Purification and properties of carotene 15,15′-dioxygenase of rabbit intestine

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Abstract Carotene 15,15′-dioxygenase, which oxidizes carotenoids to retinal, has been purified up to 200-fold from rabbit intestine by ammonium sulfate fractionation, heat treatment, and acetone precipitation. With β-apo-10′-carotenol as the substrate, the purified enzyme has a pH optimum of 7.8, a K_m of 6.7×10^{-5} M, and a V_{max} at 37°C of 9 nmoles of retinal/mg protein/hr. The purified enzyme is inhibited by ferrous ion-chelating agents such as α,α′-dipyridyl and *o*-phenanthroline, and by sulfhydryl-binding agents such as iodoacetamide, *N*-ethylmaleimide, and *p*-chloromercuribenzoate. The latter inhibitory effects are reversed by reduced glutathione. The cleavage of β-apo-10′-carotenol is competitively inhibited by its acetylenic analog, 15,15′-dehydro-β-apo-10′-carotenol. The enzyme is present in the intestinal mucosa of several mammals, the chicken, the tortoise, and a freshwater fish, but it is absent from cat intestinal tissue.

Supplementary key words β-apo-10′-carotenol · β-carotene cleavage · 15,15′-dehydro-β-apo-10′-carotenol · retinal · thiobarbituric acid method · mammals · fish · tortoise · chicken · cat

CAROTENE 15,15′-DIOXYGENASE, an enzyme initially found in the cytoplasm of rat liver and intestine, catalyzes the conversion of all-*trans*-β-carotene into 2 moles of retinal (1–3). In addition to β-carotene, several other carotenoids and β-apo-carotenol derivatives are cleaved to yield retinal (4), and a rough correlation has been shown to exist between the rate of retinal formation and the biological activity of a given carotenoid (5). In the present paper a procedure is described for purifying the carotene cleavage enzyme up to 200-fold from rabbit intestine. Several properties of the purified enzyme are defined, and the activity of the enzyme in the intestinal mucosa of various species is assessed.

MATERIALS AND METHODS

Preparation of β-apo-10′-carotenol

β-Apo-10′-carotenal (10 mg) dissolved in 3 ml of 95% ethanol was reduced by the addition of 5 mg of sodium borohydride in 0.2 ml of water. Within 10 min the characteristic absorption peaks (403 and 420 nm) of β-apo-10′-carotenol (6) reached maximal values. The solution was extracted with diethyl ether, washed thoroughly with distilled water, dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo; the residue was dissolved in petroleum ether. The petroleum ether extract was chromatographed on thin-layer plates of alumina G, using 2% (v/v) acetone in petroleum ether as the developing solvent. β-Apo-10′-carotenol (R_F 0.18) was the major, if not sole, component present, the carotenal (R_F 0.61) being virtually absent. The β-apo-10′-carotenol band was scraped off the plate and was quantitatively extracted with acetone. It was found to have an $E^{1\%}_{1\,cm}$ value of 1850 at 403 nm in petroleum ether.

Preparation of 15,15′-dehydro-β-apo-10′-carotenol (DAC)

DAC was prepared from its corresponding aldehyde and purified in the same way as β-apo-10′-carotenol. DAC was found to have an $E^{1\%}_{1\,cm}$ value of 1830 at 383 nm in petroleum ether, and its spectral characteristics agreed very well with published values (6). The formulas for β-carotene, β-apo-10′-carotenol, DAC, and retinal are given in Fig. 1.

Abbreviations: TLC, thin-layer chromatography; DAC, 15,15′-dehydro-β-apo-10′-carotenol; SDS, sodium dodecyl sulfate; GSH, reduced glutathione; TBA, thiobarbituric acid.

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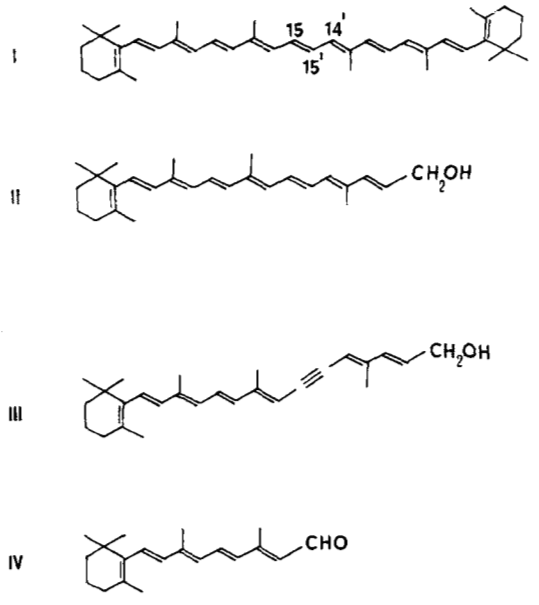

FIG. 1. Formulas of all-*trans* isomers of β-carotene (I), β-apo-10′-carotenol (II), 15,15′-dehydro-β-apo-10′-carotenol (III), and retinal (IV).

Enzyme assay

The standard solution contained 100 nmoles of substrate, 25 μmoles of SDS, 2 mg of GSH, and about 2 mg of enzyme protein in a final volume of 2 ml of 0.1 M potassium phosphate buffer, pH 7.8. The reaction was initiated by the addition of substrate. In special studies the range of substrate concentration was 5–40 nmoles/ml, the range of enzyme concentration was 0.5–10 mg/ml, and the volume was 2–10 ml; the SDS and GSH concentrations were maintained constant. Protein was measured by the method of Lowry et al. (7). After incubation for 1 hr at 37°C, the standard assay reaction was stopped by the addition of 5 ml of acetone. The resultant precipitate was centrifuged off, and the supernatant solution was extracted twice with 10-ml portions of diethyl ether. The ether extract was washed, dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo, and the residue was redissolved in a small volume of petroleum ether. The petroleum ether extract was chromatographed on thin-layer plates of alumina G, using 2% acetone in *n*-hexane as the solvent system. The retinal band (R_F value 0.66) was scraped off, extracted with acetone, and estimated by the thiobarbituric acid procedure (8), except that no thiourea was added. The enzymatic product and a known sample of all-*trans*-retinal had essentially identical spectra in petroleum ether (9), behaved the same in TLC systems, and formed TBA complexes with the same spectrum. Under the conditions of the assay, the TBA complex of retinal was found to have an $E^{1\%}_{1\,cm}$ value of 2163 at 520 nm.

Electrophoresis and ultracentrifugation

Enzyme fractions were electrophoresed on polyacrylamide gel in the presence of SDS, essentially as described by Weber and Osborn (10), and stained with Coomassie brilliant blue. The 45–60% acetone fraction was dialyzed against 0.05 M potassium phosphate buffer, pH 7.8, for 6 hr in the cold and then analyzed in the Beckman Spinco model E analytical ultracentrifuge at 56,000 rpm for 4 hr.

Materials

The authors are greatly indebted to Professor O. Isler of Hoffmann-La Roche and Co., Basle, for the generous gifts of β-apo-10′-carotenal and 15,15′-dehydro-β-apo-10′-carotenal. Other materials were the same as those described earlier (4). All chemicals and solvents, which were used without further purification, were of reagent grade.

RESULTS

Enzyme purification

The mucosa was scraped from a slit intestine (pylorus to the lower ileum) of a rabbit that had been fasted overnight. It was homogenized with 8 vol of 0.1 M potassium phosphate buffer, pH 7.8, and then centrifuged at 44,000 *g* for 1 hr. The residue was discarded, and the supernatant solution was subjected to ammonium sulfate precipitation. The enzyme activity resided in the 0–60% saturated ammonium sulfate pellet, which could be stored at −20°C for considerable periods without appreciable loss of activity. In studying the distribution of the enzyme in various species, enzyme purification was generally carried this far.

The ammonium sulfate pellet was dissolved in 0.1 M potassium phosphate buffer, pH 7.8, to give a final protein concentration of 15 mg/ml. It was heated with continuous stirring to 55°C, kept at 55°C for 3 min, quickly cooled to 2°C, and centrifuged at 15,000 rpm for 10 min. The pellet was discarded. At this stage the enzyme was also quite stable when stored at −20°C.

The supernatant solution from the previous step, which had a protein concentration of about 6 mg/ml, was further fractionated with cold acetone (−30°C) into 0–45% and 45–60% fractions. The pellet precipitating at 45–60% acetone had all the cleavage enzyme activity. At this stage the enzyme activity was rapidly lost during storage. However, the enzyme could be stabilized for storage at −20°C by dissolving the 45–60% acetone pellet in a suitable volume of 0.1 M potassium phosphate buffer, pH 7.8, and precipitating it again by adding ammonium sulfate to 60% saturation.

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TABLE 1. Summary of enzyme purification

Fraction	Protein	Total Activity	Specific Activity[a]	Purification
	mg			
Crude homogenate	13,400	118	0.0088	1
Supernatant solution	4,400	66	0.015	1.7
0–60% saturated ammonium sulfate	2,000	60	0.03	3.4
Heat stable (55°C, 3 min)	800	152	0.19	21.4
45–60% acetone (ammonium sulfate stabilized)	128	269	2.10	238

[a] The specific activity is expressed as nmoles of retinal formed from β-apo-10′-carotenol/mg protein/hr at 37°C, pH 7.8.

Since the total enzyme activity increased markedly in the latter steps, the true overall purification could not be simply assessed. The specific activity of the crude homogenates also varied considerably from rabbit to rabbit, sometimes being less than 0.005 and occasionally as high as 0.20. Enzyme purification from an "average" intestine, with an initial specific activity of 0.01, was therefore selected for presentation in Table 1.

In polyacrylamide gel electrophoresis of the 45–60% acetone fraction, a major protein band and four minor bands, two of which moved faster and two slower than the major band, were detected. The ultracentrifugal pattern of the same fraction showed a single but rather diffuse band. The most highly purified fraction, therefore, is far from homogeneous.

Attempts to purify the enzyme further by gel filtration were not successful. Although 0.2 M ammonium sulfate tends to protect the enzyme from inactivation, protein fractions eluted from Sephadex G-200 columns with ammonium sulfate solutions were devoid of enzyme activity. Large losses in the activity of the rat and hog mucosal enzymes also occurred on Sephadex G-200 and DEAE-Sephadex columns (11, 12).

Effect of varying parameters of the enzyme reaction

With β-apo-10′-carotenol as substrate and with a 1-hr incubation period, the reaction rate was directly proportional to the purified enzyme concentration in the range of 0–2 mg of protein/incubation mixture. With the same substrate and 2 mg of purified enzyme, the reaction rate was proportional to incubation time up to 1 hr. At higher protein concentrations or longer incubation times, the rate tended to reach a plateau. The enzyme activity was quite sensitive to pH, as shown in Fig. 2, and consequently all assays were conducted at pH 7.8. With increasing substrate concentrations (0–40 nmoles of β-apo-10′-carotenol/ml), the enzyme activity gave a typical Michaelis-Menten curve (Fig. 3*A*). From a Lineweaver-Burk plot of the data (Fig. 3*B*), a K_m value of 6.7×10^{-5} M and a V_{max} value of 9.1 nmoles of retinal formed/mg protein/hr at 37°C were calculated.

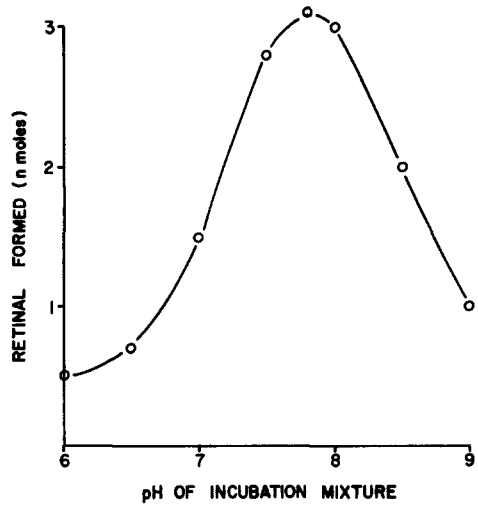

FIG. 2. Effect of pH on enzyme activity.

Effect of DAC and other inhibitors

Although DAC, the acetylenic analog of β-apo-10′-carotenol, is not cleaved by the enzyme to give any detectable product, it markedly inhibits the cleavage of β-apo-10′-carotenol, as shown in Table 2. DAC seems to compete with the substrate, inasmuch as an increase in the ratio of substrate to DAC offsets the inhibition.

Crude and partially purified preparations of the enzyme isolated from several sources are inhibited by chelators of ferrous ions and by sulfhydryl-binding agents (2, 11, 12). Purified rabbit intestinal enzyme behaves similarly, as shown in Table 3. GSH offsets the inhibitory effect of *p*-chloromercuribenzoate, whether added before or after the inhibition.

Carotene cleavage activity in several species

The activity of the cleavage enzyme was also determined in partially purified preparations of intestinal mucosa from several other vertebrates. As shown in Table 4, the specific activity of the mucosal enzyme varies from relatively high values in the guinea pig to zero in the cat. In addition, the relative specific activity of a given enzyme towards β-carotene, the most abundant natural substrate, and β-apo-10′-carotenol also varies greatly, being highest in the chicken and lowest in the rabbit (Table 4).

DISCUSSION

In nature, vitamin A is not synthesized directly from acetate but is derived solely by the cleavage of carotenoids, which contain an unsubstituted retinyl or

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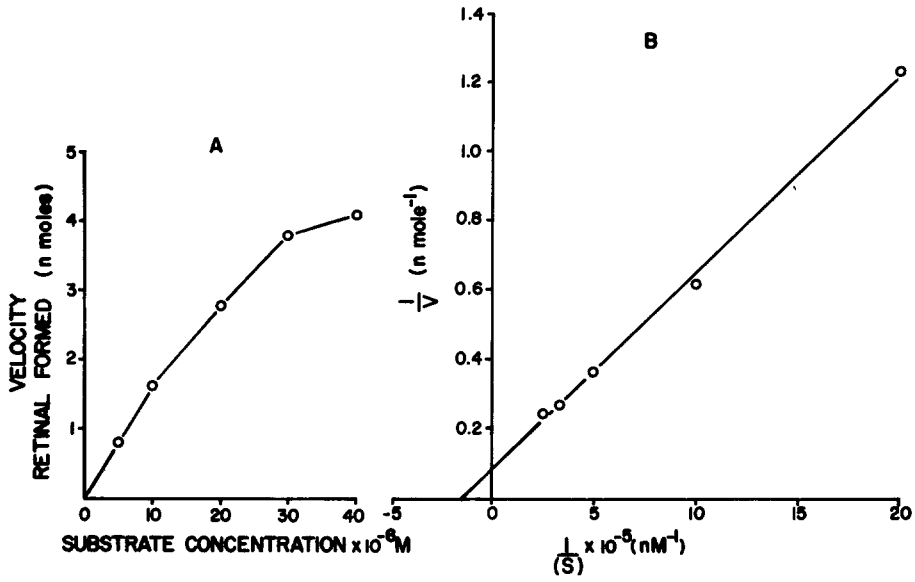

FIG. 3. Effect of substrate concentration on enzyme activity. The indicated concentrations of β-apo-10′-carotenol were incubated with 2 mg of purified enzyme under standard conditions. *A*, reaction velocity vs. substrate concentration; *B*, Lineweaver-Burk plot of *A*.

3-dehydroretinyl moiety. Consequently, the enzyme or enzymes catalyzing this reaction play an indispensable role in the maintenance of animal life. Although enzymes that cleave carotenoids at other loci may well exist in plants (13), carotene 15,15′-dioxygenase is the only enzyme of its class that has thus far been characterized in animals.

In this study the cleavage enzyme was purified a maximum of roughly 200-fold from rabbit intestine by using a few simple fractionation procedures. Since the total activity increased almost fourfold during purification, possibly due to the removal of inhibitors, and since the initial specific activity of the crude homogenate varied greatly from rabbit to rabbit, the true purification may be 50-fold or even less in some cases. The purified enzyme (45–60% acetone fraction) is clearly not homogeneous, inasmuch as it contains one major and four detectable minor electrophoretic bands and shows a broad, albeit single, sedimentation peak. Attempts to identify the band or bands that have enzyme activity are now underway.

The catalytic properties of the purified rabbit enzyme are similar in most regards to those reported for the rat intestinal enzyme (11) and the hog intestinal enzyme (12). The purified rabbit enzyme has a pH optimum of 7.8, a

TABLE 2. Inhibitory effect of DAC on β-apo-10′-carotenol cleavage

DAC	β-Apo-10′-carotenol	Relative Activity
M	M	
1×10^{-3}	0	0
0	5×10^{-5}	100
1×10^{-5}	5×10^{-5}	74
5×10^{-5}	5×10^{-5}	50
1×10^{-4}	5×10^{-5}	40
1×10^{-3}	5×10^{-5}	2
1×10^{-4}	1.5×10^{-4}	85
1×10^{-4}	3×10^{-4}	100

The purified enzyme (1.5 mg) was preincubated for 10 min with DAC before adding the substrate under standard assay conditions.

TABLE 3. Effect of chelating agents and sulfhydryl-binding agents on β-apo-10′-carotenol cleavage

Compound Added	Final Inhibitor Concentration	Relative Activity
	M	
None	0	100
α,α′-Dipyridyl	1×10^{-3}	25
1,10-Phenanthroline	1×10^{-3}	10
Iodoacetamide	1×10^{-3}	50
N-Ethylmaleimide	1×10^{-4}	25
	1×10^{-3}	5
p-Chloromercuribenzoate (PCMB)	1×10^{-4}	75
	1×10^{-3}	20
GSH[a] + PCMB	1×10^{-3}	100
PCMB + GSH[b]	1×10^{-3}	100

The purified enzyme (1.5 mg) was preincubated for 10 min at 37°C with the indicated final concentration of each inhibitor before the addition of substrate. Otherwise, assay conditions were standard.

[a] GSH (final concentration, 1×10^{-2} M) was added to the enzyme solution before preincubation with PCMB.

[b] GSH (final concentration, 1×10^{-2} M) was added to the enzyme solution after preincubation with PCMB.

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TABLE 4. Activity of the intestinal cleavage enzyme in several species

Species	Specific Activity		β-Carotene/ β-Apo-10′-carotenol
	β-Carotene	β-Apo-10′-carotenol	
Guinea pig	0.10	0.54	0.19
Rabbit	0.02	0.25	0.08
Tortoise (*Domania subtryuga*)	0.03	0.08	0.38
Monkey	0.015	0.06	0.25
Freshwater fish (*Clarias batrachus*)	0.05	0.05	1.00
Chicken	0.02	0.007	2.86
Cat	0	0	

β-Carotene or β-apo-10′-carotenol (2×10^{-5} M) was incubated in a 10-ml volume, but otherwise under standard assay conditions, with 20–100 mg of the 0–60% saturated ammonium sulfate fraction prepared from the intestinal mucosa of the listed species. The specific activity is expressed as nmoles of substrate cleaved/mg protein/hr. The highest values obtained for each species are presented.

K_m for β-apo-10′-carotenol of 6.7×10^{-5} M, and a V_{max} of 9 nmoles of β-apo-carotenol cleaved/mg protein/hr at 37°C. In comparison, the 27-fold purified hog intestinal enzyme has a pH optimum of 7.8–8.2, a K_m for β-carotene of 1.3×10^{-6} M, and a V_{max} of 0.8 nmole of β-carotene cleaved/mg/hr at 37°C (12), whereas the 2–4-fold purified rat intestinal enzyme has a pH optimum of 7.5–8, a K_m for β-carotene of 3×10^{-6} M, and a V_{max} of 0.57 nmole of β-carotene cleaved/mg/hr at 37°C (11). Since β-apo-10′-carotenol is 12.5 times more readily cleaved than β-carotene by the rabbit enzyme, the V_{max} values of these three purified preparations towards β-carotene are roughly the same. The purified rabbit mucosal enzyme, like all other cleavage enzymes studied, is inhibited by sulfhydryl-binding agents and by ferrous ion chelators. The hog enzyme (12), however, seems to be more sensitive to sulfhydryl-binding reagents than either the rabbit enzyme or the rat enzyme (11). We might conclude, however, that the cleavage enzymes thus far studied from various sources are remarkably similar in their general catalytic properties, with only trivial qualitative differences.

Of the inhibitors tested, 15,15′-dehydro-β-apo-10′-carotenol is clearly the most interesting. The 15,15′-acetylenic linkage is not cleaved by the enzyme, nor are products of any kind detected. It seems to inhibit the cleavage of β-apo-10′-carotenol competitively, however, and might be considered as the first specific inhibitor of carotenoid cleavage. This inhibitory action of DAC might be related to the specificity of the enzyme. Although the cleavage enzyme acts on carotenoids of various chain length and with different substituents (4, 5), it does seem to be highly specific for the 15,15′-ethylenic bond. Interestingly, the nearest substituents to this double bond are in the β-position, whereas all other double bonds in the conjugated system of carotenoids are adjacent to an α-methyl group. If an α-substituent protects a double bond from enzymatic attack, and conversely its absence allows cleavage, then the biological inertness of all desmethyl derivatives of vitamin A (14) may well be due to their degradation rather than to a structural requirement for side-chain methyl groups. Consequently, the rather strict structural requirements for biological activity determined by measuring the growth response to diverse vitamin A analogs may not necessarily reflect the specificity of the cellular response system.

Whether one or several enzymes are responsible for the conversion of all biologically active carotenoids into retinal in vertebrates is still uncertain. For example, the epoxy-carotenoids, some of which have biological activity without being cleaved by carotene 15,15′-dioxygenase (4, 5), may well be metabolized by other pathways (15, 16).

Carotenoid cleavage activity has been found in most species studied, the rat, hog, guinea pig, rabbit, monkey, tortoise, fish, and chicken, and we would indeed expect to find it in all species for which carotenoids have provitamin A activity. The domestic cat, in which the enzyme cannot be detected, is therefore an interesting exception. As early as 1931 cats were shown to become vitamin A deficient on a vitamin A-free diet supplemented with β-carotene (17), an observation which was later confirmed and extended (18). Although several possible explanations might be given for this observation, such as the nonabsorption or rapid degradation of carotenoids, the defect can now be attributed to the lack of cleavage enzyme activity in the cat intestine. If other organs also lack the enzyme, which seems likely in view of the above-cited nutritional studies (14, 15), the cat's survival would depend on its carnivorous food habits. The mink has also been reported to lack the ability to convert carotenoids into vitamin A (19).

In this regard it is interesting that the herbivores examined, the guinea pig and the rabbit, have the highest activities, at least with respect to β-apo-10′-carotenol as the substrate, the omnivores, the tortoise, fish, and chicken, have intermediate values, and the only carnivore studied, the cat, has none at all. With respect to β-carotene, however, the relationship is much less clear.

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