH3 was assessed by immunoprecipitating proteins bound to T3 with an antibody, αT3 (Fitzgerald Industries International, Concord, MA, U.S.A.). In 500 μl of RALDH3 reaction buffer, 50 μg of cell extract supernatant was incubated in the presence or absence of 100 μM T3 for 30 min on ice. T3-bound protein was immunoprecipitated with anti-αT3 (1:100000) for 1 h on ice and bound to Protein G beads (20 μl of a 50% slurry) by incubating on a rotating wheel at 4°C for 1 h. The beads were pelleted by centrifugation (14000 g, 4°C, 2 min) and washed in 1 ml of IP (immunoprecipitation) buffer with six cycles of 10 min rotation and centrifugation (14000 g, 4°C, 2 min). The beads were then resuspended in SDS sample buffer, boiled for 5 min, centrifuged briefly and the supernatant was separated by denaturing 10%-(w/v)-PAGE. Proteins on the gel were transferred to a nitrocellulose membrane and the Western blot probed with an antibody to the histidine tag (αHis). This approach specifically identified histidine-rich proteins to which T3 is bound.

Dehydrogenase assay
In a total reaction volume of 1 ml, 2–200 nM enzyme was preincubated with 500 μM NAD+ in reaction buffer (50 mM Hepes/50 mM MgCl2/5 mM DTT) for 10 min at 37°C. The reaction was initiated by the addition of 0–100 μM substrate and the rate of NADH production at 37°C was measured as a rate

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of ΔA_{340}/s using a Hitachi 3010 UV–visible spectrophotometer. For inhibitor assays, RALDH3 was pre-incubated with a range of inhibitor concentrations at 37°C for 10 min and the reaction was initiated by the addition of both substrate and cofactor. For substrates with sub-micromolar $K_m$ values, the dehydrogenase assay was performed using an Applied Photophysics (Leatherhead, Surrey, U.K.) SX+18MV-R stopped-flow reaction analyser. The reactants were allowed to equilibrate to 37°C in the chamber for 10 min before firing. Each reaction was measured for 20–50 s as ΔA_{340}/s. The reaction was repeated four times for each data point and each experiment repeated three times. Kinetic data were analysed using DeltaGraph 4.5 to provide values of $K_m$ and $V_{max}$. 

**Determination of dissociation constants of enzyme–ligand complexes**

These were determined from changes in intrinsic tryptophan fluorescence. RALDH3 has six tryptophan residues, and at least one residue (Trp 389) is part of the substrate-binding tunnel. The fluorescence of 0.4 µM protein in a total volume of 3 ml in a 10 mm quartz cuvette was measured by excitation of 280 nm and emission at 340 nm at 20°C in a Hitachi 2010 spectrophotometer. The change in fluorescence (ΔF) was measured after sequential addition of substrate, with account taken of the change in concentration. The dissociation constant ($K_d$) was calculated using eqn (1) and DeltaGraph 4.5:

$$B = \frac{[B_{max1} \times L]}{(K_d1 + L)} + \frac{[B_{max2} \times L]}{(K_d2 + L)}$$  

where $B$ is ΔF, $B_{max}$ is the maximal ΔF, $L$ is the ligand concentration and $K_d$ is the dissociation constant. The first term of the right-hand side of the equation represents specific binding (specific ΔF) and the second incorporates non-specific binding (non-specific ΔF). These terms are plotted separately as a function of substrate concentration.

**pH-dependence studies**

These were carried out over the pH range 6.25–10.5 at pH intervals of 0.25 using a substrate concentration (100 µM) that was 100 times the $K_m$ values to provide values of $v_i$ that approximate closely to $V_{max}$ values. Values of $K_{cat}$ were calculated by dividing these $V_{max}$ values by [RALDH]. Buffers used were Mes (pH 6.0–7.25), Heps (pH 7.0–8.75) and Caps [3-(cyclohexylamino)-propane-1-sulphonic acid; pH 9.75–10.25].

**Computer evaluation of pH-dependent kinetic data**

These were evaluated using the basis described in [16]. pH-dependent kinetic studies, sometimes involving a multiplicity of reactivity states, require a rapid method of evaluating a series of kinetic models differing in the number and reactivity of the reactive states (see [17]). This is the situation that obtains in the present work. A useful method is provided by the multitasking application program SKETCHER [18,19]. SKETCHER permits rapid estimation of characterizing parameters in the general set of equations for the various models by means of interactive manipulation of calculated curves. This approach allows different models to be readily distinguished and the parameters thus obtained provide provisional estimates for analysis by weighted non-linear regression. The regression values are generally close to the SKETCHER values, particularly in the case of the complex models, when one or more of the parameters is known from an independent experiment, which obviates problems deriving from multiple local minima.
one side of the substrate access tunnel are disordered in the binary complex, whereas the corresponding residues in RALDH1 are well defined. The lack of insertions and deletions, together with the high sequence identity, increase confidence in the overall features of the model which is necessarily very closely similar to RALDH1 (Figure 1A shows the subunit structure). Because the RALDH1 structure is a binary complex with NAD$^+$, the binary complex of RALDH3 and NAD$^+$ can also be readily modelled. This we call the out conformation. The in conformation of the coenzyme can be modelled using the phosphate positions defined in the RALDH2 binary complex and predicting the position of the nicotinamide moiety. The catalytic machinery of RALDH3 is closely similar to that of RALDH1, comprising the nucleophile Cys$^{314}$, the predicted general base Glu$^{280}$, and Gln$^{181}$, which may contribute to transition-state stabilization. The authors of the RALDH1 structure [8] suggest that the disorder present in the position of the residue equivalent to Glu$^{280}$ and the observation that NAD$^+$ binds in two distinct modes indicates that flexibility is a key facet of the reaction mechanism. In our RALDH1-based structure of RALDH3, the C-4 atom of the nicotinamide is too far away (7.1 Å) from Cys$^{314}$ for direct hydride transfer from a thiohemiacetal intermediate to occur during the dehydrogenase reaction; this is the NAD$^+$ out position. In the NAD$^+$ in conformation modelled on the RALDH2–NAD$^+$ phosphate positions the distance is closer at 3.8 Å. These observations are in agreement with a reaction mechanism in which the nicotinamide can move in and out of the active-site pocket, anchored to the protein by the adenine moiety, with the nicotinamide phosphate and ribose acting as a flexible linker. Glu$^{280}$ can also be in a proximal or distal position with respect to Cys$^{314}$. In the Glu in position the thiohemiacetal would be dehydrogenated to form the thiol ester intermediate, and in the Glu out position the enzyme–thiol ester would be hydrolysed. The nicotinamide moiety of NAD$^+$ proximal to, and Glu$^{280}$ distal from, Cys$^{314}$ would be expected to increase the availability of the cysteine thiolate and also allow hydride transfer from Cys$^{314}$ thiohemiacetal to the C-4 atom of the nicotinamide of the NAD$^+$, leading to formation of the thiol ester intermediate. Electrostatics calculations support the argument for the enhanced availability of Cys$^{314}$ when NAD$^+$ is in and Glu$^{280}$ is out. The nicotinamide may then move away from, and Glu$^{280}$ towards, Cys$^{314}$, allowing Glu$^{280}$ to fulfil the role of general base catalyst by orientating and activating a water molecule to hydrolyse the thiol ester bond. The size and shape of the RALDH3 substrate-access tunnel is similar to that of RALDH1.
with aromatic amino acid residues lining the tunnel. As shown in Figure 1(B), the eight-carbon chain binds in the tunnel and the β-ionone ring is relatively exposed on the surface of the enzyme. This may explain the similar affinities that RALDH3 has for octanal and retinal. One clear difference between the access tunnels is the substitution of Phe471 in RALDH3 for Leu488 in RALDH1. The NAD$^+$ cofactor and Cys314 nucleophile are at the bottom of the substrate-access tunnel (Figure 1B).

The tetrahedral intermediate state in deacylation

On the basis of the model of retinal binding to RALDH3 (Figure 1B), a model of a tetrahedral intermediate can readily be constructed. C-15 of the thiol ester intermediate is attacked by a base-activated water molecule, and the electrons move from carbonyl–oxygen double bond of the intermediate to form the oxynion of the quasi-tetrahedral transition state (Figure 1C). Glu280 acts as the base activating and orientating the water molecule, and the model suggests that the oxynion may be stabilized by interactions with Asn481 and by the amide group of Cys314.

Establishment of reaction conditions for RALDH3 catalysis assay

Reaction conditions for a general kinetic assay of the oxidation of a number of substrates catalysed by RALDH3 were established using octanal as substrate (see the subsection on substrate specificity). Increase in the concentration of MgCl$_2$ over the range 0–50 mM produced an increase in activity that became essentially constant above 10 mM. MgCl$_2$ had been shown to stimulate catalysis by mouse RALDH2, but to inhibit catalysis by RALDH1 [1,7]. Assay conditions at 37 $^\circ$C for particular concentrations of NAD$^+$ that produced optimal activity (50 mM Hepes buffer, pH 8.5, containing 20 mM MgCl$_2$, 2 mM DTT, 150 mM KCl and 1 mM EDTA) were adopted as a standard assay for the study of substrate specificity. Because sequence analysis suggested a possible ATP/GTP-binding site in RALDH3, the influence of MgCl$_2$ on the enzyme. This may explain the similar affinities that RALDH3 has for benzaldehyde, all-trans-Retinal, trans-13-cis-Retinal, trans-11-cis-Retinal, all-trans-retinal, NAD$^+$ and NADH, with both wild-type enzyme and the F471L mutant. Examples of kinetic saturation curves and binding curves are shown in Figure 2, and parameter values for all of the 11 data sets are listed in Table 2. The kinetic studies were carried out at pH 8.5 as discussed above under Michaelis–Menten conditions, i.e. using [S]$_0$ $\gg$ [E]$_0$, where [S]$_0$ is initial substrate concentration and [E]$_0$ is total enzyme concentration, and measuring initial rates (v). When the concentration of the pathway substrate was varied, the concentration of NAD$^+$ was fixed at close-to-saturating concentration and vice versa when the concentration of NAD$^+$ or NADH was varied. Hyperbolic saturation curves such as those in Figures 2(A) and 2(B) were obtained and the data were used to obtain the values of $K_m$, $k_{cat}$, $k_{cat}/K_m$, and $K_d$ shown in Table 2.

Studies on wild-type RALDH3

The catalytic effectiveness ($k_{cat}/K_m$) of wild-type RALDH3 towards non-physiological aldehydes varies markedly with chain length of the substrate. Of the aldehydes investigated, the value of $k_{cat}/K_m$ is smallest for benzaldehyde (1.9 $\times$ 10$^4$ M$^{-1}$·s$^{-1}$), increases 7-fold to 1.36 $\times$ 10$^5$ M$^{-1}$·s$^{-1}$ for hexanal and by a

![Figure 2 Examples of (A) kinetic saturation curves at 37 $^\circ$C and pH 8.5 and (B) binding curves](image)

(A) Saturation of RALDH3 (10 nM) by NAD$^+$ with an octanal concentration of 100 $\mu$M.
(B) Specific binding of octanal by the RALDH3 F471L mutant.

Table 2 Kinetic and binding parameters at pH 8.5 and 37 $^\circ$C for wild-type RALDH3 and the RALDH3 F471L mutant

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$·s$^{-1}$)</th>
<th>$K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>all-trans-Retinal</td>
<td>Wild-type</td>
<td>0.2</td>
<td>3.9</td>
<td>1.95 $\times$ 10$^7$</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>0.2</td>
<td>3.9</td>
<td>1.95 $\times$ 10$^7$</td>
<td>ND</td>
</tr>
<tr>
<td>Octanal</td>
<td>Wild-type</td>
<td>0.7</td>
<td>4.8</td>
<td>6.9 $\times$ 10$^4$</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>0.9</td>
<td>5.4</td>
<td>6.0 $\times$ 10$^4$</td>
<td>0.16</td>
</tr>
<tr>
<td>Hexanal</td>
<td>Wild-type</td>
<td>22.1</td>
<td>0.3</td>
<td>1.4 $\times$ 10$^6$</td>
<td>ND</td>
</tr>
<tr>
<td>Decanal</td>
<td>Wild-type</td>
<td>6.5</td>
<td>0.4</td>
<td>6.15 $\times$ 10$^4$</td>
<td>ND</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>Wild-type</td>
<td>103.0</td>
<td>0.2</td>
<td>1.9 $\times$ 10$^3$</td>
<td>ND</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>Wild-type</td>
<td>52.8</td>
<td>3.3</td>
<td>1.0 $\times$ 10$^5$</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>43.1</td>
<td>3.1</td>
<td>1.0 $\times$ 10$^5$</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>NADH</td>
<td>Wild-type</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>39.74 ± 2.22</td>
</tr>
<tr>
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<td>Mutant</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>25.60 ± 3.76</td>
</tr>
</tbody>
</table>

S.E.M. values for the kinetic parameters were less than 10 % of the values given. Abbreviation: ND, not determined.
further 500-fold to $6.86 \times 10^6 \text{M}^{-1} \cdot \text{s}^{-1}$ for octanal. An additional increase in chain length to decanal results in a decrease in $k_{\text{cat}}/K_m$ to $6.15 \times 10^4 \text{M}^{-1} \cdot \text{s}^{-1}$. The variation in $k_{\text{cat}}/K_m$ for most of these substrates derives mainly from the variation in $K_m$ (6.5–103 µM), variation in $k_{\text{cat}}$ being smaller (0.2–0.4 s$^{-1}$). For the most effective of these substrates (octanal), however, the large increase in $k_{\text{cat}}/K_m$ derives from a combination of a much smaller $K_m$ (0.7 µM) and a much larger $k_{\text{cat}}$ (4.8 s$^{-1}$). Insight into the major recognition determinant of RALDH3 is obtained by comparing the values of the Michaelis–Menten parameters for octanal with those for the naturally occurring retinal, namely all-trans-retinal. $K_m$ for the retinal is only about one-third of that for octanal, with $k_{\text{cat}}$ similar (3.9 s$^{-1}$) to that for octanal, and thus $k_{\text{cat}}/K_m$ (1.95 × 10$^6$ M$^{-1}$ · s$^{-1}$) is only about three times greater than that for octanal (6.86 × 10$^6$ M$^{-1}$ · s$^{-1}$). Both substrates have an eight-carbon chain, although whereas that in octanal is fully saturated and hence conformationally flexible, that in the retinal is rigid and planar, as a result of the four double bonds in the conjugated system, and possesses two methyl substituents. The surprising result is that the $\beta$-ionone ring does not appear to be a significant recognition site for RALDH3. Rather, the major requirement appears to be an eight-carbon chain whose kinetic effectiveness through binding decreases both when shortened to six carbon atoms and lengthened to 10 carbon atoms. This result is in accord with the model of Figure 1(B), which shows that the eight-carbon chain binds in the access tunnel, whereas the $\beta$-ionone ring is relatively exposed to the solvent. Comparison of the sequence of RALDH3 with those of other class 1 ALDHs led to the classification of this enzyme as an NAD-dependent dehydrogenase. In the present work the saturation of RALDH3 by NAD$^+$ was established (Figure 2A) and $k_{\text{cat}}/K_m$ for NAD$^+$ was shown to be about 200 times smaller than for all-trans-retinal and about 70 times smaller than for octanal (Table 2). Preliminary work showed that NAD$^+$ is at least 100 times more effective in the catalysed oxidation of octanal than NADP$^+$. To complement the kinetic studies, the dissociation constant ($K_d$) for the complexes of RALDH3 with octanal, NAD$^+$ and NADH were determined by using the intrinsic tryptophan fluorescence of the enzyme as described in the Materials and methods section. Although ALDH from sheep liver has been reported to adhere to an ordered sequential mechanism [11], and this seems often to be assumed for other ALDHs, the similarity of the $K_d$ values for NAD$^+$ (0.16 µM) and octanal (0.25 µM) in the RALDH3-catalysed product demonstrates a substantial degree of randomness in this mechanism. The NADH product of the forward reaction binds much less tightly (approx. 40 µM) than NAD$^+$, and the differences between the $K_m$ and $K_i$ values for the forward reaction, particularly for those of NAD$^+$ ($K_i = 0.16 \mu M$; $K_m = 52.8 \mu M$) demonstrate that $K_m$ is not purely a binding constant, but is perturbed by one or more additional rate constants, e.g. $k_{\text{cat}}$ as in $(k_{\text{cat}} + k_{\text{i}})/k_{\text{i}}$.

**Effect of mutation of Phe$^{471}$ to leucine**

The model of RALDH3 (Figure 1A) discussed above highlights the differences between the structures of RALDH3 and RALDH1, in particular the nature of residue 471 in RALDH3 (458 in RALDH1). This residue in the model of RALDH3 is phenylalanine, whereas in RALDH1 it is leucine, which provides a more spherical and less rigid steric bulk than phenylalanine. The possibility that access to the catalytic-site cysteine residue (Cys$^{314}$) might be differentially affected in RALDH3s 3 and 1 by residue 471 (458) was investigated in the present work by mutation of Phe$^{471}$ to leucine in RALDH3. The kinetic and binding parameters for the RALDH3 F471L mutant are compared with those for the wild-type enzyme in Table 2. The largest change resulting from the change of phenylalanine to leucine at position 471 is that the enzyme discriminates somewhat more effectively in favour of all-trans-retinal relative to octanal ($k_{\text{cat}}/K_m$ (retinal)/$k_{\text{cat}}/K_m$ (octanal)) $5.2 \times 10^6$/$6.0 \times 10^6 = 8.7$ for the mutant and $1.95 \times 10^6$/6.9 × 10$^6 = 2.8$ for the wild-type enzyme]. The values of $K_m$ and $k_{\text{cat}}$ for octanal are closely similar for wild-type and mutant enzymes, and $k_{\text{cat}}/K_m$ for the retinal is approximately three times larger for the mutant than for the wild-type. The $k_{\text{cat}}$ values for octanal, NAD$^+$ and NADH change only slightly as a result of the mutation. It is concluded, therefore, that decreasing the steric bulk of the side chain of residue 471 results in only a small increase in kinetic specificity in favour of the larger substrate (retinal) and emphasizes that the major recognition determinant in RALDH3 is the eight-carbon chain.

**Inhibition of RALDH3 by T$_3$**

Residues 91–107 in RALDH1 have been identified as a thyroid-hormone-binding site [10]. This region is within the NAD$^+$-binding domain which accounts for the competitive nature of the inhibition by T$_3$ of NAD$^+$ binding to RALDH1 [10,14]. In the present work, sequence alignment of RALDH1 and RALDH3 showed that RALDH3 contains a region with 80% sequence identity with the T$_3$-binding site of RALDH1. Kinetic analysis using a fixed concentration of substrate (octanal) and different concentrations of NAD$^+$ at several fixed concentrations of T$_3$ identified this hormone as a competitive inhibitor (see Figure 3). $K_{\text{app}}$ is the observed value of $K_m$ in the presence of a given concentration of inhibitor, I. The value of $K_i$ for the T$_3$ inhibition of RALDH3, calculated to be 37.7 µM by using eqn (2) and the parameters given in the legend of Figure 3, is somewhat larger than the values reported for the analogous inhibition of RALDH1 (7.4 µM) and ALDH2 (12 µM) [10,14]:

$$K_i = (K_m \cdot [I])/(K_{\text{app}} - K_m)$$  \hspace{1cm} (2)

Direct evidence that T$_3$ binds to RALDH3 was obtained by immunoprecipitation using an antibody to T$_3$, coupled to Protein G beads, followed by an antibody to the histidine-rich region...
of RALDH3. Proteins with a histidine-rich region and immuno-precipitated by αT3 were detected on the Western blot with the αHis. Hence T3 specifically binds to RALDH3, in accord with the observation of the competitive inhibition described above. Inhibition of RALDH1 activity by T3 has been reported previously (K_i = 7.4 µM) [10] and is five times more effective than that obtained for RALDH3 in the present study (K_i = 37 µM). It seems possible that this is one mechanism that might be used to maintain precise control of retinoic acid levels in the retina.

Kinetically influential ionizations of RALDH3

The importance of the protonation state of specific side chains in the enzyme mechanism make pH-dependent kinetic studies a valuable contributor to the characterization of enzyme function. The principal objective of such studies is the determination of pK_a values approximating to those of individual ionizing groups and rate constants characteristic of reactions of specific ionic forms of the free enzyme molecule and its intermediates on the catalytic pathway. The former can be obtained by the study of the pH-dependence of k_cat/K_m and the latter by analogous study of k_cat.

In the present work, using octanal as substrate, it was possible to use the condition [S]_o >> K_m over an appropriate pH range, but not [S]_o << K_m. As a consequence the study was restricted to the pH-dependence of k_cat. It was established that, at pH values 7.0, 8.5 and 10.5, the initial rates were closely similar, with [S]_o = 100 µM and [S]_o = 1 mM, and thus approximated closely to V_max values. Division of these by [E]_i provided values of k_cat over the approximate pH range 6–10. A typical data set is shown in Figure 4. In the approximate pH range 6.0–9.0 the data appear to adhere to a sigmoidal ionization curve. By contrast, at pH values above approx. 9.4, the values of k_cat decrease steeply with increase in pH in a manner characteristic of a co-operative denaturation process (results not shown). Closer examination of the data over the pH range 6.0–9.0 using SKETCHER (see the Materials and methods section) demonstrates that the pH-dependence of k_cat does not adhere to a single ionization curve, but rather corresponds to the kinetic influence of two ionizations with macroscopic pK_a values of 5.0 and 7.5 respectively (eqn 3). The good fit of the data to the equation for two kinetically influential ionizations (equation 3) is shown in Figure 4.

\[
k = \frac{\tilde{k}_1}{1 + \frac{[H^+]}{K_1}} + \frac{\tilde{k}_2}{1 + \frac{[H^+]^2}{K_1K_II} + \frac{[H^+]}{K_II}}
\]

Mechanistic implications

Evidence from studies on several ALDHs using kinetics, fluorescence spectroscopy, affinity labelling and site-directed mutagenesis (see [25]) have provided a useful mechanistic model for the investigation of aldehyde dehydrogenases. Major features of the GAPN mechanism are summarized below and related to the results obtained for RALDH3 in the present work.

For GAPN, cofactor binding induces a conformational change within the active centre that leads to increased reactivity of the catalytic-site cysteinyl thiol group and a shift in its pK_a from 8.5 to 6.1, detected by the use of thiol-specific time-dependent inhibitors. The kinetically influential ionizations observed in catalysis by GAPN have pK_a values of 6.2 and 7.5 in acylation and 6.1 and 7.4 in deacetylation. The apparently analogous ionizations observed in deacetylation (k_cat) for RALDH3 have pK_a values of 5.0 for the minor ionization \([\tilde{k}_a(1) = 0.16 s^{-1}]\) and 7.5 for the major ionization \([\tilde{k}_a(2) = 2.14 s^{-1}]\). For GAPN, the ionization with pK_a 7.5 in acylation and pK_a 7.4 in deacetylation was assigned to the carboxy group of the invariant glutamic acid residue reported as Glu_268 [25,26], but now known to be Glu_250 [28,29]. The catalytic base and the catalytic-site thiol group in GAPN were designated Glu_268 and Cys_230 respectively [26,27] by using the numbers of the conserved residues in ALDHs (class I and class II). These residues are Glu_250 and Cys_244 in the crystal structure of GAPN [28,29]. The evidence for this assignment is strong: mutation of Glu_268 (i.e. Glu_250) to alanine transforms the double-sigmoidal pH-dependence of the rate constant for acylation (with pK_a values 6.2 and 7.5) to a single sigmoidal curve with pK_a 6.2. This mutation did not significantly affect the efficiency of the acylation process, but resulted in a large decrease (by at least three orders of magnitude) in the rate of deacetylation. The low residual rate did not permit observation of any ionization phenomena.

In the case of RALDH3 the values of k_cat for the various substrates (Table 2) support the view that deacetylation may be rate-determining for the specific substrates, all-trans-retinal and octanal, whereas for the non-specific substrates with much smaller k_cat values, a step prior to deacetylation may be rate-determining.

The modelled structure of the quasi-transition state for deacylation (Figure 1C) suggests that the orientation and activation of the water molecule (base catalysis) is brought about by Glu_280 in the in conformation where it is adjacent to Glu_488. This result provides the simplest interpretation for the double sigmoidal pH-dependence of k_cat, the rate constant for deacylation. The Glu_280/ Glu_488 couple constitutes a two-site acid, protonic dissociation from which would occur in two stages (Scheme 1). The kinetically determined pK_a values are macroscopic (molecular) constants pK_1 and pK_II, which are related to the microscopic (group) constants pK_A, pK_B, pK_A', pK_B' of Scheme 1 by the well-known expressions eqns 4 and 5 (see, e.g., [27]).
The relationships between the microscopic (group) acid dissociation constants \( K_A, K_B, K'_A, K'_B \) and the macroscopic (molecular) constants \( K, K' \) characteristic of the two stages of ionization of the dicarboxy couple \( \text{Glu}^{488} \) and \( \text{Glu}^{488} \) \( \text{H}_2 \) to \( \text{X}_2 \) - to \( \text{X}^{+} \) are given by eqns (4) and (5) in the text.

\[
K_1 = K_A + K_B
\]

\[
K_1 = K'_A K'_B/(K'_A + K'_B)
\]

If the upper route of Scheme 1 predominates, \( \text{CO}_2\text{H} \), represents \( \text{Glu}^{488} \) and only a small fraction of \( \text{Glu}^{280} \) becomes the catalytic base \( [\text{CO}_2\text{H}] \) as the pH is increased across \( pK_a \) (5.0). Most of \( \text{Glu}^{280} \) becomes \( \text{Glu}^{280} \text{CO}_2\text{H}^{-} \) as the pH is increased across \( pK_B \) (7.5), which will approximate to the intrinsic \( pK_a \) of this group in the presence of \( \text{Glu}^{488} \text{CO}_2\text{H}^{-} \), i.e. \( pK_B \). An analogous interpretation is not available for the minor ionization \( (pK'_a 6.1) \) in GAPN because the structure [28,29] does not contain another carboxy side chain in the vicinity of \( \text{Glu}^{280} \). The fact that both \( pK_a \) values are relatively high for carboxy groups, even after the perturbation downwards in the case of \( \text{Glu}^{488} \text{CO}_2\text{H} \) by its proximity to \( \text{Glu}^{280} \), may be explained by the hydrophobic nature of the environment of the carboxy couple in RALDH3 provided principally by the side chains of \( \text{Trp}^{199} \), \( \text{Leu}^{139} \), \( \text{Phe}^{777} \) and \( \text{Tyr}^{497} \). Examples of carboxy groups with high \( pK_a \) values resulting from hydrophobic environments include those in lysozyme (pK \( \alpha \), 6.5), \( \alpha \)-lactoglobulin (pK \( \alpha \), 7.5) and lysozyme–glycochitin complex (pK \( \alpha \), approx. 8.2) [30].

### Concluding comment

The combination of modelling and kinetic and binding studies produced a number of advances in understanding RALDH3 and its relationship to some other ALDHs. Surprisingly, the major recognition determinant of RALDH3 is the eight-carbon chain and not the \( \beta \)-ionone ring of all-trans-retinal, which modelling suggests protrudes into solvent. This explains why this enzyme, like RALDH1, but unlike RALDH2, does not discriminate markedly in favour of the physiological substrate relative to non-physiological aldehydes. Also, surprisingly, RALDH3 appears to follow a random kinetic mechanism rather than the ordered sequential mechanism often associated with ALDH enzymes. Inhibition of RALDH3 by T3 in competition with NAD\(^+\), predicted by the modelled structure and characterized kinetically, might contribute to control of the concentration of retinoic acid in the retina, in view of the marked difference between RALDH1 and RALDH3 in binding T3. The modelled structure of RALDH3 underscores the notion that the double-sigmoidal pH-dependence of \( k_{cat} \), suggested to be the rate constant for the rate-determining deacylation of specific thioacylezyme intermediates, is due to successive protonic dissociations from the \( \text{Glu}^{280} \text{Glu}^{488} \) couple with \( \text{Glu}^{280} \text{CO}_2\text{H}^{-} \) acting as the catalytic base.

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