Biosynthesis of All-trans-retinoic Acid from Retinal

RECOGNITION OF RETINAL BOUND TO CELLULAR RETINOL BINDING PROTEIN (TYPE I) AS SUBSTRATE BY A PURIFIED CYTOSOLIC DEHYDROGENASE*

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An NAD-dependent rat liver cytosolic dehydrogenase accepted as substrate retinal generated in situ by microsomes from retinol bound to excess CRBP (cellular retinol binding protein, type I). This activity, which was not retained by anion-exchange chromatography at pH 9.15, was designated P1. P1 activity increased 2.5-fold, with no statistically significant change in its K or Hill coefficient, in liver cytosol from rats fed a retinoid-deficient diet. Orally dosed retinoic acid partially suppressed the increase. Activities chromatographically similar to hepatic P1 were observed in cytosols from rat kidney and testes. P1, purified from rat liver cytosol, had a pl of ~8.3, migrated as a tetramer (214 kDa) on a Sephadex G-200 column, and had a subunit molecular mass of 55 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. With free retinal it catalyzed a maximum rate of retinoic acid synthesis of 265 nmol/min/mg of protein and exhibited allosteric kinetics with a Hill coefficient of 1.5 ± 0.13 (mean ± S.D., n = 4). Substrate inhibition was noted with retinal concentrations greater than 6 μM. The purified enzyme not only recognized retinal generated by microsomes as substrate, but also recognized retinal bound to CRBP. The rates of retinoic acid synthesis from CRBP-retinal, with a series of increasing apoCRBP concentrations, exceeded the rates that would be supported by the free retinal present. The CRBP-retinal complex exhibited allosteric kinetics (K, 0.13 μM; Hill coefficient, 1.75; averages of duplicates) in the presence of excess apoCRBP (the ratio total CRBP/total retinal at each concentration of retinal was 2). This enzyme is likely to play a significant role in retinoic acid synthesis in vivo, because it participates in the synthesis of retinoic acid from a physiologically occurring form of retinol (holoCRBP), reflects retinoid status, and is distributed in extrahepatic tissues in addition to liver. These results also suggest a novel role for CRBP in retinoid metabolism, facilitating the conversion of retinal into retinoic acid.

Two sequential reactions starting from retinol, with retinal as intermediate, constitute one pathway of retinoic acid synthesis. Close control mechanisms probably govern this pathway because discrete phases of differentiation and development require retinoic acid, but its presence out of temporal sequence or in inappropriate loci is teratogenic (1-3). Yet detailed information has not been available concerning enzymes committed specifically to the synthesis of retinoic acid. A problem confounding insight into this process has been apparently nonspecific metabolism in vitro of free retinol and retinal by multiple cytosolic and microsomal dehydrogenases and oxidases (4-7). Elucidation of the mechanisms that regulate retinoic acid homeostasis will require ultimate identification of the enzymes committed to retinoid metabolism.

HoloCRBP1,2 represents the most abundant form of unesterified retinol in many tissues (8, 9). In such cases, it would also probably be expressed in the most abundant substrate for retinoic acid synthesis. Indeed, a microsomal NADP-dependent retinol dehydrogenase, expressed in liver and in extrahepatic tissues, accepts holoCRBP as substrate (10). Direct interaction between holoCRBP and this dehydrogenase circumvents diffusion of retinol through the aqueous phase and protects retinol from reactions with enzymes that do not recognize the high-affinity-specificity retinoid binding protein, such as NAD-supported microsomal dehydrogenases or NADPH-supported monoxygenases (7, 10). Microsomes, however, do not convert the retinal they produce from holoCRBP into retinoic acid at high rates in the presence of apoCRBP, reflecting both low microsomal retinoidal dehydrogenase activity (6) and probable binding of retinoic acid to apoCRBP. One pathway of retinoic acid synthesis consistent with these observations would involve recognition by a cytosolic retinoidal dehydrogenase(s) of CRBP-retinal generated from CRBP-retinol in microsomes. This report describes the purification of a rat liver cytosolic NAD-dependent dehydrogenase that synthesizes retinoic acid from free retinal, retinal generated in situ by microsomes from holoCRBP, and CRBP-bound retinol. Retinoid deficiency enhances the activity in liver, and similar activities are expressed in testes and kidney. These data indicate that CRBP-bound retinol can function as substrate for a cytosolic retinoidal dehydrogenase and are consistent with a physiological role for this dehydrogenase in retinoic acid synthesis.

EXPERIMENTAL PROCEDURES

General—Retinol and retinal, purchased from Eastman Kodak, were used within 2 weeks after purification by normal-phase HPLC (11). Other chemicals were purchased from Sigma. Chromatography resins and FPLC columns were purchased from Pharmacia LKB Biotechnology Inc. Protein was determined by the dye binding

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1 The abbreviations used are: CRBP, cellular retinol binding protein (type I); DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2 HoloCRBP refers to CRBP occupied by retinol. CRBP occupied by retinal is referred to as CRBP-retinal.
method with bovine serum albumin as standard (12), or in the case of lower protein amounts, by absorbance at 280 nm. CRBP was expressed in *Escherichia coli* with the vector pMONCRBP (13) and purified and quantified as described (14). Kinetic data were expressed in a buffer of 20 mM Hepes, 150 mM KCl, 1 mM EDTA, and 2 mM DTT, pH 8.6, at 37 °C. With free retinal, substrate was added in 2 μl of dimethyl sulfoxide to a final volume of 500 μl. The incubations were initiated by addition of 2 mM NAD (final concentration) and protein and were done for 20 min. When CRBP-bound retinal was substrate, preincubations of retinal and apoCRBP were done at 25 °C for 30 min to allow CRBP-retinal complex formation. The final volumes, temperatures, times, and ratios of total CRBP to total retinal for the incubations are noted in the figure and table legends. These assays were initiated with 2 mM NAD and protein. Controls were assays done either without protein or without cofactors. At the end of the incubations sufficient water was added to raise volumes to 500 μl, if necessary. Reactions were quenched by addition of 0.25 N KOH to raise the pH to 12. The proteins were separated by 7.5% (1, 3, 3-tetramethyl-5-indanyl)-5-methyl-cta-2,4,6-triene acid, was added in 5 μl of ethanol. After extracting neutral retinoids with 2.5 ml of hexane, the pH of the aqueous phase was adjusted to <2 with 4 N HCl. Retinoid acid and the internal standard were extracted with a second 2.5-ml portion of hexane. The hexane from extraction of the aqueous phase was removed under a stream of nitrogen, and 110 μl of hexane was added to each sample. The samples were analyzed by HPLC.

**HPLC Analyses**—Retinoid acid was quantified by normal-phase HPLC with a Du Pont Zorbax-Sil Reliance cartridge column (0.4 4 cm) as described (16). Retinoid acid eluted in 3.3 min at a flow rate of 2 ml/min with dichloroethane/hexane/acetetic acid (9.65/90/0.35). Absorbance was monitored at 340 nm with a Waters model 484 Tunable Absorbance Detector.

**Preparation of Cytosol and Microsomes**—Male Sprague-Dawley rats with an average weight of 250 g (Harlan, Indianapolis, IN) were killed by decapitation after an overnight fast. Livers, kidneys, and testes were excised and rinsed with cold saline. Kidneys and testes were decapsulated. 25–30% homogenates were made in a buffer of 10 mM Hepes, 0.25 M sucrose, 2 mM DTT, and 1 mM EDTA, pH 7.5, from the minced livers with a Wheaton Instruments overhead homogenizer. The homogenate was spun at 15000 × g for 15 min. The supernatant was spun at 28,000 × g for 15 min. The supernatant from this spin was spun at 105,000 × g for 1 h to produce cytosol and microsomes.

**Purification of the Retinal Dehydrogenase**—Liver cytosol was concentrated using an Amicon Diaflo concentrator (PM10 filter) and exchanged with 25 mM ethanolamine, 2 mM DTT, pH 9.15 (buffer A). In this and all following buffer exchanges in preparation for ion-exchange chromatography, the final conductivities of the exchanged samples were within 0.2 microsiemens of the replacement buffer. The bound protein was then eluted with 1 M NaCl in buffer A (3 column volumes). Protein bound to the column was then eluted with 1 M NaCl in buffer A (~3 column volumes). The retinal dehydrogenase activity not retained was designated P1 and that eluting in the NaCl wash was designated P2. Each was individually concentrated as described above. P1 was exchanged with buffer B (25 mM piperazine, 2 mM DTT, pH 7.5) and was applied to a Q-Sepharose column (2.5 18.5 cm). The column was washed with buffer B until protein elution ceased (~2 column volumes). The bound protein was eluted with a linear gradient of 0–500 mM NaCl in buffer B (2-liter total volume). The active fractions, which were not retained in the salt gradient, were pooled, concentrated, and exchanged with buffer C (20 mM Hepes, 2 mM DTT, pH 6.8). This sample was applied to a Mono S FPLC column (0.5 × 5.0 cm) and washed with buffer C (10 column volumes). The bound protein was eluted with a linear gradient of 0–300 mM NaCl in buffer C (30 ml total volume). The uncleaved protein was designated P1a; the activity that bound was designated P1b. P1a and P1b were each concentrated and stored at −70 °C. Final purification of P1a was achieved with a Superose-12 FPLC column (1 × 30 cm) eluted with buffer C containing 150 mM KCl and adjusted to pH 8.0. Active fractions were concentrated using a 10-kDa limit Centricon filter from Amicon.

**Molecular Mass Determination**—The native molecular mass of P1a was determined by elution from a Sephadex G-200 column (1.7 × 48.5 cm) with 20 mM Hepes, 150 mM KCl, 2 mM DTT, pH 8.0. The column was standardized with a series of mass markers: blue dextran (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). In addition to these standards, the Superose-12 column was used in the last purification step to also be standardized with β-galactosidase (116 kDa) and ovalbumin (45 kDa).

**Effect of Dietary Retinoid Status on the Activity of P1**—Vitamin A-deficient (A−) and vitamin A-deficient retinoic acid-supplemented (A+/RA+) rats were obtained as described previously (17). Purified diets, prepared by the modified AIN-76A rodent diet formula (18, 19), were purchased from Dyets, Inc., Bethlehem, PA. The A− diet contained 1200 retinol equivalents/kg as retinyl palmitate. The A+ diet was the same as the A− diet except retinyl palmitate was omitted. Twenty-five-day-old male Sprague-Dawley rats were divided randomly into 2 groups of 13 and the rats in each group were fed one of the diets. At 82 days old, four rats who had ceased gaining weight were separated from the A− group and were given daily supplements of 100 μg of retinoid acid in 0.1 ml of Wesson oil orally. All rats were sacrificed by decapitation after an overnight fast when they were 73 days old. The livers from each group were pooled, homogenized, and the P1 fractions were prepared from cytosol as described above under "Purification of the Retinal Dehydrogenase," up to and including the first, pH 9.15, anion-exchange column.

**Anion-exchange FPLC**—Cytosol from each tissue was concentrated and exchanged as described above. Samples were loaded onto a Mono Q HR 5/5 column (0.5 × 5 cm, Pharmacia) and eluted for 5 min with buffer A to elute P1 activity, followed by 5 min with 20 mM Tris- HCl, pH 7.5, and then 15 min with 200 mM NaCl in buffer D, to elute P2 activities. The P2 samples were exchanged with 20 mM Hepes, 2 mM DTT, pH 7.5 (buffer E), and reapplied to the same anion-exchange column equilibrated with buffer E. After 5 min a linear gradient was run over 20 min to 250 mM NaCl in buffer E. Analyses were done at a flow rate of 1 ml/min with the collection of 1-ml fractions. Protein Gels—SDS-PAGE mini slab gels were run as described by Laemmli (20) with the Might Small 11 slab gel electrophoresis unit from Hoefer Scientific. The low range SDS-PAGE molecular weight standards from Bio-Rad were used as markers. Gels were silver-stained as described by Wray et al. (21).

**In vivo Focusing with Acrylamide**—Focusing was done with a 1-mm 1% agarose gel slab with Pharmalyte, pH 3–10, on a flat bed style LK 2117 Multipher II electrophoresis unit powered by the LKB 2297 Macrodrive 5 unit. The Broad pi Calibration kit, pH 3–10, from Pharmacia provided standards. The gel was prefocused for 10 min at 10 °C with a voltage of 150 mV (150 V, 1.5 watts) at 10 °C prior to application of the sample and standards. The samples were applied ~6.5 cm from the cathode and focused approximately 1500–2000 volts/h (1500 V, 150 mV, 15 watts). After focusing, the gel was cut into two longitudinal sections. The section with the pi standards and one lane of the sample was stained for protein with Coomassie Blue R-250. The second section containing the lane of focused retinal dehydrogenase, was cut into 0.5-cm horizontal pieces, and retinal dehydrogenase activity was measured in each piece.

**RESULTS**

**Retinal Generated in Microsomes from HoloCRBP as Substrate for Cytosolic Retinoic Acid Synthesis**—To determine whether the retinal produced by microsomes from holoCRBP supports cytosolic retinoic acid synthesis, cytosol was partitioned into two fractions by batch elution anion-exchange chromatography (Q-Sepharose at pH 9.15) as described under "Purification of the Retinal Dehydrogenase." With 2 μM free retinal as substrate and NAD as cofactor, retinal dehydrogenase activity was noted in the fraction not retained by the resin, designated P1, and also in the fraction that eluted only with high salt, designated P2, which contained most of the protein (Table I). P1 and P2 were tested for their ability to synthesize retinoic acid from retinal generated in situ from holoCRBP; liver microsomes, and NADP (Fig. 1). Low HCl (100 μM) promoted P1 activity, and relatively high rates of retinoic acid synthesis, whereas even high amounts of P2 (800 μg) provided only very low retinoic acid (~4 pmol). These data indicate that microsomal retinal synthesis from holoCRBP can provide substrate for retinoic acid synthesis:
produce retinoic acid without cofactor. Adding either NAD or NADP (open circles) with 200 pg of microsomal protein. Incubation time was 20 min. The conditions were examined for retinoic acid synthesis from the combination of NAD and NADP provided a small amount of retinoic acid partially, but significantly, reduced the enhancement in activity resulting from retinoid deficiency, without affecting the Hill coefficient or K. Oral dosing with retinoic acid partially, but significantly, reduced the enhancement in activity resulting from retinoid deficiency, without affecting the Hill coefficient or K. These data indicate that P1 activity is enhanced when retinoid concentrations are low, possibly via increased amounts of enzyme, as would be expected for an enzyme in the pathway of retinoic acid biosynthesis.

Expression of P1-like Activity in Kidney and Testes—Cytosols prepared from the livers, testes, and kidneys of the same rats were applied to a Mono Q FPLC column at pH 9.15 to compare the proportion of P1 activity in extrahepatic tissues with that in liver. With 2 μM free retinol as substrate, P1 provided 67, 85, and 67% of the total P1 and P2 retinal

![Graph](image)

Table I

| Sample       | Protein (mg) | Specific activity (nmol/min/mg) | Increase |
|--------------|-------------|--------------------------------|----------|
| Cytosol      | 3941 (100%) | 2333 (100%)                    | 0.6      |
| Mono Q, pH 9.1 | 646 (16.4%) | 1195 (51%)                     | 1.9      |
| P1 (non-retained) | 1971 (50%) | 641 (27%)                      | 0.3      |
| P1 (retained) | 8.8 (0.4%)  | 181 (8%)                       | 21       |
| P1 along with both cofactors. These data suggest that a microsomal NADP-dependent retinol dehydrogenase and a cytosolic NAD-dependent dehydrogenase contribute to a pathway of retinoic acid synthesis from holoCRBP. It is probable that CRBP-bound retinol supported cytosolic retinoic acid synthesis under these conditions, because CRBP was present in excess of the total retinol and retinal, and CRBP binds retinol with high affinity (Kd of 50–100 nM) (13, 22).

Effect of Dietary Retinoid Status on P1 Activity—The effects of dietary retinoid status were determined on P1 activity to provide further support for a contribution of P1 to retinoic acid synthesis. P1 prepared from the liver cytosol of rats fed a diet normal in vitamin A exhibited allosteric kinetics (Table III). Retinoid deficiency, caused by a vitamin A-deficient diet, A-/RA+, rats fed a vitamin A-deficient diet supplemented orally with 100 μg of retinoic acid daily.

Entries with similar superscripts are significantly different from each other p < 0.05.

| Group        | Vmax (nmol/min/mg) | K (nmol/min/mg) | Hill* |
|--------------|--------------------|-----------------|-------|
| A+/A-        | 3.8 ± 0.2          | 0.7 ± 0.2       | 1.7 ± 0.3 |
| A-/RA+       | 9.4 ± 1.0          | 1.2 ± 0.4       | 1.7 ± 0.3 |
| A+/RA+       | 7.0 ± 0.9          | 0.8 ± 0.3       | 1.9 ± 0.4 |

*“Hill” represents the Hill coefficient.

Fig. 1. Retinoic acid synthesis from retinal generated in microsomes from holoCRBP. Retinal was generated in situ from 5 μM CRBP-retinol, 2 μM apoCRBP, rat liver microsomes (150 μg of protein), and 2 mM NADP. Either P1 (open circles) or P2 (filled circles) was added to test for retinoic acid synthesis (y axis). In addition to the reagents stated above, 2 mM NAD was also added, as cofactor for the retinal dehydrogenase. Reactions were done for 30 min in a final volume of 200 μl using the standard conditions described under “Experimental Procedures.”

Table II

| Picosmol of retinoic acid (mean ± S.D., n = 4) | 0 | 0 | 5 ± 0.4 | 0 | 0 | ~2 | 0 | 9 ± 5 | 20 ± 2 | 49 ± 4 |
|---|---|---|---------|---|---|-----|---|---|--------|--------|
| Micro | ++ | + | + | + | + | + | + | + | + | + |
| P1 | – | – | – | – | – | + | + | + | + | + |
| NAD | – | – | – | + | + | + | + | + | + | + |
| NADP | – | – | – | + | + | + | + | + | + | + |

tively to a cytosolic dehydrogenase.

The conditions were examined for retinoic acid synthesis from holoCRBP, microsomes, and P1 (Table II). No retinoic acid was produced from holoCRBP by microsomes in the absence of cofactors or with either NADP or NAD, but the combination of NAD and NADP provided a small amount of microsomal retinoic acid synthesis. P1 alone did not produce retinoic acid from holoCRBP nor did P1 produce retinoic acid in the presence of either NAD or NADP. A combination of P1 and both NAD and NADP did produce low, but detectable, retinoic acid. The combination of P1 and microsomes did not produce retinoic acid without cofactor. Adding either NAD or NADP enhanced retinoic acid synthesis from the combination of P1 and microsomes, but NADP alone was twice as effective as NAD alone. Maximum retinoic acid synthesis from holoCRBP occurred only in the presence of both microsomes and P1 along with both cofactors. These data suggest that a microsomal NADP-dependent retinol dehydrogenase and a cytosolic NAD-dependent dehydrogenase contribute to a pathway of retinoic acid synthesis from holoCRBP. It is probable that CRBP-bound retinol supported cytosolic retinoic acid synthesis under these conditions, because CRBP was present in excess of the total retinol and retinal, and CRBP binds retinol with high affinity (Kd of 50–100 nM) (13, 22).

Effect of Dietary Retinoid Status on P1 Activity—The effects of dietary retinoid status were determined on P1 activity to provide further support for a contribution of P1 to retinoic acid synthesis. P1 prepared from the liver cytosol of rats fed a diet normal in vitamin A exhibited allosteric kinetics (Table III). Retinoid deficiency, caused by a vitamin A-deficient diet, A-/RA+, rats fed a vitamin A-deficient diet supplemented orally with 100 μg of retinoic acid daily.

Entries with similar superscripts are significantly different from each other p < 0.05.

| Group        | Vmax (nmol/min/mg) | K (nmol/min/mg) | Hill* |
|--------------|--------------------|-----------------|-------|
| A+/A-        | 3.8 ± 0.2          | 0.7 ± 0.2       | 1.7 ± 0.3 |
| A-/RA+       | 9.4 ± 1.0          | 1.2 ± 0.4       | 1.7 ± 0.3 |
| A+/RA+       | 7.0 ± 0.9          | 0.8 ± 0.3       | 1.9 ± 0.4 |

*“Hill” represents the Hill coefficient.

A+, rats fed a diet normal in vitamin A; A-, rats fed a vitamin A-deficient diet; A-/RA+, rats fed a vitamin A-deficient diet supplemented orally with 100 μg of retinoic acid daily.

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dehydrogenase activity in liver, kidney, and testes cytosol, respectively. The P2 (retained) activity that had been eluted with 500 mM NaCl was exchanged with salt-free buffer at pH 7.5 and reapplied to the anion-exchange FPLC column. The number and nature of P2 components was tissue-dependent (Table IV). These data show that patterns of constitutive retinal dehydrogenase activities in cytosols of rat tissues are tissue-specific, but P1 provides the quantitatively major activity in at least two extrahepatic tissues as well as in liver.

**Purification of P1**—The purification of P1 is summarized in Table I. P1, obtained from the pH 9.15 anion-exchange column, was applied to a second anion-exchange column at pH 10 (Fig. 2). With the higher pH, most of the proteins were retained, and a single peak of retinal dehydrogenase activity, distinct from the major protein peaks, eluted between 200-250 mM NaCl. The pooled fractions were applied to a cation-exchange resin at pH 6.8, which separated the activity into two parts. The first, P1a, was not retained, whereas the second, P1b, was retained and eluted at 200 mM NaCl. The proportion of units in P1a varied from 30% of the total of P1a and P1b, as shown in Table I, to 96, 95, and 99% in three subsequent purifications. Additionally, rechromatography of P1b through the cation-exchange column, after exhaustive exchange with salt-free buffer, retained less than half of the P1b, as though P1b were a modified (or modifiable) form of P1a, rather than a distinct isozyme. Therefore attention focused on P1a. Size-exclusion chromatography over Superose-12 achieved final purification of P1a (Figs. 3 and 4). Purified P1a had a 2300-fold higher specific activity than P1b in cytosol, when the contributions were taken into account of P2 to total cytosolic retinal dehydrogenase activity and P1b to the pool of P1, indicating that it represents ~0.04% of cytosolic protein.

**Characteristics of Purified Retinal Dehydrogenase**—The Pl of P1, obtained from the cation-exchange column, was ~8.3 by agarose gel electrophoresis (Fig. 5). The dehydrogenase migrated on Superose-12 as a native species with a molecular mass of 65 kDa (Fig. 3) but migrated on a G-200 column with a molecular mass of ~214 kDa. The subunit molecular mass of P1a was 55 kDa (Fig. 4), suggesting that the results from the Superose-12 column were anomalous and that the retinal dehydrogenase exists as a tetramer. P1a, from the Superose-12 column and with free retinal as substrate, was linear in activity with time to 20 min and with protein in the range of 10-500 ng (Fig. 6, A and B). It had a pH optimum at 8.5 (Fig. 6C) and required NAD, rather than NADP, as cofactor (Fig. 6D). It exhibited allosteric kinetics with free retinal as substrate, with a K of 0.76 μM ± 0.35 (mean ± S.D., n = 4) and a Hill coefficient of 1.5 ± 0.13 (mean ± S.D., n = 4). In all four trials to obtain the kinetic constants of P1a, greater than 6 μM free retinal exhibited depressed activity, consistent with substrate inhibition (Fig. 7A). Therefore kinetic constants were calculated only from the data up to and including 6 μM retinal. Purified P1a recognized retinal generated in situ by microsomes as did the crude P1 fraction. Incubating 10 and 15 μg of P1a from the pH 6.8 cation-exchange column, with
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Fig. 5. Isoelectric focusing of the retinal dehydrogenase activity in P11. An aliquot of the activity not retained by the cation-exchange column (12 μg of P11a protein) was focused on a 1% agarose gel in the pH range 3 to 10 as described under "Experimental Procedures." One lane was cut into 0.5-cm sections which were minced and assayed for retinal dehydrogenase activity under standard conditions with 2 μM retinal for 45 min. The pI (value in parentheses) standards (filled circles, right axis) were: carbonic anhydrase I (6.6), and lactate dehydrogenase (8.6). Retinoic acid produced (open circles, left axis) is plotted versus the 0.5-cm section of gel.

Fig. 6. The effects of time, protein, pH, and cofactors on retinoic acid synthesis catalyzed by P11a. The activity of P11a from the final purification step (Superose-12 column) was examined with 2 μM retinal under standard conditions with 0.2 μg of protein, unless otherwise stated. A, time course; B, protein effects; C, effects of pH (triangles represent a no protein control); D, effects of NAD (circles) and NADP (triangles).

10 μM holoCRBP, 1 μM apoCRBP, microsomes (250 μg of protein), and 2 mM each NAD and NADP for 30 min produced 115 and 131 pmol of retinoic acid, respectively, versus ~11 pmol in the absence of P11a.

Recognition of CRBP-Retinal as Substrate by P11a—In the experiments in which retinoic acid was generated from holoCRBP by microsomes and P11, total CRBP was in excess of total retinoids. Under such conditions, retinal would be bound to CRBP. This suggested that P11a recognizes as substrate the complex CRBP-retinal. Two approaches were taken to test this. The first determined the relationship between the rate of retinal conversion into retinoic acid as a function of increasing retinal concentrations in the presence of 2 mM equivalents of apoCRBP at each retinal concentration. These conditions provide increasing concentrations of CRBP-retinal but low and essentially constant concentrations of free retinal, because the K_d of retinal binding to CRBP is 50–100 nM (13, 22). For example, with total retinal concentrations of 0.2–0.8 μM, the concentrations of free retinal in the presence of 2 mM equivalents of CRBP range from ~60 to ~80 nM (using K_d = 100 nM), but those of CRBP-retinal range from 145 to 920 nM, respectively. A relationship between increasing concentrations of CRBP-retinal and the rate of retinoic acid formation implies recognition of CRBP-retinal by P11a (Fig. 7B). As with free retinal, allosteric kinetics were observed for CRBP-retinal with a K_d determined in two separate experiments, of 0.13 μM (0.1 and 0.16 μM) and a Hill coefficient of 1.75 (1.8 and 1.7).

The effect of increasing apoCRBP on the rate of retinoic acid formation from free retinal also was measured to confirm whether P11a recognized CRBP-retinal as substrate. In the presence of CRBP, the conversion of retinal into retinoic acid did not depend on the concentration of free retinal (Fig. 8). In this experiment 115 pmol of retinoic acid were produced from 2 μM retinal in the absence of CRBP. In the presence of 4 μM apoCRBP, 97 pmol of retinoic acid were produced. The 2000 nM concentration of free retinal at 0 μM CRBP would decrease to 90 nM with the addition of 4 μM CRBP, as calculated from a K_d of 100 nM for retinal binding to CRBP. From the Hill equation and the kinetic constants of free retinal and P11a, this would have resulted in a rate decrease from 79% of V_max to 2% of V_max, a 40-fold decrease, if free retinal were the only substrate. At 10 μM CRBP, 88 pmol of retinoic acid were observed, but the concentration of free retinal had decreased to 25 nM. This would have decreased the rate of retinoic acid synthesis to 0.3% of V_max, a 260-fold difference, had free retinal been the sole substrate.

DISCUSSION

The present work was undertaken with the goals of determining whether retinal generated in microsomes from
hologoCRBP could support retinoic acid synthesis and whether CRBP-retinal could serve as substrate for retinoic acid synthesis. The latter might be useful, in addition to providing insight into CRBP functions, to help distinguish retinaldehyde dehydrogenases that catalyze retinoic acid synthesis in vitro from the multiple activities in cytosol that recognize free retinal in vitro (25, 24). Consistent with our previous results (24), anion-exchange chromatography provided unretained, P1, and retained, P2, retinal dehydrogenase activity. P2 was not the main focus of this work, because it did not account for the majority of units, and it was not as efficient as P1 in recognizing retinal generated in situ from microsomes and hologoCRBP. Effort was focused on the P1 retinal dehydrogenase, because it represents the majority of units, it converts the retinal generated from hologoCRBP into retinoic acid in the presence of excess CRBP, dietary retinoid status influenced its activity, and extrahepatic tissues as well as liver expressed it. These characteristics are consistent with a retinal dehydrogenase that could function in the pathway of retinoic acid metabolism under physiological conditions.

The purification of P1 took advantage of its unusual interactions with ion-exchange resins. A necessary step was anion-exchange chromatography at pH 10, after separation of the activity from the majority of protein by anion exchange at lower pH. Attempts to eliminate this column resulted in grossly impure material, even after the other steps. Two of the steps, the first anion-exchange step at pH 9.15 and the cation-exchange step at pH 8.5, were essentially graduating elutions that provided high recovery of activity. Usually, proteins not retained on anion exchange at high pH would be retained on cation exchange at low pH, especially those with isoelectric points of ~8.3. The reasons for the behavior of P1a on the cation-exchange column are not obvious. The relationship between P1a and P1b was also not obvious. The contribution of P1b to the P1 pool was inconsistent, suggesting that P1b could be an inducible isozyme or an artifact of the purification procedure. More puzzling was the apparent change in affinity of P1b for the cation-exchange resin upon reapplication, an observation seemingly inconsistent with an inducible isozyme. This may indicate that a post-translational modification has occurred. Future work will attempt to resolve this issue.

With respect to subunit molecular weight and function as a multimer, the retinal dehydrogenase reported here typifies the general structures of aldehyde dehydrogenases. On the other hand, very little of the total aldehyde dehydrogenase activity occurs in normal rat liver cytosol; the majority of constitutive aldehyde dehydrogenase activity occurs in the microsomes and mitochondria (25). Nevertheless, up to eight different cytosolic enzymes have been detected by isoelectric focusing gels stained with propanal as substrate, all with neutral to acidic pI points (26). From its decidedly basic pI, the P1 retinal dehydrogenase seems to be unique among known rat tissue aldehyde dehydrogenases. In contrast, basic pI aldehyde dehydrogenases have been observed in human and mouse liver and in limited extrahepatic tissues of these species. By isoelectric point, the rat retinal dehydrogenase is similar to human ALDH IV (27) and the various forms of mouse AHD-2 (28). Co-identity is not certain, for one reason, because other activities with pI points between 7.8 and 8.5 are also expressed in mouse and human. It is not known which, if any, of the reported human forms recognize retinal as substrate. The mouse AHD-2 has a low $K_m$ (<1 μM) for retinal and provides the majority of mouse cytosolic retinal dehydrogenase activity, but other mouse forms also metabolize free retinal in vitro with low $K_m$ values (23, 24). Another open question is the apparently restricted tissue distribution of ALDH IV and AHD-2. This could mean either that different isozymes contribute to retinoic acid synthesis in different tissues or that AHD IV and AHD-2 are not the human and mouse equivalents of P1, and P1 itself is distributed ubiquitously. P1-like activity in rat testes and kidney may indicate wider distribution of this form in the rat than either AHD-2 in the mouse or ALDH IV in the human.

CRBP has not been regarded as a retinal binding protein because retinal cannot compete with equimolar concentrations of retinol for binding to limited concentrations of CRBP (29). Nor has retinal been observed as an endogenous ligand of CRBP, but this is not surprising, because concentrations of retinal are low in most tissues and the $K_m$ for retinal from CRBP is rapid.3 Although the affinity of CRBP for retinol is at least an order, perhaps 2 orders, of magnitude higher than the affinity for retinal, apoCRBP does bind retinal with high affinity (13, 22) presenting the possibility that CRBP could affect retinal metabolism as well as that of retinol. Two mutually supportive approaches were taken in this work to test recognition of CRBP-retinal by a retinal dehydrogenase. One varied the concentration of CRBP-retinal while maintaining an essentially fixed concentration of free retinal (Fig. 7B); the other varied the concentrations of free retinal while maintaining a fixed concentration of CRBP-retinal (Fig. 8). A rate of retinoic acid synthesis in the presence of CRBP independent of the concentrations of free retinal and dependent on the concentrations of CRBP-bound retinal supports the conclusion that CRBP-retinal itself was recognized as substrate. CRBP-retinal had about 6-fold higher affinity for the enzyme than did free retinal, supporting a role for CRBP-retinal in the pathway of retinoic acid synthesis in vivo.

One of two mechanisms could describe the CRBP-enzyme interaction. One would involve direct transfer of retinoid from the binding protein to the enzyme via protein-protein interaction, with ultimate release of the product from the enzyme. Alternatively, reactions could occur while the retinoid remains bound to the binding protein, with ultimate release of the

3 K. C. Posch, R. D. Burns, and J. L. Napoli, unpublished results.
product from the binding protein. Future elucidation of the structures of CRBP and the dehydrogenases that recognize CRBP will be required to resolve these two possibilities.

There are other examples of involvement in or moderation of retinoid dehydrogenations by retinoid binding proteins. Two retinol dehydrogenations have been identified that do not require diffusion of substrate from a retinoid binding protein prior to enzymatic recognition. The stimuli of the present work, an NADP-dependent microsomal retinol dehydrogenation that occurs in several tissues, uses the ubiquitous 15-kDa holoCRBP as substrate (10). The other is an NAD-dependent microsomal enzyme in retinal pigment epithelium that converts 11-cis-retinol to bound to the 33-kDa cellular retinol binding protein (CRALBP), specific to eye, into 11-cis-retinal (30). A third binding protein, CRBP (type II), a 16-kDa intestinal protein, allows bound retinol to be reduced into retinol, but neither retinol nor retinal can be oxidized enzymatically when bound to CRBP (type II) (31). Participation as substrate in the conversion of retinol into retinoic acid represents a new function for a retinoid binding protein.

Recent experimental results have thrust CRBP to the forefront of cellular retinol metabolism. Retinol in equilibrium with the serum retinol binding protein can diffuse rapidly through the aqueous phase and accumulate in membranes (32, 33), quite possibly with the degree of retinol uptake by cells, depending on cellular concentrations of CRBP (34). The holoCRBP formed serves as a substrate for a lecithin-retinol acyltransferase (35, 36) and protects retinol from esterification by an acyl-CoA-retinol acyltransferase (37). ApoCRBP also stimulates the hydrolysis of membrane-bound retinyl esters (14), by a cholate-independent retinyl ester hydrolase (38, 39). The holoCRBP formed supports retinoic acid synthesis by providing substrate for an NADP-dependent microsomal retinol dehydrogenase that produces retinal and by protecting retinol from conversion into retinol by microsomal NAD-dependent dehydrogenase(s) and monoxygenases. The present work suggests an extension of the role of CRBP to that of providing substrate for cytosolic retinoic acid synthesis from the retinol produced in microsomes from CRBP-retinol. Altogether, the rate of accretion or mobilization of retinyl esters and the rate of retinoic acid synthesis would seem to be influenced, in part, by the relative amounts of apoCRBP and holoCRBP. The transition of retinoids through the pathway from esters through production of retinol would occur via a series of protein-protein interactions without release of retinoids into the aqueous phase. A summary of CRBP roles in retinoid metabolism is depicted (Fig. 9). Perhaps this model of lipid binding proteins participating in the metabolism of their ligands may be applicable generally to delineating the function(s) of other members of this family, i.e., the fatty acid binding proteins may similarly mediate fatty acid uptake and metabolism (40).

FIG. 9. Summary of CRBP affects on retinol metabolism. HoloCRBP is substrate for retinyl ester synthesis catalyzed by a microsomal enzyme lecithin-retinol acyltransferase (LRAT) and is also substrate for a microsomal NADP-dependent retinol dehydrogenase (ROH-DHase) that catalyzes the synthesis of retinal. The present work shows that CRBP-retinal serves as substrate for a cytosolic retinol dehydrogenase (RCHO-DHase). The apoCRBP generated stimulates microsomal retinyl ester hydrolysis from a cholate-independent retinyl ester hydrolase (REH).

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