The biosynthesis of the hormone retinoic acid from retinol (vitamin A) involves two sequential steps, catalyzed by retinol dehydrogenases and retinal dehydrogenases, respectively. This report describes the cloning of a cDNA encoding a heretofore unknown aldehyde dehydrogenase from a rat testis library and its expression in Escherichia coli. This enzyme has been designated retinal dehydrogenase, type II, RalDH(II). The deduced amino acid sequence of RalDH(II) had the highest identity with mammalian aldehyde dehydrogenases that feature low $K_m$ values ($\mu M$) for retinal: human ALDH1 (72.2%), rat microsomal dehydrogenases, type I (71.5%), bovine retina (72.7%), and mouse AHD-2 (71.5%). RalDH(II) expressed in E. coli recognizes as substrates free retinal, with a $K_m$ of $-0.7 \mu M$, and cellular retinol-binding protein-bound retinal, with a $K_m$ of $-0.2 \mu M$. RalDH(II) also can utilize as substrate retinal generated in situ by microsomal retinol dehydrogenases, from the physiologically most abundant substrate: retinol bound to cellular retinol-binding protein. Rat testis expresses RalDH(II) mRNA most abundantly, followed by (relative to testis): lung (6.7%), brain (6.3%), heart (5.2%), liver (4.4%), and kidney (2.7%). RalDH(II) does not recognize citral, benzaldehyde, acetaldehyde, and propanal efficiently as substrates, but does metabolize octanal and decanal efficiently. These data support a function for RalDH(II) in the pathway of retinoic acid biogenesis.

The hormone RA induces a variety of biological responses by modulating gene expression during development and postnatally, to control differentiation or entry into apoptosis of diverse cell types in numerous organs (1–3). Vertebrates require RA for normal hematopoiesis, reproduction, bone remodelling, and sustaining epithelia (4, 5). Yet, excessive RA causes toxicity.

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The nucleotide sequences reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U60063 (RalDH(II)).

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The abbreviations used are: RA, all-trans-retinoic acid; CRBP, cellular retinol-binding protein; IPTG, isopropyl-$\beta$-thiogalactoside; PCR, polymerase chain reaction; RalDH(II), retinal dehydrogenase type II; RalDH(II)/rS, recombinant RalDH(II) expressed from the first ATG with an N terminus histidine tag: RoDH, retinal dehydrogenase; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); nt, nucleotides(s).

**MATERIALS AND METHODS**

A cDNA Encoding RalDH(II)—Rat testis RNA served as the template for reverse transcriptase-PCR with the two primers, 5'-TTGTCAGAGG/GAAGG/AACAGG/AAAGC-3' and 5'-AC(T/C)GGT(T/C)CCCAAT/A/GAT- CTCCTCA-3'. RNA (3.5 $\mu g$) was allowed to react with 3 $\mu l$ of random

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hexamer (Invitrogen), 10 units of RNase ribonuclease inhibitor and 45 units of avian myeloblastosis virus reverse transcriptase (Promega) in a total volume of 30 μl for 2 h at 39 °C. The reaction mixture was diluted 1/20 with water, and 1/10 of the diluted solution was added to a PCR reaction mixture consisting of (final concentrations): 1 μM each primer, 1.5 mM MgCl₂, 0.2 mM each dNTP, and 2 units of Taq DNA polymerase (Promega). The 30-μl reaction mixture was incubated in the presence of 0.16 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin for 10 min. After incubation, 15 μl of the reaction mixture was used to amplify the coding region of RalDH(II) by PCR (final conditions): 1.5 mM MgCl₂, 0.2 mM each dNTP, and 2 units of DNA polymerase (Promega) under the same conditions. Probes were purified with a 5% deoxycyclin chain termination using the fmol™ DNA Sequencing System (Promega).

Northern Blots—The RNA blot was prepared with adult male rat tissue poly(A)⁺ as described previously (11). The probe for RalDH(II), a 796-nucleotide BssHII/EcoRI fragment from a unidirectional deletion mutant with cDNA from RalDH(II), was linearized with Scal, which cut at nucleotide 1512 to produce a 341-nucleotide fragment consisting of nt 1513 through 1853. This fragment included 291 nt of 3'-untranslated cDNA. The 341-nucleotide fragment was used as template for transcription of 32P-labeled antisense RNA polymerase (Stratagene), ranging from 0.2- to 20-fold the Kᵥ₁ value. Kinetic constants were determined under initial velocity conditions linear with time and protein.

Rat Tissues—Microsomes and RNA were prepared as described previously from the tissues of male rats (~250 g) fed a chow diet (11).

Preparation of CRBP—CRBP was generated in E. coli with the vector pMONCRBP (19), as described (9). The concentration of functional apo-CRBP was determined by saturating an aliquot with retinol, separating free and bound retinol by size-exclusion chromatography, and determining the Aᵥ₂₅₀/Aᵥ₃₉₀ ratio. Holo-CRBP denotes CRBP saturated with retinol, whereas CRBP-retinal denotes CRBP saturated with retinal.

RESULTS AND DISCUSSION

Nucleotide and Deduced Amino Acid Sequences of RalDH(II)—We selected testis RNA and cDNA, respectively, as templates for synthesizing a probe and for library screening to avoid bacterial heptane aldehyde dehydrogenases. Primers were designed for reverse transcriptase-PCR from two sequences of amino acids in rat phenobarbital-inducible aldehyde dehydrogenase (residues 267–273 and 399–404), which are highly conserved regions of the mammalian members of the aldehyde dehydrogenase superfamily (20). Reverse transcriptase-PCR with testis RNA produced a 0.4-kb probe (the anticipated size), with a nucleotide sequence only ~70% identical with known aldehyde dehydrogenases (Fig. 1, nt 864 through 1270). Library screening with this probe identified a 2.2-kb clone, which was subcloned to yield pBC/RalDH(II). This clone also included 0.8 kb of 5’ flanking sequence and 20 amino acid histidine tag onto the N terminus used in RalDH(II) or L.
lar" (i.e., (K/R), (D/E), (T/S), (Y/F), (N/Q), or (L/I/V/M)), in at least 87% of this superfamily. By analogy to the human aldehyde dehydrogenases, Cys-313 and Glu-279 probably have catalytically essential roles. Mammalian aldehyde dehydrogenases also require the Glu residue at 498, for reasons not yet known. The cofactor binding site sequence, GXXTXXG, begins at Gly-256.

The protein encoded by pBC/RalDH(II) has the highest amino acid identities and similarities, in parentheses respectively, with chicken (73, 86%), bovine retina (73, 87%), human ALDH1 (72, 87%), rat RalDH(I) (72, 85%), mouse AHD-2 (72, 85%), and rat phenobarbital-inducible (71, 85%) aldehyde dehydrogenases (22–27). Thus, the overall amino acid sequence similarity, as well as the conservation of specific amino acid residues, denote a heretofore unrecognized aldehyde dehydrogenase. The high amino acid similarity with rat RalDH(I), mouse AHD-2, bovine retina aldehyde dehydrogenase, and human ALDH-1 is especially intriguing, because these enzymes catalyze the conversion of retinal into RA with $K_m$ values $<1 \mu M$, except for bovine retina aldehyde dehydrogenase, which has a $K_m$ value $<9 \mu M$ (17, 27–30). These enzymes, however, have not been tested for recognition of retinal in the presence of CRBP or retinal generated in situ from holo-CRBP.

Distribution of RalDH(II) mRNA—Northern blot analysis of RalDH(II) expression in rat testis revealed a 2.2-kilobase mRNA, but did not reveal RalDH(II) mRNA in several other adult rat tissues (data not shown). RNase protection assays, however, showed widespread expression of mRNA in adult rat tissues in addition to testis (Fig. 2). Extratesticular tissues expressed RalDH(II) at a fraction of the magnitude of expression in testis (relative expression, 100%): lung (6.7%), brain (6.3%), heart (5.2%), liver (4.4%), and kidney (2.7%).

**Fig. 1.** Nucleotide and deduced amino acid sequences of RalDH(II). Underlined nucleotides identify the second possible start site and the polyadenylation signal, respectively. Boldface identifies the 23 invariant amino acids in the members of the aldehyde dehydrogenase superfamily; underlined amino acid residues indicate those strictly conserved residues or "invariant similarities" (i.e., (K/R), (D/E), (T/S), (Y/F), (N/Q), or (L/I/V/M)) of at least 87% of mammalian, bacterial, yeast, and plant aldehyde dehydrogenases (23).
Chromatography to a single band on SDS-PAGE. About 1 mg of purified RalDH(II)/rL was obtained from a 100-ml incubation.

RalDH(II)/rL and Retinal—RalDH(II)/rL catalyzed the conversion of 2 μM retinal into RA at a rate linear for at least 12.5 min and up to 0.4 μg of purified protein and functioned optimally—pH 8.5 (not shown). RalDH(II)/rL recognized NAD with a K_m of 70 ± 0.6 μM and NADP with a K_m of 400 ± 7 μM (± S.E., Enzfitter (31)). The V_max in the presence of NAD was 5-fold higher than that with NADP. The apparent K_m of RalDH(II)/rL for free retinal (i.e. unbound with CRBP) was 0.7 ± 0.3 μM, and the V_max was 105 ± 4 nmol/min/mg of protein (± S.D., n = 3) (Fig. 4, top panel). The kinetic constants were re-evaluated in the presence of a 2 molar excess of apo-CRBP at each retinal concentration, which generated an apparent K_m of 0.2 ± 0.06 μM and a V_max of 62 ± 15 nmol/min/mg of protein (n = 3) (Fig. 4, bottom panel). CRBP binds retinal with a K_d between 50 and 100 nM (19, 32). In the presence of a 2 molar excess of CRBP, the concentration of unbound retinal would range from 0.042 μM, at a total retinal concentration of 0.1 μM, to 0.1 μM at a total retinal concentration of 6 μM (calculating from a K_d of 100 nM), i.e. it would remain practically constant. In contrast, the CRBP-retinal concentration would range from 0.58 μM to 5.9 μM. The Michaelis-Menten relationship must therefore occur between CRBP-retinal and the rate of RA production, suggesting that RalDH(II)/rL recognizes retinal in the presence of CRBP as substrate. Because retinal has very poor solubility in aqueous media and there occurs an excess of CRBP relative to retinal in vivo, retinal in cells would likely occur bound to CRBP, in equilibrium with membranes and/or proteins. The ability of RalDH(II)/rL to catalyze the synthesis of RA in the presence of CRBP shows that it functions under conditions that more closely model physiological conditions than retinal dispersed in the aqueous medium.

Because holo-CRBP is the most abundant form of retinol in vivo, and NADP-dependent microsomal RoDHs are the most active retinol dehydrogenases with respect to holo-CRBP as substrate (33), the ability was determined of RalDH(II)/rL to utilize as substrate retinal generated in situ by microsomal RoDHs from holo-CRBP. Apo-CRBP also was included to ensure complete binding of retinol (K_d = 0.1–1 nM (32)) and...
because this combination of holo-CRBP and apo-CRBP most closely approximates conditions in vivo. Generation of RA from the retinal produced in situ from holo-CRBP by microsomal RoDHs increased with increasing amounts of RalDH(II)/rL titrated into the incubation mixture (Fig. 5). Maximum RA synthesis from holo-CRBP occurred only in the presence of both microsomes and RalDH(II)/rL along with both cofactors, whereas measurable but much less RA production was observed with a combination of microsomes and RalDH(II)/rL, if one cofactor were omitted (Table I). Consistent with previous results, microsomes alone in the presence of both NADP and NAD produced little RA (17, 34). These data show that the NAD-supported RalDH(II)/rL can use retinal generated by the microsomal, NADP-dependent RoDHs to biosynthesize RA.

RalDH(II)/rL Substrate Specificity—RalDH(II)/rL does not recognize citral as substrate and inefficiently catalyzes the dehydrogenations of acetaldehyde, benzaldehyde, and propenal (Table II). These are the prototypical substrates used to assay and classify the mammalian aldehyde dehydrogenases. Thus, RalDH(II) differs from many other members of the superfamily not only in primary amino acid sequence, but also in substrate specificity. Medium-chain aldehydes, however, were metabolized efficiently by RalDH(II). Of the eight aldehyde dehydrogenase substrates assayed, octanal and decanal had the most favorable $V_{\text{max}}/K_m$ values, whereas retinal had the lowest $K_m$. Although the $V_{\text{max}}/K_m$ for retinal was 3- to 4-fold lower than the values for ocetal and decanal, the apparent $K_m$ for retinal was an order of magnitude lower. Thus, RalDH(II) can catalyze the dehydrogenation of retinal at much lower concentrations than the other substrates assayed. It should not surprise that medium-chain aldehydes would be accommodated by an active site that recognizes retinal. Octanal and decanal are similar in length to the side chain of retinal and enjoy greater flexibility. Perhaps their greater flexibility and more simple structures (lack of double bonds, lack of methyl groups) and the absence of a conjugated carbonyl account for their greater rates of dehydrogenation, even though they are apparently accommodated less efficiently by the active site.

Characteristics of RalDH(II)/rS—RalDH(II)/rS, the shorter protein expressed in E. coli from the second possible initiator, had enzymatic characteristics similar to those of RalDH(II)/rL. It was NAD-dependent, had a pH optimum ~8.5, recognized free retinal with a $K_m$ ~1 $\mu$M, and was inefficient in metabolizing benzaldehyde, acetaldehyde, propanal, and hexanal, but metabolized octanal and decanal, efficiently with $V_{\text{max}}/K_m$ ratios of 175 and 148, respectively. The shorter N terminus, therefore, does not affect activity markedly. This affords the possibility that alternative forms of RalDH(II) are translated in different cell types or under different conditions.

Concluding Summary—The designation of the isozyme reported here as RalDH(II) seems justified by several criteria. Firstly, the amino acid sequence of RalDH(II) has the greatest identity and similarity with RalDH(II) and other aldehyde dehydrogenases that catalyze the conversion of retinal into RA.

**TABLE I**

| Addition  | Picoles of RA (mean ± SD, n = 3) |
|-----------|----------------------------------|
|           | 1 ± 0 | 0 | 0 | 0 | 0 | 6 ± 1 | 8 ± 1 | 45 ± 11 |
| Microsomes | +     | - | - | - | - | +     | +     | +       |
| RalDH(II)  | -     | + | + | + | + | +     | +     | +       |
| NAD        | +     | - | - | - | - | +     | -     | -       |
| NADP       | +     | - | - | - | - | -     | +     | +       |

**TABLE II**

| Aldehyde  | $K_m$ $\mu$M | $V_{\text{max}}$ nmol/min/mg | $V_{\text{max}}/K_m$ |
|-----------|--------------|-----------------------------|---------------------|
| Citral    | NA $^*$     | NA $^*$                     | NA $^*$             |
| Acetaldehyde | 645        | 139                          | 0.2                 |
| Benzaldehyde | 305        | 200                          | 0.7                 |
| Propanal  | 61           | 145                          | 2.4                 |
| Hexanal   | 28           | 654                          | 23                  |
| 2-trans-retinal | 0.4 ± 0.1 | 20 ± 5                      | 49 ± 6              |
| Octanal   | 5            | 759                          | 152                 |
| Decanal   | 3            | 653                          | 214                 |

$^*$ NA, no activity detected with substrate concentrations up to 10 $\mu$M.

Secondly, the enzyme recognizes “free” retinal with a low $K_m$, and also recognizes retinal in the presence of CRBP with a low $K_m$. Thirdly, RalDH(II) can use retinal generated in situ from holo-CRBP/apo-CRBP by microsomal RoDHs as substrate for RA synthesis. These conditions approximate those that occur in RA-producing tissues. Fourthly, although RalDH recognizes aldehydes other than retinal, their structures are simpler than that of retinal. Their ability to enter into an active site that accommodates retinal would not be unusual, whereas it would be more unusual for the more rigid, branched, and polysaturated molecule, retinal, to adapt to a nonspecific enzyme with a low $K_m$ and a physiologically effective $V_{\text{max}}$.

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