Holocellular Retinol Binding Protein as a Substrate for Microsomal Retinal Synthesis†

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ABSTRACT: Holocellular retinol binding protein (holo-CRBP) was substrate for retinal synthesis at physiological pH with microsomes prepared from rat liver, kidney, lung, and testes. Four observations indicated that retinal synthesis was supported by holo-CRBP directly, rather than by the unbound retinol in equilibrium with CRBP. First, the rate of retinal synthesis with holo-CRBP exceeded the rate that was observed from the concentration of unbound retinol in equilibrium with CRBP. Second, NADP was the preferred cofactor only with holo-CRBP, supporting a rate about 3-fold greater than that of NAD. In contrast, with unbound retinol as substrate, similar rates of retinal formation were supported by either NAD or NADP. Third, the rate of retinal synthesis was not related to the decrease in the concentration of unbound retinol in equilibrium with holo-CRBP caused by increasing the concentration of apo-CRBP. Fourth, the rate of retinal synthesis increased with increases in the concentration of holo-CRBP as a fixed concentration of unbound retinol was maintained. This was achieved by increasing both apo-CRBP and holo-CRBP, but keeping constant the ratio apo-CRBP/holo-CRBP. Retinal formation from holo-CRBP displayed typical Michaelis–Menten kinetics with a K_m about 1.6 μM, less than the physiological retinol concentration of 4–10 μM in the livers of rats fed diets with recommended vitamin A levels. The V_{max} for retinal formation from holo-CRBP was 14–17 pmol min^{-1} (mg of protein)$^{-1}$, a rate sufficiently high to generate adequate retinal to contribute significantly to retinoic acid synthesis. Neither 860 mM ethanol nor 40 μM ketoconazole inhibited retinal formation from holo-CRBP, excluding ethanol-oxidizing enzymes and cytochrome P-450 isozymes from involvement. These data suggest a role for CRBP as a substrate in retinoic acid biogenesis.

Retinoid-dependent processes such as stem cell differentiation are supported directly by retinoic acid (Williams & Napoli, 1985), the most potent known physiologically occurring metabolite of retinol (Strickland & Sawey, 1980; Lotan, 1980; Williams et al., 1987; Darman et al., 1988). The synthesis of retinoic acid is likely to be a key and closely regulated step of retinol metabolism, because retinoic acid acts during specific phases of differentiation and development (Lotan, 1988) as a transcription modulating agent (Chiocca et al., 1988; Morita et al., 1989). Retinoic acid synthesis occurs by a two-step process: generation of retinal from retinol followed by conversion of the retinal into retinoic acid. Recently, the synthesis of retinoic acid from retinol was studied with retinol concentrations in the physiological range. Retinoic acid synthesis was observed in many cell types (Napoli, 1990a) and was catalyzed by enzymes that were distinct from the alcohol dehydrogenases that catalyze ethanol catabolism (Napoli, 1986a; Siegenthaler et al., 1990). The rate-determining step was the first one, the conversion of retinol into retinal. Yet enzymes specific for the synthesis of retinal from retinol have not been characterized in depth, nor have they been identified unequivocally. Obstacles have included multiple activities, nonspecific lipid dehydrogenases utilize unbound retinoids as substrate in vitro (Napoli & Race, 1987), and relatively low rates of metabolism: retinoic acid is a quantitatively minor metabolite of retinol (Williams & Napoli, 1985; Napoli, 1986a).

Considerable evidence suggests that intermediates pass through metabolic pathways by direct transfer between proteins, rather than by diffusion through the aqueous phase (Clegg, 1984; Srivastava & Bernhard, 1986; Bernhard, 1988). If this occurs for retinoic acid synthesis, the first protein in the series would be the ubiquitous cellular retinol binding protein (CRBP),[1] which binds cellular retinol specifically (Ong et al., 1982). CRBP belongs to a family of low molecular weight, lipid binding proteins that includes the fatty acid binding proteins, cellular retinoic acid binding protein, and the sterol carrier proteins (Clark & Armstrong, 1989; Matarese et al., 1990). The proteins in this class may have the common function of mediating the transport and/or metabolism of their ligands. Direct evidence of a role for CRBP in retinol metabolism has been reported. Esterification of CRBP-bound retinol by liver microsomes is catalyzed by LRAT, but not by ARAT (Ong et al., 1988; Yost et al., 1988). Unbound retinol, in contrast, is esterified by both liver microsomal ARAT (Ross, 1982) and LRAT (Ong et al., 1988; Yost et al., 1988). CRBP may also interact with membrane-associated retinyl ester hydrolase(s) to stimulate the hydrolyses of retinyl esters (Ottonello, 1987). If holo-CRBP, rather than unbound retinol, is a physiological substrate in retinoic acid synthesis, it should help distinguish retinoid-specific dehydrogenases from nonspecific dehydrogenases.

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[1] Abbreviations: ARAT, acyl-CoA:retinol acyltransferase; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; DTT, dithiothreitol; FPLC, fast-protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; LRAT, lecithin:retinol acyltransferase; 4-MP, 4-methylpyrazole; PMSF, phenylmethanesulfonyl fluoride.

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This report will show that physiological concentrations of holo-CRBP are substrate for NADP-dependent microsome-mediated retinal synthesis. This reaction has a K_m and a V_{max} that are consistent with a physiological role for it in the biogenesis of retinoic acid.

Experimental Procedures

Materials. Radioinert retinoids were purchased from Eastman Kodak and were purified by HPLC. [11,12-^{3}H]-Retinol (60 Ci/mmol) was purchased from Amersham. Pyridine nucleotide cofactors and most other reagents were purchased from Sigma.

Preparation of Rat Liver CRBP. CRBP was purified from rat liver by a combination, with modifications, of two previously published procedures (Ong & Chytil, 1980; Ross, 1982). All operations were performed at 4 °C. Rat liver (2 kg, Pel Freeze) was processed as described by Ong and Chytil (1980) up to and including the Cm-cellulose step. The pH of the filtrate obtained from the Cm-cellulose step was adjusted to 8.3 with 6 N NaOH. [^{3}H]Retinol (63 μCi), to facilitate monitoring column fractions, and PMSF (0.5 mM) were added, and the mixture was allowed to equilibrate overnight. The filtrate was then diluted with distilled water to a conductivity less than 3 mS and was loaded onto a DE-52 column (5 × 44 cm) equilibrated with 50 mM Tris–acetate, pH 8.3. The column was washed with equilibration buffer until less than 1 mg of protein eluted per milliliter of effluent. A linear gradient to 500 mM Tris–acetate was then run with a total of 4 L of buffer. Seventeen-milliliter fractions were collected: CRBP eluted between fractions 139 and 150. The pool was concentrated with an Amicon PM10 filter, 0.5 mM PMSF was added, and the concentrate was applied to a Sephadex G-50 column (5 × 115 cm) equilibrated with 50 mM Tris-HCl, 200 mM NaCl, 2 mM DTT, and 0.01% sodium azide, pH 7.5. Sixteen–milliliter fractions were collected: CRBP eluted in fractions 66–96. The pool was concentrated with an Amicon YM5 filter and frozen. Before use, CRBP was further purified in 2–5-mg aliquots by size-exclusion FPLC over a TSK GW3000SW column (0.8 × 30 cm) eluted with 20 mM Hepes, 150 mM KCl, and 2 mM DTT, pH 7.5 (Rainer et al., 1983). CRBP eluted in 12 mL. The ratio A_{350}/A_{280} was 0.96. An aliquot (0.2 mg of protein in 0.2 mL of buffer) was allowed to equilibrate with radioinert retinol (62.5 nmol). Holo-CRBP was separated from excess retinol by the same size-exclusion FPLC procedure. The ratio A_{350}/A_{280} was 1.4, indicating that 82% of the protein in the original sample was CRBP, which consisted of 68% holo-CRBP and 32% apo-CRBP as calculated from the maximum ratio of 1.7 for pure CRBP saturated with retinol (Ong & Chytil, 1980). To obtain different proportions of holo-CRBP and apo-CRBP, sufficient 100% holo-CRBP (generated from the mixture by saturation with retinol and purification by size-exclusion FPLC) was added to the original mixture to provide the desired ratio.

Preparation of Escherichia coli CRBP. CRBP was expressed in *E. coli* with the vector pMONCRBP, a gift from Dr. Marc Levin, and purified as described (Levin et al., 1988). Before the Sephadex G-50 step, the sample was divided into two portions. One was saturated with retinol to provide holo-CRBP; the other was used to provide apo-CRBP. Separate G-50 columns (5 × 100 cm) were used to purify each. The holo-CRBP obtained had a ratio of A_{350}/A_{280} to 1.5, indicating that it was 88% pure. The amount of apo-CRBP was verified by saturating an aliquot of it with retinol, separating bound from free by FPLC as described above, and determining the A_{350}/A_{280} ratio.

Rat Tissue Microsomes for Enzyme Assays. Tissues were obtained from male Sprague-Dawley rats (Harlan, Indianapolis, IN) that had been starved overnight and were sacrificed by decapitation. Tissues were rinsed in ice-cold saline. Testes and kidneys were decapsulated. Parenchyma of lung was scrapped from the bronchial tubes. The tissues (1 g/4 mL) were homogenized in buffer A (10 mM Hepes, 250 mM sucrose, 1 mM EDTA, and 2 mM DTT, pH 7.5). Microsomes were prepared by differential centrifugation as described (Napoli & Race, 1987) and were washed by rehomogenizing them in buffer A and repelleting them for 1 h at 105000*g*. The washed microsomes were rehomogenized in buffer A (20 mg of protein/mL). Homogenates and microsomes were stored in small aliquots at −80 °C. Protein was measured by the dye binding method with bovine serum albumin as standard (Bradford, 1976).

Enzyme Assays. Assays were done in duplicate at 37 °C in buffer B (2 mM DTT, 150 mM KCl, and 20 mM Hepes) at the times, pH, and concentrations of cofactors indicated in the figure and table legends in a final volume of 200 μL when CRBP was substrate and 500 μL when unbound retinol was substrate. Generally, duplicates were within 10% of their average. Unbound retinol was added in 2 μL of dimethyl sulfoxide, whereas CRBP was added in buffer B. Unless stated otherwise, when CRBP was used as substrate, it was that purified from rat liver and consisted of 5 μM holo-CRBP and 2.4 μM apo-CRBP, and will be referred to as 5 μM holo-CRBP. Controls consisted of incubations done in the absence of protein, or with boiled protein with cofactors, or with native protein in the absence of cofactors. Neither retinal nor retinoic acid was detected in any of these controls with holo-CRBP as substrate. In contrast, retinal was detected with unbound retinol as substrate, regardless of the type of control. Reactions were quenched by addition of sufficient 0.025 N KOH/ethanol to raise the pH to at least 12 (Napoli, 1986b, 1990b). Buffer B was added to increase the volume to 500 μL where appropriate, and the neutral retinoids were extracted with 2.5 mL of hexane (hexane-I). The aqueous phase was then adjusted to less than pH 2 with 4 N HCl, and retinoic acid was extracted with a second 2.5-mL portion of hexane (hexane-II). The solvents were evaporated under a stream of nitrogen.

HPLC Assays. Retinal (hexane-I) was eluted from a normal-phase HPLC column (DuPont Zorbax-Sil Reliance cartridge, 0.4 × 4 cm) at a flow rate of 2 mL/min with an 8-min linear gradient of tetrahydrofuran/hexane (1/99) to tetrahydrofuran/hexane (15/85), followed for 5 min with initial conditions. Retinal eluted in ~4.95 min, and retinol eluted in ~8.1 min. A standard curve of 12 points from X = 1–50 pmol retinal vs Y = peak height (in centimeters) was linear with a slope of 0.355 ($r > 0.998$). Sensitivity was at least 1 pmol (0.4-cm peak height). Overall retinal recovery was 50% from incubation through HPLC. Retinoic acid (hexane-II) was eluted in ~3.2 min from the normal-phase HPLC column in separate runs with 0.35% acetic acid in dichloroethane/hexane (1/9) at a flow rate of 2 mL/min as described previously (Napoli, 1990a; Napoli & Race, 1990). Retinoids were monitored with a Waters Model 484 tunable absorbance detector set at 370 nm for retinal assays and at 340 nm for retinoic acid assays.

Kinetic Data. Kinetic data were fitted to Michaelis–Menten and Lineweaver–Burke plots with the microcomputer program "Enzfitter" and are expressed as the calculated mean ± the standard error (Leatherbarrow, 1987).

Results

The formation of retinal from retinol delivered on CRBP by microsomes is shown in Figure 1. These results suggested

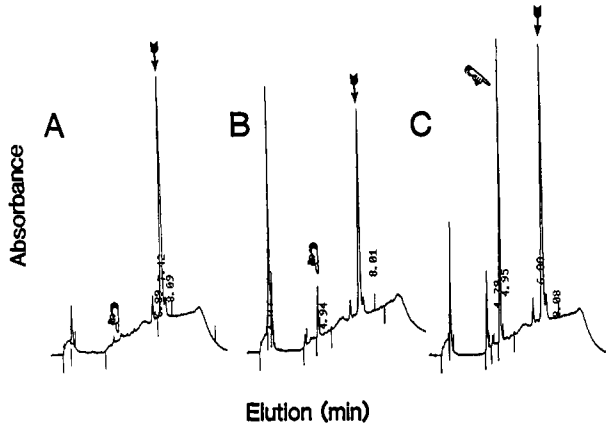

FIGURE 1: HPLC analysis of retinal produced from liver microsomes. Five micromolar holo-CRBP was incubated for 30 min with 100 μg of protein at pH 7.5 and with (A) no cofactor, (B) 2 mM NAD, or (C) 2 mM NADP. The amounts of retinal detected were 0, 5, and 35 pmol, respectively (4.95 min, indicated by the symbol). The latter eluting peak is retinol (~8.1 min, indicated by the arrow).

Table I: Synthesis of Retinal from Holo-CRBP or Free Retinol by Rat Liver Homogenate and Microsomes[a]

substrate	fraction	cofactor none	cofactor NAD	cofactor NADP
holo-CRBP	homogenate	0, 0[b]	7, 2	18, 4
	microsomes	0, 0	14, 4	34, 45
free retinol	homogenate	19, 9	86, 77	93, 99
	microsomes	14, 8	85, 121	74, 95

[a] The first set of numbers in each category is the picomoles of retinal produced in experiment 1, and the second set represents the picomoles produced in experiment 2. Each value is the average of duplicate reactions conducted with 500 μg of protein for 30 min with 5 μM substrate. The concentration of NAD(P) used was 4 mM. Experiment 1 was done at pH 8.0, and experiment 2 was done at pH 7.5. [b] Less than 0.5 pmol.

that when retinol is delivered bound to CRBP, NADP is the preferred cofactor and retinal synthesis is not observed in the absence of added cofactor. Nor was retinal observed in controls consisting of boiled protein or no protein when cofactor is present (data not shown). These chromatograms also illustrate the sensitivity of the normal-phase HPLC system used for retinal analyses and its ability to resolve retinal from retinol. As anticipated from the data of Figure 1, retinol bound to CRBP was not converted into retinal by either the homogenate or the microsomes in the absence of cofactor, and NADP was the preferred cofactor for retinal synthesis from both homogenates and microsomes (Table I). Microsomes, however, had higher activity than the homogenate. In contrast, relatively high concentrations (4 mM) of either NAD or NADP supported equally well the conversion of a low concentration of unbound retinol (5 μM) into retinal by rat liver homogenates or microsomes. With unbound retinol as substrate, microsomes did not have higher activity than the homogenate, and retinal was detected in the "no cofactor" control, albeit at least 5-fold less than in the experimental.

The microsomal-catalyzed rate of retinal synthesis from retinol in the presence of CRBP, relative to the rate from retinol alone (Table I), indicated that the unbound retinol equilibrating with CRBP was not the substrate for retinal synthesis in this in vitro experiment. To provide further evidence for this conclusion, the effect on retinal synthesis was tested of decreasing the unbound retinol concentration by increasing the apo-CRBP concentration, which decreases markedly the concentration of unbound retinol without having a measurable effect on the concentration of holo-CRBP. For

Table II: Effects of CRBP on Retinal Synthesis[a]

reagents (μM) CRBP (total)	retinol (total)	retinol (unbound)	retinal V_{max} (pmol min^{-1} mg^{-1})
6.0	5.0	0.08	8.3
7.4	5.0	0.03	10
none	5.0	5.0	20
none	0.2	0.2	3.3

[a] Reactions were conducted at pH 8.0 for 30 min with 100 μg of microsomal protein and 1 mM NADP. CRBP and total retinol were the concentrations added, whereas the amount of unbound retinol in the incubations with CRBP was calculated. Results are the means of duplicates. Controls have been subtracted from the data with unbound retinol.

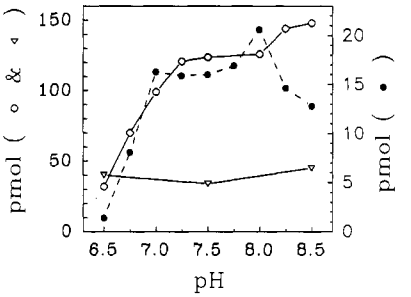

FIGURE 2: Effect of pH on retinal synthesis from retinol by liver microsomes. The substrate used was either 5 μM unbound retinol (open circles, 200 μg of protein) or 5 μM holo-CRBP (filled circles, 100 μg of protein). Reactions were done in duplicate for 30 min with 2 mM NADP. The control for unbound retinol was boiled microsomes (open triangles).

example, with a holo-CRBP concentration of 5 μM, increasing the apo-CRBP concentration from 1 to 2.4 μM decreases the unbound retinol concentration nearly 3-fold, as calculated from $[\text{retinol}]_f = [\text{retinol}]_t K_d/(K_d) + [\text{CRBP}]_t - [\text{retinol}]_t$ using a K_d of 16 nM for CRBP and retinol (Ong & Chytil, 1980), where f = free and t = total. The decrease in unbound retinol concentration, however, did not result in a decrease in the rate of retinal synthesis (Table II). Additionally, the results of Table II confirm that unbound retinol concentrations considerably higher than those that had been present were required to provide the rate of retinal synthesis observed in the presence of CRBP. Thus, the data in Tables I and II and Figure 1 indicate that holo-CRBP is a substrate for microsomal retinal synthesis.

The pH optimum of the NADP-supported reaction ranged between 7 and 8.25 when holo-CRBP was substrate. With unbound retinol, retinal synthesis did not decrease with an increase in pH to 8.5, as it did with holo-CRBP (Figure 2). The reactions with unbound retinol or holo-CRBP were linear about 30 min. With holo-CRBP, the reaction was linear to 100 μg of microsomal protein, whereas with unbound retinol the formation of retinal was linear to 200 μg of protein. As noticed in the previous experiments, another difference between the two substrates was the amount of retinal formed in the absence of active protein. With holo-CRBP as substrate, retinal was not detected in the absence of microsomes, but with unbound retinol, retinal was detected routinely in the controls.

The ability of NADP to support microsomal retinal synthesis was compared to that of NAD. With unbound retinol, the K_m for NADP (10 ± 4 μM) was much lower than that for NAD (270 ± 100 μM), but the rate supported by each cofactor was not significantly different [4 ± 0.2 vs 5.7 ± 1.3 pmol min^{-1} (mg of protein)$^{-1}$, respectively]. In contrast, with holo-CRBP as substrate, the K_m for NADP (30 ± 7 μM) was lower than that for NAD (280 ± 80 μM), and the maximum

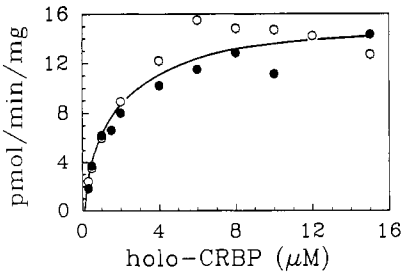

FIGURE 3: Relationship between the holo-CRBP concentrations and the rate of retinal synthesis. Incubations were done at pH 7.5 for 30 min with 100 μg of protein and 2 mM NADP. The ratio total CRBP/retinol ratio was either 1.1 (open circles) or 1.5 (filled circles).

velocity supported by each cofactor was different [7.4 ± 0.3 vs 2.2 ± 0.2 pmol min^{-1} (mg of protein)$^{-1}$ for NADP and NAD, respectively].

The change in rate of retinal synthesis with increasing concentration of unbound retinol was determined for comparison with the kinetic constants with holo-CRBP as substrate. The K_m for free retinol in two separate experiments (9–10 concentrations between 0.5 and 30 μM) was 3.47 ± 1 and 4.75 ± 1.27 μM, and the V_{max} for each was 139 ± 19 and 146 ± 12 pmol min^{-1} (mg of protein)$^{-1}$, respectively. The average of the two determinations [K_m = 4.1 μM and V_{max} = 143 pmol min^{-1} (mg of protein)$^{-1}$] was used in the calculations below.

A Michaelis–Menten relationship was observed between the concentrations of holo-CRBP and the rates of retinal synthesis (Figure 3). This relationship was examined with two different ratios of apo-CRBP/holo-CRBP for two reasons: to demonstrate reproducibility of the kinetic constants and to provide additional evidence that holo-CRBP itself was substrate. With apo-CRBP present at 20% of the concentrations of holo-CRBP (total CRBP/retinol ratio = 1.1), the K_m was 1.64 ± 0.2 μM, and with apo-CRBP present at 50% of the concentrations of holo-CRBP (total CRBP/retinol ratio = 1.5), the K_m was 1.57 ± 0.14 μM. The V_{max} in the former case was 16.5 ± 0.9 pmol min^{-1} (mg of protein)$^{-1}$ and in the latter case was 14.2 ± 0.6 pmol min^{-1} (mg of protein)$^{-1}$. These results show that retinal synthesis was dependent on holo-CRBP, not on unbound retinol, because with a fixed ratio of apo-CRBP/holo-CRBP the concentration of unbound retinol does not increase as the concentrations of apo-CRBP and holo-CRBP increase. An increase in the ratio of total CRBP/retinol from 1.1 to 1.5 would decrease the concentration of unbound retinol 5-fold, from 160 to 32 nM, over the range of holo-CRBP concentrations. With these concentrations of free retinol, calculating from the kinetic constants of free retinol, the maximum rates of retinal synthesis would have been no higher than 5.4 and 1.1 pmol min^{-1} mg^{-1}, respectively, if free retinol had been substrate.

The synthesis of retinal from holo-CRBP was not affected by a series of agents that inhibit ethanol metabolism and/or cytochrome P-450 catalyzed reactions. Neither 4-methylpyrazole nor ketoconazole affected the conversion of holo-CRBP into retinal (Table III). Ethanol did not inhibit the reaction, but very high concentrations caused 2-fold stimulation. The reaction was not dependent on DTT and was not inhibited by the sulfhydryl group complexing reagent disulfiram.

The NADP-dependent conversion of CRBP-delivered retinol into retinal was not restricted to liver microsomes (Table IV). Microsomes prepared from kidney had activity similar to liver microsomes, and the activities in kidney and liver were about 2-fold greater than that of lung microsomes. Lung microsomal activity was about 3-fold greater than testes microsomal activity.

Table III: Effects of Inhibitors on Microsomal Retinal Synthesis from Holo-CRBP[a]

dithiothreitol concn (mM)	inhibitor	pmol/retinol
2	none	22, 27
2	10 mM 4-MP	26, 25
2	100 mM 4-MP	31, 27
2	300 mM ethanol	36, 30
2	860 mM ethanol	59
2	10 μM ketoconazole	32, 32
2	40 μM ketoconazole	34, 39
0	none	28, 25, 48
0	10 μM disulfiram	32, 28, 61
0	100 μM disulfiram	30, 48

[a] Reactions were conducted at pH 7.5 for 30 in with 100 μg of microsomal protein, 5 μM holo-CRBP, and 2 mM NADP. Each number represents an individual experiment done in duplicate. The results in each column under "pmol/retinal" were obtained in the same experiment.

Table IV: Tissue Distribution of Holo-CRBP/Retinal Synthesis Activity[a]

tissue	NAD	NADP
liver	12, 10	92, 49
kidney	21, 21	66, 76
lung	5, 3	34, 36
testes	3, 5	11, 12

[a] Incubations were done for 20 min with 2 mM cofactor at pH 7.5 with 500 μg of microsomal protein and 5 μM CRBP. The two sets of values for each cofactor represent the picomoles of retinal formed in two different experiments, each done in duplicate.

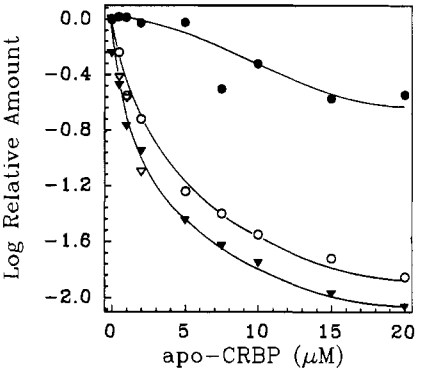

FIGURE 4: Effect of apo-CRBP on the synthesis of retinal or retinoic acid from holo-CRBP. The syntheses from holo-CRBP of retinal by microsomes (filled circles) and retinoic acid by cytosol (open triangles) are shown with increasing concentrations of apo-CRBP. Both the apo-CRBP and holo-CRBP were from *E. coli*. Cytosol-produced retinoic acid was not detected with apo-CRBP concentrations greater than 2 μM (not plotted). Microsomes (100 μg of protein, 2 mM NADP) and cytosol (500 μg of protein, 2 mM NAD) were incubated for 30 min at pH 8.0. With no added apo-CRBP, an average of 51 pmol of retinal was produced in microsomes, but retinoic acid was below detection limits, whereas with cytosol and no added apo-CRBP, 20 pmol of retinoic acid was produced, but retinal was below detection limits. Also represented are the calculated amounts of unbound retinol in equilibrium with holo-CRBP (open circles) and the ratio of the calculated amount of retinal that would have been produced from the free retinol present in equilibrium with holo-CRBP to the amount of retinal actually observed (filled triangles). With these incubation conditions, 28 pmol of retinal would have been anticipated at no added CRBP.

After these experiments had been completed, the pMONCRBP vector was obtained, and CRBP was expressed in *E. coli*. The *E. coli* expressed CRBP was used to compare microsomal and cytosolic synthesis of retinal and retinoic acid

from holo-CRBP in the presence of increasing concentrations of apo-CRBP (Figure 4). Consistent with the previous data, microsomal retinal synthesis from holo-CRBP was not affected by increasing the apo-CRBP concentration to 5 μM (i.e., a ratio of total CRBP/retinol of 2.0), which results in a decrease of the free retinol concentration from 283 nM (no added apo-CRBP) to 16 nM (5 μM apo-CRBP), i.e., an 18-fold decrease. Calculating from the kinetic constants obtained with free retinol, had only free retinol been substrate, the retinal produced at zero added apo-CRBP should have been 28 pmol total under the experimental conditions, rather than the average of 51 pmol actually observed (see the legend to Figure 4). This would have decreased to 1.8 pmol with the increase in apo-CRBP concentration to 5 μM. Although decreases in retinal synthesis were observed with increases in apo-CRBP concentration beyond 5 μM, these did not correlate with the decreases in unbound retinol, or the calculated rate that would have been observed had only free retinol been substrate. For example, at 20 μM apo-CRBP, the unbound retinol concentration had decreased to 4 nM, i.e., 70-fold relative to zero added apo-CRBP, which should have resulted in a 65-fold reduction in retinal produced, but retinal synthesis decreased only 3-fold. These data confirm that holo-CRBP is a substrate and indicate that high concentrations of apo-CRBP, generally beyond those that occur physiologically, compete with holo-CRBP. In contrast to the results with microsomes, the rate of retinoic acid synthesis by cytosol from holo-CRBP seemed to be dependent on the concentration of unbound retinol. Indeed, at 5, 10, 15, and 20 μM apo-CRBP, no retinoic acid was detected.

Discussion

This report shows that holo-CRBP is a substrate for retinal synthesis in microsomes prepared from liver, kidney, lung, and testes. The conclusion that holo-CRBP is substrate, rather than the unbound retinol in equilibrium with the binding protein, is supported by four independent observations: the rate of retinal formation from holo-CRBP exceeded the rate supported by the free retinol present in equilibrium with CRBP (Table II), was 3-fold higher with NADP as cofactor, in contrast to the results with unbound retinol (Tables I and IV, Figure 4), was unaffected by decreasing unbound retinol concentration with apo-CRBP concentrations in the physiological range when holo-CRBP concentration was fixed (Table II, Figure 3), and increased with increasing holo-CRBP concentration as unbound retinol concentration remained constant (Figure 4).

Our results do not exclude the possibility that free retinol is also a substrate in vivo. The results do, however, indicate the importance of direct transfer of retinol from CRBP to the dehydrogenase. Retinol does not require dissociation from CRBP and random diffusion through the aqueous medium, but apparently can be transferred by a protein/protein interaction between the dehydrogenase and holo-CRBP. CRBP belongs to a family of lipid binding proteins that includes other retinoid binding proteins, such as CRBP (type II) and CRABP (types I and II). Each member binds specific lipids and is characterized by a "β-clam" pocket formed by orthogonal β-sheets. Each ligand occupies the interior of its unique binding protein with its functional group, e.g., the hydroxyl group of retinol in the case of CRBP, oriented toward the interior of the protein (Jones et al., 1988). This and the protection of retinol bound to CRBP from oxidation by the NAD-dependent microsomal dehydrogenase activity and cytosolic dehydrogenases that recognize unbound retinol (Napoli & Race, 1987, 1990; Posch et al., 1989) argue for specificity in the interaction between the NADP-dependent microsomal activity and CRBP.

Retinol concentrations in the livers of rats fed diets adequate in vitamin A vary between 4 and 10 μM (Williams et al., 1984; Yost et al., 1988). The concentration of CRBP in liver is about 6–7 μM, and retinol not bound to CRBP is bound to the serum retinol binding protein (Yost et al., 1988). Therefore, the use of holo-CRBP as substrate by an NADP-dependent dehydrogenase with a K_m below the physiological concentration of the substrate implies that microsomal retinal generation by this path is important physiologically. A probable function of this reaction would be to generate retinal as an intermediate in retinoic acid biosynthesis. In preliminary experiments, addition of cytosol to CRBP/microsomal incubations resulted in a decrease in retinal, which was accompanied by an equimolar increase in retinoic acid. This observation is being pursued with the working hypothesis that retinal generated in the endoplasmic reticulum from holo-CRBP contributes physiologically to retinoic acid synthesis.

The activity reported here seems to be distinct from the cytochrome P-450 family of enzymes because it was not inhibited by ketoconazole, a potent inhibitor of cytochromes P-450 that suppresses a spectrum of reactions, including steroid, retinoic acid, and vitamin D metabolism (Mason et al., 1985; Napoli, 1986a; Williams & Napoli, 1987; Hollis, 1990). Lack of inhibition by ethanol at concentrations 60000-fold greater than the retinol concentration, an ethanol concentration that would have been lethal in vivo, excludes cospecificity of the reaction with ethanol-metabolizing enzymes, as does the insensitivity to 4-methylpyrazole, an inhibitor of ethanol metabolism (Li & Theorell, 1969; Cornell et al., 1983). Stimulation by very high ethanol concentrations could be the result of solvent effects on the microsomal vesicles and/or the conformation of the proteins involved in retinal synthesis. Another potent inhibitor of ethanol metabolism, disulfiram, also did not affect retinal synthesis. These results indicate that microsomal retinol metabolism by the NADP-dependent pathway is not likely to be affected by ethanol intake nor mediated by the microsomal ethanol-oxidizing P-450 isozyme.

There are two other examples of specific binding protein mediated redox reactions of retinoids with probable physiological significance. Saari and Bredberg (1982) showed that an NADH-dependent microsomal enzyme in retinal pigment epithelia reduced 11-*cis*-retinal, bound to cellular retinal binding protein, to 11-*cis*-retinol. The 11-*cis*-retinol remained bound and was reoxidized to 11-*cis*-retinal in the presence of NAD. Kakkad and Ong (1988) showed that retinal bound to CRBP (type II) was reduced by an intestinal microsomal reductase supported by either NADH or NADPH. The retinol remained bound but could not be reoxidized in the presence of NAD or NADP. The dehydrogenase reported here differs from these two by the retinoid binding protein it recognizes, its tissue distribution, and an order of magnitude higher affinity for NADP than for NAD. An NADP-dependent, *all-trans*-retinol dehydrogenase has been reported in microsomes of bovine rod outer segments (Futterman, 1963; Nicotra & Livrea, 1982). The work was done with high substrate concentrations ($\sim$100 μM), so the physiological import of the activity is not clear, and holo-CRBP was not tested as substrate for this enzyme. An NAD-dependent microsomal retinol dehydrogenase activity has been observed in rat liver, but it has not been examined with holo-CRBP as substrate (Leo et al., 1987; Napoli & Race, 1990). The NAD-dependent activity was characterized with substrate concentrations of 500

μM retinol and reportedly has a K_m of 120 μM for unbound *all-trans*-retinol (Leo et al., 1987). This is at least 3 orders of magnitude greater than the concentration of unbound retinol in liver, making its physiological significance uncertain. The modest activity in the present work with NAD when holo-CRBP is substrate could be the result of NAD-supported NADP synthesis, low affinity for NAD by an enzyme that uses NADP, or low affinity for holo-CRBP by the enzymes that are supported by NAD.

Multiple cytosolic dehydrogenases convert retinol into retinoic acid in vitro (Napoli & Race, 1987; Posch et al., 1989). These dehydrogenases include alcohol dehydrogenases that are involved in ethanol metabolism as well as enzymes that are distinct from the alcohol dehydrogenases that catalyze ethanol metabolism. The latter conclusion is supported by the presence of cytosolic retinol dehydrogenases in mutant deer mice that do not express alcohol dehydrogenase that have low K_m's ($\sim$4 μM) for unbound retinol and are insensitive to 4-methylpyrazole (Posch et al., 1989). These cytosolic retinol dehydrogenases are also distinct from the NADP-dependent microsomal dehydrogenase. Besides the different subcellular localization, they use NAD as cofactor, are sensitive to low concentrations of disulfiram, and are more active in the presence of DTT. In vitro, CRBP seems to protect retinol from the vast majority, if not all, of the cytosolic dehydrogenases, including the alcohol dehydrogenases that are distinct from those that metabolize ethanol and other aliphatic short- and long-chain alcohols. Some of these cytosolic retinol dehydrogenases, however, might also contribute physiologically to retinoic acid synthesis by using the small amount of unbound retinol in cytosol, and could be especially important in cells that do not express CRBP.

Since its discovery by Bashor et al. in 1973, the function of CRBP has been a matter of investigation and discussion. Some evidence suggests that it may stimulate the hydrolyses of retinyl esters (Ottonello et al., 1987) and transport the released retinol into the nucleus (Liau et al., 1985). CRBP is recognized as a substrate for esterification of retinol in liver by LRAT and restricts retinol from ARAT-mediated liver microsomal esterification (Ong et al., 1988; Yost et al., 1988). The present work indicates yet another function: the oxidation of its retinol by an NADP-dependent microsomal enzyme and restriction from NAD-dependent microsomal or cytosolic oxidation. It is important to note, however, that whereas holo-CRBP is a *physiological* substrate, it is not an *obligatory* substrate for retinol esterification or oxidation. In both cases, the enzymes that recognize holo-CRBP also recognize free retinol. Nevertheless, it is reasonable to expect that this multifunctional, ubiquitous protein is a major component in the pathways of retinol metabolism and contributes to the net directional flux of retinoids down specific metabolic pathways. Increases in apo-CRBP could accelerate retinyl ester hydrolysis, and, conversely, increases in holo-CRBP could accelerate retinol esterification. The K_m and V_{max} of CRBP-mediated retinol esterification by liver microsomes are 1–4 μM and 50–150 pmol min^{-1} (mg of protein)$^{-1}$, respectively (Ong et al., 1988; Yost et al., 1988). The K_m of 1.6 μM and V_{max} of about 15 pmol min^{-1} (mg of protein)$^{-1}$ for retinol dehydrogenation indicate that this reaction could occur concurrently with esterification. Retinal synthesis and thereby retinoic acid synthesis would be controlled by the availability of holo-CRBP and by the rate of retinol dehydrogenation.

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Registry No. NADP, 53-59-8; NAD, 53-84-9; retinal, 116-31-4; retinol, 68-26-8; retinoic acid, 302-79-4.

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Approaches to Predicting Effects of Single Amino Acid Substitutions on the Function of a Protein†

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ABSTRACT: The relative activities of 313 mutants of the gene V protein of bacteriophage f1, assayed in vivo, have been used to evaluate two approaches to predicting the effects of single amino acid substitutions on the function of a protein. First, we tested methods that only depend on the properties of the wild-type and substituting amino acids. None of the properties or measures of the functional equivalence of amino acids we tested, including the frequency of exchange of amino acids among homologous proteins as well as changes in side-chain size, hydrophobicity, and charge, were found to be more than weakly correlated with the activities of mutants. The principal reason for this poor correlation was found to be that the effect of a particular substitution varies considerably from site to site. We then tested an approach using the activities of several mutants with substitutions at a site to predict the activity of another mutant, and we find that this is a relatively good indicator of whether the other mutant at that site will be functional. A predictive scheme was developed that combines the weak information from the models depending on the properties of the wild-type and substituting amino acids with the stronger information from the tolerance of a site to substitution. Although this scheme requires no knowledge of the structure of a mutant protein, it is useful in predicting the activities of mutants.

It is not possible, at present, to predict with certainty whether a protein differing from a natural protein by an amino acid substitution is likely to be functional, even if detailed structural information on the wild-type protein is available. Nevertheless, it is widely thought that chemically conservative amino acid substitutions, such as one in which a leucine is replaced with an isoleucine, will generally not affect the function of a protein, while other less conservative replacements are more likely to affect protein function. Although this idea has never been systematically tested, it is supported by the observation that amino acid side chains that are chemically similar to one another are much more likely to be found at corresponding positions in structurally or functionally related proteins than dissimilar ones (Dayhoff et al., 1978). For example, leucine and isoleucine are found to substitute for one another more than 10 times as frequently as valine and arginine.

We have set out to evaluate two simple approaches for predicting whether a protein with an amino acid substitution is likely to be functional. One approach makes the assumption that the activity of a protein with an amino acid exchange relative to the wild-type protein is related to some measure of the functional equivalence of the wild-type and substituting amino acid side chains. An approach of this type has been implicitly used in many schemes to align amino acid sequences (Gribskov et al., 1987; Pearson & Lipman, 1988), to predict structures and functions of proteins (Chou & Fasman, 1978; Garnier et al., 1978; Eisenberg et al., 1982; Kidera et al., 1985), and to design new proteins with structures similar to natural ones (DeGrado, 1988). It seems certain that a mutant protein which differs from the wild type by an exchange that occurs very frequently among homologous proteins is more likely to be functional than one which differs by an infrequently observed exchange. It is not clear, however, whether this difference is sufficiently large to be of any use in predicting whether a mutant protein will be functional. A second approach incorporates information on the tolerance of each site in a protein to substitution. For a number of proteins, some sites are much more tolerant of amino acid substitutions than others (Loeb et al., 1989; Bowie & Sauer, 1989; Bowie et al., 1990; Kleina & Miller, 1990). In this second type of model,

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