Rat Liver Cytosolic Retinal Dehydrogenase: Comparison of 13-*cis*-, 9-*cis*-, and *all-trans*-Retinal as Substrates and Effects of Cellular Retinoid-Binding Proteins and Retinoic Acid on Activity†

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ABSTRACT: A basic p*I* retinal dehydrogenase has been purified recently that accounts for ~90% of the *all-trans*-retinal dehydrogenase activity of rat liver cytosol. In this work, we show that this enzyme also accounts for ~90% of the 9-*cis*-retinal dehydrogenase activity of rat liver cytosol. The partially-purified enzyme displayed allosteric kinetics for 9-*cis*-retinal [$K_{0.5}$ = 5.2 μM, Hill coefficient = 1.4, V_{max} = 7.85 nmol min^{-1} (mg of protein)$^{-1}$] with the ratio $V_{max}/K_{0.5}$ = 1.5. The latter is similar to that of 2.1 for *all-trans*-retinal [$K_{0.5}$ = 1.6 μM, Hill coefficient = 1.4, V_{max} = 3.4 nmol min^{-1} (mg of protein)$^{-1}$]. Competition between *all-trans*- and 9-*cis*-retinal occurred only when micromolar concentrations of both were present, indicating that the dehydrogenase could catalyze both *all-trans*- and 9-*cis*-retinoic acid syntheses simultaneously at the nanomolar amounts of the retinals that are likely to occur physiologically. Although reactions of *all-trans*- and 9-*cis*-retinoids were catalyzed with similar efficiencies, 13-*cis*-retinal was not an efficient substrate. This retinal dehydrogenase was not feedback-inhibited by *all-trans*- or 9-*cis*-retinoic acid, nor by holocellular retinoic acid-binding protein, but was stimulated modestly by apocellular retinoic acid-binding protein, an effect not observed in the presence of cellular retinol-binding protein. These data indicate that products, *via* feedback inhibition, do not regulate retinoic acid synthesis by this dehydrogenase. This dehydrogenase may serve as a common enzyme in the conversion of *all-trans*- and 9-*cis*-retinal into their acids.

Retinoic acid (RA)[1] is a potent modulator of development that affects multiple organs and structures (Summerbell & Maden, 1990; Richman, 1992; Morris-Kay, 1993). For example, it programs positional information in limb generation and is crucial to nervous system, cardiovascular, urogenital, and cranial facial development. In mature vertebrates, RA is essential for normal hematopoiesis, reproduction, bone remodeling, and sustaining normal epithelia (Wolf, 1984; Goss & McBurney, 1992). Yet, excessive RA is teratogenic (Satre & Kochhar, 1989) and is also toxic postnatally (Wolf, 1984). RA concentrations *in vivo*, therefore, are likely to be closely controlled through a combination of regulating synthesis, controlling catabolism and managing the contentrations of free retinoids by sequestering them with specific binding proteins.

A pathway of atRA synthesis involves both retinoid-specific enzymes and retinoid-specific binding proteins (Napoli, 1993). In multiple tissues, holoCRBP is recognized by a microsomal NADP-dependent retinol dehydrogenase, which uses it as substrate to form *all-trans*-retinal (Posch *et al.*, 1991). The *all-trans*-retinal then serves as an intermediate in atRA synthesis by cytosolic dehydrogenase(s) (Posch *et al.*, 1992). HoloCRBP seems to be involved not only in delivering retinol to the microsomal dehydrogenase but also in denying enzymes that cannot recognize the binding protein access to retinol, protecting retinol from artifactural oxidation, and transferring retinal from microsomes to cytosol (Napoli *et al.*, 1992; Posch *et al.*, 1992). ApoCRBP also influences RA synthesis by stimulating a bile salt-independent membrane-bound retinyl ester hydrolase, resulting in the hydrolysis of endogenous retinyl esters (Boerman & Napoli, 1991), and by inhibiting lecithin–retinol acyltransferase (Herr & Ong, 1992), which recognizes holoCRBP as substrate for esterifying retinol (Ong *et al.*, 1988; Yost *et al.*, 1988). Both effects of apoCRBP would increase the amount and proportion of holoCRBP available for RA synthesis, and would decrease the degree and rate of retinol storage as retinyl esters.

The major cytosolic retinal dehydrogenase in adult rat liver has been purified recently (Posch *et al.*, 1992). It is a quantitatively minor cellular protein composed of four 55-kDa subunits. Thus, it is typical of aldehyde dehydrogenases, which are composed generally of multimers of 55-kDa subunits (Lindahl, 1992). From its isoelectric point of 8.3, the enzyme was a previously undetected rat aldehyde dehydrogenase (Cao *et al.*, 1989). This p*I* and composition are similar to those of the mouse liver cytosolic aldehyde dehydrogenase isozyme AHD2, which also recognizes *all-trans*-retinal as substrate (Lee *et al.*, 1991), but are distinct from the rat phenobarbital-induced aldehyde dehydrogenase, a dimer with a p*I* of 6.8 (Dunn *et al.*, 1989). The phenobarbital-induced aldehyde dehydrogenase had been proposed as a retinal dehydrogenase, on the basis of pharmacological retinal concentrations (Leo

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[1] Abbreviations: BSA, bovine serum albumin; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein (type I); DTT, dithiothreitol; fplc, fast protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hplc, high-performance liquid chromatography; 9cRA, 9-*cis*-retinoic acid; atRA, *all-trans*-retinoic acid; 13cRA, 13-*cis*-retinoic acid; RA, retinoic acid; RAR, retinoic acid receptor(s); RXR, retinoid X receptor.

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et al., 1989). Mouse AHD2 and the rat retinal dehydrogenase have been studied with *all-trans*-retinal as substrate, but not with any of its geometric isomers. Geometric retinoid isomers are important because RXRs recognize a geometric isomer of atRA, 9cRA, as their endogenous ligand and discriminate against atRA, whereas the RARs recognize both atRA and 9cRA equally well (Levin *et al.*, 1992; Heyman *et al.*, 1992; Allenby *et al.*, 1993). Although enzymatic pathways for 9cRA biosynthesis have not been established, 9-*cis*-carotenoids occur in fruits and vegetables, and 9-cis-carotenoids as well as 9-*cis*-retinol accumulate in animal organs (Brown, 1959; Chandler & Schwartz, 1987; Ben-Anotz *et al.*, 1988; Stahl *et al.*, 1993). These might support 9cRA synthesis, if the appropriate enzymes exist.

This report further characterizes the rat liver cytosolic dehydrogenase with respect to substrate specificity and the impact of free RA and CRABP on RA synthesis. The dehydrogenase catalyzed the conversion of *all-trans*-retinal and 9-*cis*-retinal into their acids with similar efficiencies, suggesting that 9-*cis*-retinal may be a source of 9cRA. RA exerted no feedback effect on its own synthesis, but apoCRABP stimulated RA synthesis under some circumstances.

EXPERIMENTAL PROCEDURES

General. *all-trans*-Retinal was purchased from Eastman Kodak. 9-*cis*-Retinal and 13-*cis*-retinal were purchased from Sigma. Each was used within 2 weeks of purification by normal-phase hplc (Napoli, 1986a). Other chemicals were purchased from Sigma. Protein was determined by the dye-binding method with BSA as standard (Bradford, 1976). CRBP, expressed in *Escherichia coli* with the vector pMON-CRBP (Levin, 1988), was purified and quantified as described (Boerman & Napoli, 1991). CRABP was expressed in *E. coli* and was purified as described (Fiorella & Napoli, 1991). Kinetic data were analyzed with the microcomputer program "Enzfitter" (Leatherbarrow, 1987). Statistical analyses were done by two-tailed unpaired *t* tests with the program "Instat".

Retinal Dehydrogenase. The retinal dehydrogenase was purified as reported from the liver cytosol of male Sprague-Dawley rats (~250 g) maintained on a vitamin A-sufficient diet, up to and including the second anion-exchange column run at pH 10 (Posch *et al.*, 1992). Briefly, cytosol (800 mg of protein) was exchanged with buffer A (25 mM ethanolamine/2 mM dithiothreitol, pH 9.15) and partitioned by anion-exchange chromatography with a Q-Sepharose column (2.5 × 19 cm) into P1 (150 mg of protein), the unretained activity, and P2 (472 mg of protein), the retained activity. The latter was eluted with 1 M NaCl in buffer A. The P1 fraction was exchanged with 25 mM piperazine and 2 mM dithiothreitol, pH 10, and applied to a second Q-Sepharose column (1.5 × 9 cm). After 66 mL, a linear gradient was run from 0 to 350 mM NaCl to elute the dehydrogenase.

Enzyme Assays. Enzyme obtained from the pH 10 anion-exchange column was used for assays, unless noted otherwise. Unless stated otherwise, assays were done in duplicate (values were within 10% of the mean) for 20 min at 37 °C in a buffer of 20 mM Hepes, 150 mM KCl, 1 mM EDTA, and 2 mM dithiothreitol, pH 8.5, in a final volume of 500 μL. Substrates were added in 2 μL of dimethyl sulfoxide. The incubations were initiated by adding 2 mM NAD. Controls were assays done without cofactor. Reactions were quenched by adding sufficient 0.025 N KOH/ethanol to raise the pH to 12. The internal standard *all-trans*-7-(1,1,3,3-tetramethyl-5-indanyl)-3-methylocta-2,4,6-trienoic acid was added in 5 μL of ethanol. After extraction of neutral retinoids with 2.5 mL of hexane,

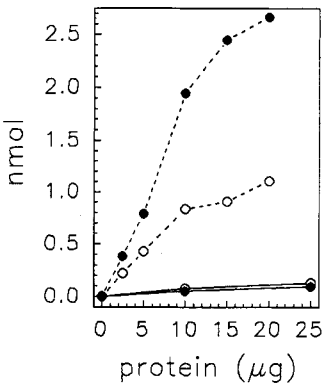

FIGURE 1: *all-trans*- and 9-*cis*-retinal dehydrogenase activities in cytosolic fractions. The rates of 9cRA and atRA formation from 20 μM 9-*cis*-retinal (filled circles) or 5 μM *all-trans*-retinal (open circles) were determined under standard conditions with protein from the wash-through, P1 (dashed lines), and the retained, P2 (solid lines), fractions generated by anion-exchange chromatography at pH 9.15.

the pH of the aqueous phase was adjusted to <2 with 4 N HCl. RAs and the internal standard were extracted with a second 2.5-mL portion of hexane. Hexane was removed under a stream of nitrogen, and 110 μL of hexane was added to each sample for injection onto hplc.

Hplc Analyses. RA was quantified as described with modifications (Napoli, 1986a, 1990a). Normal-phase hplc was done with two DuPont Zorbax-Sil Reliance cartridge columns (0.4 × 4 cm each) connected in series with a guard cartridge. 9cRA eluted in 6.9 min, atRA eluted in 7.4 min, and the internal standard eluted in 9.4 min. Alternatively, a single-cartridge column with a guard column was used, from which 13cRA eluted in 3.65 min, 9cRA in 3.9 min, atRA in 4.2 min, and the internal standard in 5.2 min. In both cases, the columns were eluted with dichloroethane/hexane/acetic acid (5/95/0.35) at 2 mL/min. 13-*cis*-Retinal and *all-trans*-retinal were eluted in 1.7 and 2.4 min, respectively, by normal-phase hplc on a single-cartridge column with a guard column by a linear gradient of 4–15% tetrahydrofuran in hexane developed in 5 min at 2 mL/min. The absorbance was monitored at 340 nm for RAs and at 370 nm for retinals with a Waters Model 484 tunable absorbance detector.

RESULTS

In preliminary experiments, relatively high activity was observed for 9-*cis*-retinal with the semipurified basic p*I* rat cytosolic retinal dehydrogenase. Therefore, the enzyme was reisolated, up to and including the second anion-exchange column, to determine whether it represented the major 9-*cis*-retinal dehydrogenase activity in cytosol. As expected from previous work (Posch *et al.*, 1992), subjecting rat liver cytosol to anion-exchange chromatography at pH 9.15 divided the *all-trans*-retinal dehydrogenase activity into two fractions. The first peak, P1, had ~14- and 38-fold greater specific activity, respectively, for *all-trans*-retinal and 9-*cis*-retinal dehydrogenation than did the retained, P2, fraction (Figure 1). In contrast to P1, which had ~3-fold greater 9-*cis*-retinal dehydrogenase activity than *all-trans*-retinal dehydrogenase activity, P2 had equivalent and low activity for both retinoids. P1 contained 88–92% of the cytosolic *all-trans*- and 9-*cis*-retinal dehydrogenase units, but only ~24% of the recovered protein.

Application of the P1 fraction to an anion-exchange column at pH 10 eluted the *all-trans*- and 9-*cis*-retinal dehydrogenase activities as a single peak, and separated the peak from most of the protein (Figure 2). The partial purification, summarized

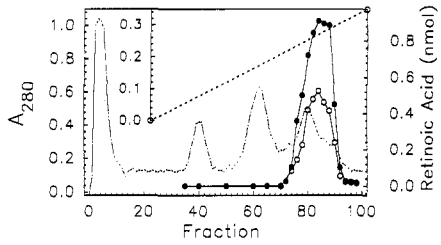

FIGURE 2: Anion-exchange chromatography at pH 10 of the P1 fraction. Fractions (3 mL each) were collected as described under Experimental Procedures. The NaCl gradient (inset) was begun at fraction 22. The left *y* axis shows protein measurements (dots), and the right *y* axis shows RA formed from *all-trans*-retinal (open circles) or 9-*cis*-retinal (filled circles). Fifty-microliter aliquots of each fraction were measured for dehydrogenase activity.

Table 1: Comparison of *all-trans*-Retinal, 9-*cis*-Retinal, and 13-*cis*-Retinal Dehydrogenase Activities in Rat Liver Cytosol during Partial Purification[a]

material	protein (mg)	activity [nmol min^{-1} mg^{-1} (units)[b]] for substrate		
		all-trans-retinal	9-*cis*-retinal	13-*cis*-retinal
cytosol	800	0.4 (320)	1 (800)	0.4 (NA)[c]
pH 9.1[d]	150	1.1 (160)	3.1 (460)	0.8 (NA)
pH 10[e]	3.8	4.3 (17.3)	20 (76)	1.0 (NA)

[a] Pools were assayed with 5 μM *all-trans*-retinal or 5 μM 13-*cis*-retinal or 20 μM 9-*cis*-retinal under standard conditions as described under Experimental Procedures. [b] The products were the homologous RAs except with 13-*cis*-retinal which yielded atRA as the product. [c] NA, not applicable. The product was atRA. [d,e] These are the activities obtained from the anion-exchange columns eluted at pH 9.15 and pH 10, respectively.

Table 2: Conversion of 13-*cis*-Retinal and 13cRA into atRA[a]

retinoid	conditions	pmol of retinoid	
		atRA	13cRA
13-*cis*-retinal	–NAD, 37 °C	ND[b]	ND
	+NAD, 37 °C	269 ± 5	ND
	+NAD, 4 °C	18 ± 1	ND
	+NAD, no protein	ND	ND
13cRA	–NAD, 37 °C	91 ± 9	424 ± 26
	+NAD, 37 °C	89 ± 2	434 ± 15
	+NAD, 4 °C	100 ± 4	417 ± 19
	+NAD, no protein	98 ± 8	416 ± 16

[a] Reactions were done under standard conditions with 6 μg of protein and either 5 μM 13-*cis*-retinal or 1.3 μM 13cRA, with the changes noted. Data are means ± SD of 3–4 replicates. [b] ND, not detected, less than 2 pmol of retinoid

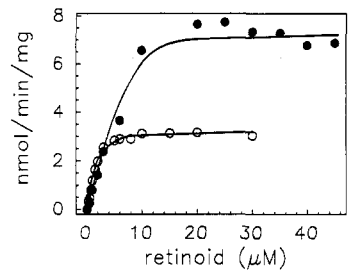

FIGURE 3: Comparison of 9-*cis*-retinal and *all-trans*-retinal as substrates for the retinal dehydrogenase. Rates of 9cRA (filled circles) and atRA (open circles) synthesis, respectively, were determined from 9-*cis*-retinal and *all-trans*-retinal under standard conditions with 4 μg of protein.

in Table 1, showed that the greater cytosolic 9-*cis*-retinal dehydrogenase activity observed remained ~3–4.5-fold higher than the *all-trans*-retinal dehydrogenase activity after each of the anion-exchange columns. 13-*cis*-Retinal also was tested as substrate with cytosol and with retinal dehydrogenase from each of the anion-exchange column pools. No 13cRA was detected after incubation with 13-*cis*-retinal. Rather, the product in the cytosol and each of the pools was atRA. The production of atRA from 13-*cis*-retinal became less efficient after each purification step, perhaps indicating that an isomerase was separated from the dehydrogenase.

Although these results are consistent with the enzyme's inability to recognize 13-*cis*-retinal as substrate, failure to detect 13cRA could also result from its instability or the instability of 13-*cis*-retinal. To exclude the former, 13cRA was incubated with the dehydrogenase under the standard incubation conditions. Seventy-nine percent of the RA added was recovered, composed of 81–83% 13cRA and 17–19% atRA (Table 2). This conversion of 13cRA into atRA was not temperature-, cofactor-, or protein-dependent, indicating that it was nonenzymatic. These data show that 13cRA would have been detected, had it been synthesized. In contrast, production of atRA from 13-*cis*-retinal was temperature-, cofactor-, and protein-dependent, consistent with an enzymatic process, and suggesting that isomerization of 13-*cis*-retinal into *all-trans*-retinal provided the substrate for atRA synthesis. This was verified by analyzing the retinoids present at the end of the incubation. From 62% to 75% of the 13-*cis*-retinal added was recovered as 13-*cis*-retinal, *all-trans*-retinal, or atRA. Under the conditions that did not produce atRA (no protein/no cofactor), 18–22% of the substrate was recovered as 13-*cis*-retinal and 78–84% as *all-trans*-retinal. Under the conditions that produced atRA, 11% of the substrate was recovered as 13-*cis*-retinal (179 ± 10 pmol, mean ± SD, n = 3), 70% was *all-trans*-retinal (1100 ± 37 pmol), and 18% was atRA (269 pmol, see Table 2). At the end of the incubation, therefore, ~0.36 μM 13-*cis*-retinal was still available as a potential substrate. The lack of 13cRA above control under these conditions indicates that 13-*cis*-retinal itself does not serve as an efficient substrate.

The enzyme might have low-affinity 13-*cis*-retinal dehydrogenase activity. This was examined by testing six concentrations of 13-*cis*-retinal from 2.5 to 30 μM for the ability to support 13cRA synthesis. No 13cRA was detected. The maximum rate of atRA synthesis was achieved at 5 μM 13-*cis*-retinal; further increases were not noted at 7.5, 10, 20, and 30 μM 13-*cis*-retinal, and the rate of atRA synthesis at 2.5 μM was 57% of that at 5 μM. These data reflect the kinetic characteristics of the dehydrogenase for *all-trans*-retinal.

With 9-*cis*-retinal as substrate the dehydrogenase exhibited allosteric kinetics with a $K_{0.5}$, determined in two separate experiments, of 5.2 μM (5.3 ± 0.8, 5.0 ± 0.8) and a Hill coefficient of 1.4 (1.4 ± 0.1, 1.4 ± 0.1) (Figure 3). The V_{max} of 7.85 nmol min^{-1} (mg of protein)$^{-1}$ (7.6 ± 0.8, 8.1 ± 0.8) was over 2-fold greater than that determined for *all-trans*-retinal [3.4 ± 0.2 nmol min^{-1} (mg of protein)$^{-1}$]. The kinetic constants with *all-trans*-retinal, $K_{0.5}$ = 1.6 ± 0.3 and a Hill coefficient of 1.4 ± 0.1, generated during this work compared well with our previously published constants of $K_{0.5}$ = 0.8 ± 0.5 and a Hill coefficient of 1.5 (Posch *et al.*, 1992).

To confirm that the same enzyme had both 9-*cis*- and *all-trans*-retinal dehydrogenase activities, *all-trans*-retinal was tested as an inhibitor of 9-*cis*-retinal dehydrogenation (Figure 4). 9cRA synthesis from 2 μM 9-*cis*-retinal was inhibited 50% by 5 μM *all-trans*-retinal and 100% by 30 μM *all-trans*-retinal, but atRA synthesis was not affected by 2 μM 9-*cis*-retinal. Increasing the 9-*cis*-retinal concentration decreased the potency of *all-trans*-retinal as inhibitor and its efficiency as substrate. 9cRA synthesis from 20 μM 9-*cis*-retinal was inhibited 50% by 6 μM *all-trans*-retinal, but only 74% by 30

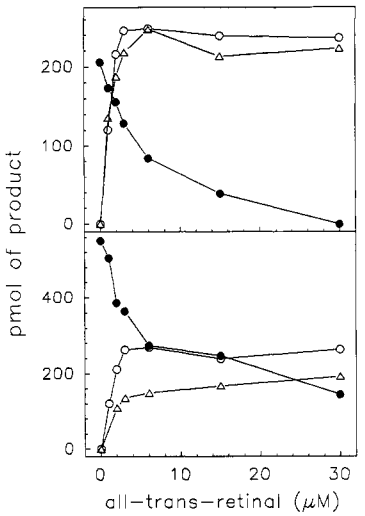

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FIGURE 4: 9-*cis*-Retinal effects on atRA synthesis from *all-trans*-retinal. The conversion of *all-trans*-retinal (*x* axis) into atRA was monitored in the absence (open circles) or presence (open triangles) of 2 μM (top panel) and 20 μM (bottom panel) 9-*cis*-retinal. The conversion of the 9-*cis*-retinal into 9cRA was also monitored (filled circles). Incubations were conducted for 15 min with 4 μg of protein.

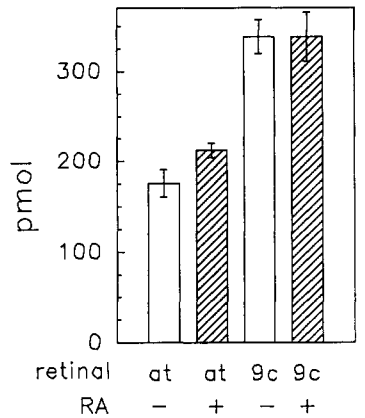

FIGURE 5: RA effects on the conversion of retinal into RA. The production was monitored of atRA from 5 μM *all-trans*-retinal (first bar) and 5 μM *all-trans*-retinal plus 0.4 μM 9cRA (second bar) and of 9cRA from 20 μM 9-*cis*-retinal (third bar) and 20 μM 9-*cis*-retinal plus 0.4 μM atRA (fourth bar). Data were obtained in 15-min incubations with 4 μg of protein and are means ± SD of triplicates.

μM *all-trans*-retinal; 20 μM 9-*cis*-retinal did inhibit atRA synthesis, with inhibition at the lower concentrations of *all-trans*-retinal (6 μM) approaching 50%.

Relatively high concentrations (0.4 μM) of either atRA or 9cRA failed to inhibit RA synthesis (Figure 5). In contrast, apoCRABP stimulated RA synthesis by ~30–50%. This outcome was not diminished by the presence of RA concentrations in the higher physiological range (Table 3). The CRABP effect was not the result of protein merely solubilizing substrate, because neither BSA nor CRBP stimulated at RA or 9cRA synthesis. Quite the contrary, CRBP decreased the rate of atRA synthesis, and BSA decreased the rate of 9cRA synthesis. CRBP also prevented CRABP enhancement of both atRA and 9cRA syntheses. The effects of apoCRABP, holoCRABP, and atRA on 9cRA synthesis then were examined over a range of concentrations (Figure 6). To generate holoCRABP, a constant ratio was maintained of CRABP/atRA, with CRABP in excess, which kept the concentration of atRA constant, while increasing the concentrations of holo- and apoCRABP. This was done to allow any impact of holoCRABP to be distinguished from those of atRA and apoCRABP alone, and to determine whether holoCRABP could ameliorate the action of apoCRABP. Half-maximum stimulation of 9cRA synthesis from 9-*cis*-retinal occurred at ~0.2 μM apoCRABP. Enhancement was also noted with the mixture of holoCRABP/apoCRABP, but the degree of stimulation seemed to reflect the concentration of apoCRABP in the mixture. Free atRA, on the other hand, caused a small decrease in 9cRA synthesis.

Table 3: Effects of Retinoid-Binding Proteins on the Conversion of *all-trans*-Retinal and 9-*cis*-Retinal into Their Respective RAs[a]

addition	% product RA	
	atRA	9cRA
none	100	100
6 μM CRABP	133	136
6 μM CRABP + 0.4 μM RA	138	149
6 μM BSA	77	10
6 μM CRABP + 6 μM BSA	60	15
6 μM CRBP	53	95
6 μM CRABP + 6 μM CRBP	68	99

[a] Data are expressed as percent RA synthesized relative to control. When *all-trans*-retinal was substrate, 9cRA was used with CRABP; when 9-*cis*-retinal was substrate, atRA was used with CRABP. 5 μM *all-trans*-retinal or 9-*cis*-retinal was used as substrate. Incubations were done for 15 min with 8 μg of protein.

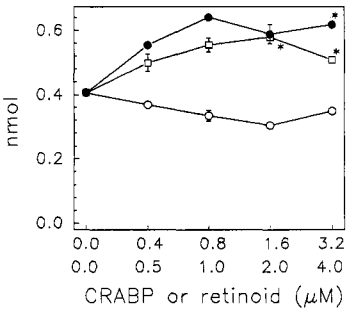

FIGURE 6: atRA and CRABP effects on 9cRA synthesis from 9-*cis*-retinal. The top numbers of the *x*-axis legends indicate the concentrations of atRA and the bottom those of CRABP. The *y* axis shows the amount of 9cRA synthesized from 20 μM 9-*cis*-retinal with increasing amounts of atRA (open circles), apoCRABP (filled circles), and both atRA and CRABP (open squares). Incubations were conducted for 15 min with 4 μg of protein. The data are expressed as the means ± SD of triplicates. Points without error bars had SD that were smaller than the points, except those denoted by asterisks, which were duplicates. Apo- and holoCRABP data, except where denoted by asterisks, were significantly different from data obtained in their absence ($p < 0.05$) or in the presence of atRA ($p < 0.005$). 9cRA synthesis in the presence of the first two concentrations of apoCRABP was significantly different from 9cRA synthesis in the presence of the first two concentrations of holoCRABP ($p < 0.05$).

DISCUSSION

The activity of a newly isolated rat cytosolic retinal dehydrogenase for *all-trans*-retinal has been compared with that for two retinol isomers, 9-*cis*-retinal and 13-*cis*-retinal. The purposes of this were to provide further insight into the role of the dehydrogenase in the biosyntheses of RAs, by determining its specificity for geometric isomers, and to explore potential pathways of 9cRA synthesis. Two anion-exchange steps, used in the original purification of the basic *all-trans*-retinal dehydrogenase, were repeated because they resolve the retinal dehydrogenase from other known cytosolic aldehyde dehydrogenases (Posch *et al.*, 1992). The greater 9-*cis*-retinal than *all-trans*-retinal dehydrogenase activity of cytosol was reflected throughout the partial purification, indicating that a single enzyme exhibits ~90% of the cytosolic *all-trans*-retinal and 9-*cis*-retinal dehydrogenase activity in the adult rat. Inhibition of 9-*cis*-retinal dehydrogenation by *all-trans*-

retinal also demonstrated that the same enzyme catalyzes both reactions.

Failure to detect 13cRA with 13-*cis*-retinal as substrate was not the result of rapid isomerization of the acid into atRA, or its demise by other mechanisms, because most of the 13cRA incubated under the same conditions was recovered intact. An average of less than 20% isomerization occurred into atRA. There was far more isomerization of 13-*cis*-retinal into *all-trans*-retinal (~80%) than of 13cRA into atRA. This 13-*cis*-retinal isomerization provided the substrate for atRA synthesis, through the pathway 13-*cis*-retinal → *all-trans*-retinal → atRA. Because 13cRA is sufficiently stable to detect, and 13-*cis*-retinal was present at the end of the incubation, and even high concentrations of 13-*cis*-retinal did not support RA synthesis, the other potential pathway, 13-*cis*-retinal → 13cRA → atRA, apparently contributed little, if anything, to atRA synthesis.

Greater recognition of the dehydrogenase for 9-*cis*-retinal *vs* 13-*cis*-retinal likely reflects the orientation of the aldehyde with respect to the side chain. In 9-*cis*-retinal, the aldehyde is oriented in the same way as *all-trans*-retinal from C(10) through C(15) and with respect to the C(20) methyl group. C(13) isomerization changes the orientation of the side chain close to the aldehyde by juxtapositioning the C(20) methyl group and the retinoid side chain. Presumably, the orientation of the aldehyde in the active site is crucial, and the 9-*cis* configuration allows sufficient insertion of the side chain with the aldehyde in the appropriate locus for efficient catalysis, whereas the change in configuration immediately after the aldehyde in 13-*cis*-retinal precludes efficient alignment. It is not unusual for proteins that bind retinoids to recognize *all-trans* and 9-*cis* isomers and discriminate against 13-*cis*-retinoids. The RARs bind atRA and 9cRA with equivalent affinity, but discriminate against 13cRA (Grettaz *et al.*, 1990; Heyman *et al.*, 1992; Allenby *et al.*, 1993), as does the epididymal retinoic acid-binding protein (Newcomer *et al.*, 1993).

The maximum rate of *all-trans*-retinal dehydrogenation was about 2-fold lower than that of 9-*cis*-retinal, but *all-trans*-retinal appeared to be favored moderately as a substrate, because the ratio $V_{max}/K_{0.5}$ for *all-trans*-retinal was 2.1 and that for 9-*cis*-retinal was 1.5. With both substrates, however, the $K_{0.5}$s were higher than the concentrations of the aldehydes are likely to be in tissues, but maximum enzyme activity was also relatively high—picomolar amounts of RAs could be produced at low nanomolar concentrations of substrates. Practically, this indicates that the dehydrogenase would support appreciable RA synthesis with the low concentrations of retinals that occur in most tissues, and both substrates could be converted into their acids simultaneously. Even though competition could occur between *all-trans*-retinal and 9-*cis*-retinal (Figure 4), it is unlikely to be important physiologically, because it would only be evident at micromolar concentrations of each retinal. At physiological concentrations of *all-trans*-retinal and those likely for 9-*cis*-retinal, there would be little competition, given the high $K_{0.5}$s relative to endogenous substrate concentrations.

Although 9cRA has been identified as the ligand for RXRs and has been detected *in vivo*, the route(s) of its synthesis has (have) not been determined (Levin *et al.*, 1992; Heyman *et al.*, 1992). It is possible that it derives enzymatically from atRA isomerization as well as nonenzymatically, but no evidence supporting an enzyme-catalyzed isomerization of atRA has been reported. It could also derive from preformed 9-*cis*-retinoids. Both 9-*cis*-retinol and 9-*cis*-β-carotene are natural products that occur in diets (Brown, 1959; Chandler & Schwartz, 1987; Ben-Anotz *et al.*, 1988). Dietary 9-*cis*-β-carotene causes accumulation of 9-*cis*-retinol in tissues (Stahl *et al.*, 1993). One function of 9-*cis*-retinal dehydrogenase activity could be to convert 9-*cis*-retinal, produced from dietary 9-*cis*-retinol,[2] or from 9-*cis*-β-carotene, into 9cRA. 9-*cis*-Retinol also may be produced from *all-trans*-retinyl esters by a pathway analogous to the one that converts *all-trans*-retinyl esters into 11-*cis*-retinol (Deigner *et al.*, 1989). Potential mechanisms of *trans* to 11-*cis* isomerization are compatible with 9-*cis* isomer production (Cañada *et al.*, 1990).

No marked inhibition of the retinal dehydrogenase was observed with atRA in concentrations greater than 3 μM. Physiological concentrations of atRA in several adult tissues vary from 20 to 130 nM, although in some tissues, *e.g.*, pancreas and in E13-E15 mouse retina, they are closer to 0.6 μM (Napoli, 1990b; McCaffery *et al.*, 1993). Therefore, atRA and 9cRA are unlikely to be physiologically-significant inhibitors of this basic p*I* cytosolic retinal dehydrogenase. These results are consistent with previous work, which showed that physilogical concentrations of atRA do not feedback-inhibit its own biosynthesis from *all-trans*-retinol in the canine kidney cell line LLC-PK_1 (Napoli, 1986b), and other work which showed no effect of atRA on the conversion of *all-trans*-retinal into atRA in ferret liver cytosol (Wang *et al.*, 1993). Lack of inhibition of RA synthesis by RA would be an important aspect of simultaneous and independent 9cRA and atRA generation, along with the lack of competition between the substrates at low substrate concentrations, if this retinal dehydrogenase were to serve as a common point in the paths of atRA and 9cRA synthesis.

CRABP was evaluated as a potential regulator of the retinal dehydrogenase because it is a logical candidate as a signal of RA status. The concentration of apoCRABP might signal RA need, the concentration of holoCRABP might signal RA sufficiency, or the ratio apoCRABP/holoCRABP might control enzyme activity to regulate the rate of RA synthesis. Only apoCRABP seemed to have an affect on RA synthesis, with no inhibition by, or competition from, holoCRABP. This was evident because the rate increase reflected the concentration of apoCRABP, whether or not holoCRABP was present. CRABP binds RA with high affinity (K_d ~10 nM), relative to the dehydrogenase (Ong & Chytil, 1978). However, because RA does not inhibit the dehydrogenase, it is not likely that apoCRABP enhanced the rate of RA synthesis by relieving feedback inhibition. Possibly, the rate of product release from the enzyme was increased. Understanding the importance of the observation with apoCRABP will require further work, including determining the cellular loci of CRABP with respect to retinal dehydrogenase. These experiments do indicate, however, that holoCRABP is not a significant signal of RA sufficiency for this retinal dehydrogenase.

Decreased dehydrogenase activity in the presence of CRBP or albumin probably is caused by substrate sequestration, albeit with an important difference. The complex CRBP–retinal acts as substrate for RA synthesis, but with a lower rate than that of free retinal, possibly because transfer of retinal from the binding protein to the enzyme becomes rate-limiting (Posch *et al.*, 1992). CRBP may obviate the CRABP effect by decreasing the concentration of free *all-trans*-retinal and also by becoming the dominant factor in limiting the rate, even in the presence of apoCRABP. Different degrees of inhibition

[2] Rat liver cytosol has much higher 9-*cis*-retinol dehydrogenase activity than *all-trans*-retinol dehydrogenase activity (unpublished observation).

with *all-trans*- and 9-*cis*-retinal could stem from the different affinities CRBP has for the two retinals. 9-*cis*-Retinoids do not bind cellular retinoid-binding proteins well compared to *all-trans*-retinoids (MacDonald & Ong, 1987; Allenby *et al.*, 1993; Fiorella *et al.*, 1993). Albumin may bind each retinoid with different affinities, and simply lower the concentration of free retinal available. The important point remains: other proteins do not stimulate RA synthesis as does CRABP.

To summarize, the major retinal dehydrogenase of rat liver can catalyze both *all-trans*-retinal and 9-*cis*-retinal dehydrogenations and could do so simultaneously at physiological concentrations of the retinals. The enzyme is stimulated by apoCRABP, an effect blocked by CRBP, but is not inhibited by RA. This dehydrogenase may serve as a common enzyme in the conversion of *all-trans*-retinal and 9-*cis*-retinal into their acids.

REFERENCES

Allenby, G., Bocquel, M.-T., Saunders, M., Kazmer, S., Speck, J., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J. F., Chambon, P., & Levin, A. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 30–34.

Ben-Anotz, A., Mokady, S., & Avron, M. (1988) *Br. J. Nutr. 59*, 443–449.

Boerman, M. H. E. M., & Napoli, J. L. (1991) *J. Biol. Chem. 266*, 22273–22278.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brown, P. S., Blum, W. P., & Stern, W. H. (1959) *Nature 184*, 1377–1379.

Cañada, F. J., Law, W. C., Rando, R. R., Yamamoto, T., Derguini, F., & Nakanishi, K. (1990) *Biochemistry 29*, 9690–9697.

Cao, Q.-N., Tu, G.-C., & Weiner, H. (1989) *Biochem. Pharmacol. 38*, 77–83.

Chandler, G. W., & Schwartz, S. J. (1987) *J. Food Sci. 52*, 669–672.

Deigner, P. S., Law, W. C., Cañada, F. J., & Rando, R. R. (1989) *Science 244*, 968–971.

Dunn, T. J., Koleske, A. J., Lindahl, R., & Pitot, H. C. (1989) *J. Biol. Chem. 264*, 13057–13065.

Fiorella, P., & Napoli, J. L. (1991) *J. Biol. Chem. 266*, 16572–16579.

Fiorella, P. D., Giguère, V., & Napoli, J. L. (1993) *J. Biol. Chem.* (in press).

Goss, G. D., & McBurney, M. W. (1992) *Crit. Rev. Clin. Lab. Sci. 29*, 185–215.

Grettaz, M., Baron, A., Sigenthaler, G., & Hunziker, W. (1990) *Biochem. J. 272*, 391–397.

Herr, F., & Ong, D. E. (1992) *Biochemistry 31*, 6748–6755.

Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. N., Eichele, G., Evans, R. M., & Thaller, C. (1992) *Cell 68*, 397–406.

Leatherbarrow, R. J. (1987) *Enzfitter: A Non-linear Regression Data Analysis Program*, Elsevier-BIOSOFT, Cambrige, U.K.

Lee, M.-O., Manthey, C. L., & Sladek, N. E. (1991) *Biochem. Pharmacol. 42*, 1279–1285.

Leo, M. A., Kim, C.-I., Lowe, N., & Lieber, C. S. (1989) *Biochem. Pharmacol. 38*, 97–103.

Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A., & Grippo, J. F. (1992) *Nature 355*, 359–361.

Levin, M. S., Locke, B., Yang, N. C., Li, E., & Gordon, J. I. (1988) *J. Biol. Chem. 263*, 17715–17723.

Lindahl, R. (1992) *Crit. Rev. Biochem. Mol. Biol. 27*, 283–335.

MacDonald, P. N., & Ong, D. E. (1987) *J. Biol. Chem. 262*, 10550–10556.

McCaffery, P., Posch, K. C., Napoli, J. L., Gudas, L. J., & Dräger, U. C. (1993) *Dev. Biol. 158*, 390–399.

Morriss-Kay, G. (1993) *BioEssays 15*, 9–15.

Napoli, J. L. (1986a) *Methods Enzymol. 123*, 112–124.

Napoli, J. L. (1986b) *J. Biol. Chem. 261*, 13592–13597.

Napoli, J. L. (1990a) *Methods Enzymol. 189*, 470–472.

Napoli, J. L. (1990b) in *Chemistry and Biology of Synthetic Retinoids* (Dawson, M. I., & Okamura, W. H., Eds.) pp 229–249, CRC Press, Boca Raton, FL.

Napoli, J. L. (1993) *J. Nutr. 123*, 362–366.

Napoli, J. L., Posch, K. C., & Burns, R. D. (1992) *Biochim. Biophys. Acta 1120*, 183–186.

Newcomer, M. E., Pappas, R. S., & Ong. D. E. (1993) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Ong, D. E., & Chytil, F. (1978) *J. Biol. Chem. 253*, 4551–4554.

Ong. D. E., MacDonald, P. N., & Gubitosi, A. M. (1988) *J. Biol. Chem. 263*, 5789–5796.

Posch, K. C., Boerman, M. H. E. M., Burns, R. D., & Napoli, J. L. (1991) *Biochemistry 30*, 6224–6230.

Posch, K. C., Burns, R. D., & Napoli, J. L. (1992) *J. Biol. Chem. 267*, 19676–19682.

Richman, J. M. (1992) *Crit. Rev. Oral Biol. Med. 4*, 93–109.

Satre, M. A., & Kochhar, D. M. (1989) *Dev. Biol. 133*, 529–536.

Stahl, W., Sundquist, A. R., Hanusch, M., Schwarz, W., & Sies, H. (1993) *Clin. Chem.* (in press).

Summerbell, D., & Maden, M. (1990) *Trends Neurosci. 13*, 142.

Wang, X.-D., Krinsky, N. UI., & Russell, R. M. (1993) *J. Nutr. 123*, 1277–1285.

Wolf, G. (1984) *Physiol. Rev. 64*, 874–937.

Yost, R. W., Harrison, E. H., & Ross, A. C. (1988) *J. Biol. Chem. 263*, 18693–18701.