Expression and Characterization of a Murine Enzyme Able to Cleave β-Carotene

THE FORMATION OF RETINOIDS*

Because animals are not able to synthesize retinoids de novo, ultimately they must derive them from dietary provitamin A carotenoids through a process known as carotene cleavage. The enzyme responsible for catalyzing carotene cleavage (β-carotene 15,15'-dioxygenase) has been characterized primarily in rat intestinal scrapings. Using a recently reported cDNA sequence for a carotene cleavage enzyme from *Drosophila*, we identified a cDNA encoding a mouse homolog of this enzyme. When the cDNA was expressed in either *Escherichia coli* or Chinese hamster ovary cells, expression conferred upon bacterial and Chinese hamster ovary cell homogenates the ability to cleave β-carotene to retinal. Several lines of evidence obtained upon kinetic analyses of the recombinant enzyme suggested that carotene cleavage enzyme interacts with other proteins present within cell or tissue homogenates. This was confirmed by pull-down experiments upon incubation of recombinant enzyme with tissue 12,000 × g supernatants. Matrix-assisted laser desorption ionization-mass spectrometry analysis of pulled-down proteins indicates that an atypical testis-specific isoform of lactate dehydrogenase associates with recombinant carotene cleavage enzyme. mRNA transcripts for the carotene cleavage enzyme were detected by reverse transcription-polymerase chain reaction in mouse testes, liver, kidney, and intestine. In situ hybridization studies demonstrated that carotene cleavage enzyme is expressed prominently in maternal tissue surrounding the embryo but not in embryonic tissues at 7.5 and 8.5 days postcoitus. This work offers new insights for understanding the biochemistry of carotene cleavage to retinoids.

Retinoids play an indispensable role in maintaining the health of higher animals (1). In vision, 11-cis-retinaldehyde serves as the chromophore for the visual pigment rhodopsin (2). Aside from this action in vision, retinoids act primarily as transcriptional regulators. All-trans- and 9-cis-retinoic acid are responsible for regulating transcription of well over 300 genes. The transcriptional actions of retinoids are mediated through the retinoic acid receptor and retinoid X receptor classes of ligand-dependent transcription factors (3–6). Although retinoids have many essential physiologic actions within the body, animals are incapable of the de novo synthesis of retinoids. Thus, all naturally occurring retinoids must be derived from carotenoids that are synthesized in plants or photosynthetic microorganisms from terpenoid precursors (7). There are more than 600 carotenoids that can be present in human diets, but only about 10% of these can be converted to retinoids (8, 9).

The biochemical processes responsible for the conversion of carotenoids to retinoids are not fully understood. Through elegant work carried out in the early and mid 1960s, an enzymatic activity able to catalyze the cleavage of β-carotene to all-trans-retinal was identified and characterized (10–13). This enzymatic activity, carotene 15,15'-dioxygenase (also referred to as carotene cleavage enzyme), was identified in the intestines of rats (10, 14, 15), hogs (16), chickens (17, 18), and humans (19), and in rat liver (10, 20, 21). Despite the research interest that has been shown in this enzyme over the past 40 years, mammalian carotene 15,15'-dioxygenase has not been purified to homogeneity, nor has it been cloned. Recently, the molecular cloning of putative β-carotene cleavage enzyme has been reported for *Drosophila melanogaster* (22) and the chicken (23). Here, we report the identification of a cDNA encoding a mouse carotene 15,15'-dioxygenase and provide data regarding the biochemical properties and expression pattern of this enzyme.

EXPERIMENTAL PROCEDURES

Carotenoids and Retinoids—All-trans- and 13-cis-retinal, all-trans- and 13-cis-retinol, and all-trans-β-carotene (β-carotene) were purchased from Sigma Chemical Co. The synthetic retinoid, all-trans-9(-4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (TMMP-ROH, 2 Ro12-0586) (24), was obtained as a generous gift from Dr. Christian Eckhoff (Hoffmann-LaRoche, Inc).

Identification and Sequence Analysis of a cDNA Encoding Carotene 15,15'-dioxygenase—The published cDNA sequence for carotene 15,15'-dioxygenase from *D. melanogaster* (accession number AJ277682) was

* This work was supported by National Institutes of Health Grants R01 EY12858, R01 DK44498, R01 DK52444, and R01 HL49879 and by a grant from the United States Department of Agriculture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We dedicate this work to the late Professors DeWitt S. Goodman and James A. Olson.

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§§ The abbreviations used are: TMMP-ROH, all-trans-9(-4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol; RT-PCR, reverse transcription-PCR; GST, glutathione S-transferase; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HPLC, high performance liquid chromatography; CRBP I, II, and III, cellular retinol-binding protein, type I, type II, and type III, respectively; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; LDH, lactate dehydrogenase.
used to screen mouse expressed sequence tag sequences deposited in GenBank® (National Center for Biotechnology Information, Bethesda, MD) to identify potential mouse homologs of the enzyme. A cDNA sequence obtained from a cDNA library prepared from mouse kidney (accession number AW044175) sharing 48% sequence identity with the D. melanogaster EST was verified and obtained from Research Genetics Inc. (Huntsville, AL). This cDNA was sequenced fully in both directions by the Columbia University Comprehensive Cancer Center Core DNA Sequencing Facility.

Expression and Purification of Carotene 15,15'-Dioxygenase—An open reading frame for the carotene 15,15'-dioxygenase cDNA described above was amplified by PCR from the BAC clone (AW044175) and subcloned into the mammalian expression vector pcDNA3 (CCE/pcDNA3) (Invitrogen, San Diego) and into the bacterial expression vector pGEX-3X (CCE/pGEX) (Amersham Pharmacia Biotech). Both subclones were sequenced to verify orientation and also the correct reading frame in the case of the pGEX-3X vector. This latter vector was used to express carotene 15,15'-dioxygenase as fusion protein with bacterial glutathione-S-transferase (GST). The recombinant fusion protein was purified by affinity chromatography on glutathione-Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Expression of a GST-containing fusion protein in Escherichia coli was confirmed by Western blot analysis as described below. CCE/ppcDNA3 was transfected into CHO cells using calcium-phosphate transfection (25), and carotene 15,15'-dioxygenase expression was verified by Northern blot analysis and in vitro enzyme activity assay.

Western Blot Analysis—Recombinant dioxygenase-GST fusion protein was subjected to SDS-PAGE on a 12% gel and blotted onto a nitrocellulose membrane at 100 V for 1 h. The blot was incubated with 10% milk blocking buffer (non-fat milk in phosphate-buffered saline (PBS); 10 mM sodium phosphate, pH 7.4, 150 mM NaCl) containing 0.3% (v/v) Tween 20 (PBST) for 1 h followed by incubation with primary goat antibody against bacterial GST protein (1:1,000 dilution, Amersham Pharmacia Biotech) for 1 h. The membrane was washed twice with PBST for 10 min and was then incubated with rabbit anti-goat IgG conjugated with alkaline phosphatase (1:10,000) for 1 h. The blot was washed twice with PBST, as described above, and signal was detected with 4-nitro blue tetrazolium and 5% 5-bromo-4-chloro-3-indolyl phosphate in buffer containing 100 mM NaCl, 100 mM Tris (pH 9.5), 5 mM MgCl₂.

Northern Blot Analysis—Total RNA was isolated from mouse tissues employing RNAzol B (Tel-test, Inc., Friendswood, TX) according to the manufacturer's instructions for RNA isolation from tissues. For this purpose, adult male C57BL/6J mice maintained throughout life on a manufacturer's instructions for RNA isolation from tissues. For this purpose, adult male C57BL/6J mice maintained throughout life on a

Expression of Recombinant Cellular Retinol-binding Proteins—Recombinant cellular retinol-binding proteins, types I, II, and III (CRBP I, CRBP II, and CRBP III) were expressed in E. coli. For expression of CRBP II, we employed a cDNA encoding rat CRBP II (26) which was cloned in a pMON vector. The rat CRBP II cDNA was provided as a generous gift by Dr. Ellen Li of Washington University, St. Louis. The conditions we employed for induction of CRBP II expression were identical to those described previously in the literature (27). Bacteria expressing the CRBP II were pelleted by centrifugation at 6,000 × g for 15 min, and the pellet was resuspended in 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 10 mM β-mercaptoethanol, 15% glycerol, 0.05% sodium azide, and 0.05 mM phenylmethanesulfonyl fluoride. Bacteria were then sonicated and centrifuged again at 10,000 × g for 15 min, and the supernatant was used for pull-down assays as described below. Both mouse CRBP I and CRBP III clones in pET11a vector (Novagen, Madison, WI) were provided as a generous gift by Dr. Silke Vogel of Columbia University. Both clones were induced to express the proteins as described by Vogel et al. (28). Expression of the CRBP I, II, and III was verified by SD-SAGE followed by Coomassie staining of the gel.

Pull-down Assays—Mouse carotene 15,15'-dioxygenase fused to GST was purified using glutathione-Sepharose and incubated with the 9,000 × g supernatant prepared from a homogenate of rat intestinal mucosa scrapings (20). For our standard assay, the enzyme source was incubated with 100 mM Tricine buffer (pH 8.0) containing 15 μM β-carotene, 0.1 mM α-tocopherol, 0.5 mM diethiothreitol, 4 mM sodium cholate, and 15 mM nicotinamide for 1 h at 37 °C in a shaking incubator. The final reaction volume was 0.2 ml.

Purification of Carotene 15,15'-Dioxygenase were measured using Bio-Rad Bradford protein assay reagents according to the manufacturer's instructions employing bovine serum albumin as standard. Specific activity was defined as pmol of retinol produced/mg of protein/h. Enzyme kinetics data were analyzed using EnzFit 5.0 software (Perrella Scientific, Inc.).

Retinoid and carotenoid contents of the assay mixtures were analyzed by reverse phase or normal phase high performance liquid chromatography (HPLC) (see below). For reverse phase HPLC analysis, the enzymatic reaction was first terminated by adding 50 μl of 37% (v/v) formaldehyde (Sigma) in water, and this mixture was allowed to incubate further at 37 °C for 10 min. Retinoids were then extracted into 500 μl of acetonitrile, and the extraction medium was incubated on ice for 5 min. After incubation, the assay mixture was centrifuged 10,000 × g for 10 min at 4 °C. An aliquot of the resulting supernatant (200 μl) was injected directly onto the reverse phase HPLC column and analyzed as described below. For normal phase HPLC analysis, the reaction was stopped by adding 500 μl of 100% ethanol, and retinoids and carotenoids were extracted into 2.5 ml of hexane. Small amounts of 13-cis-retinol or TMMP-ROH were added to the extraction mixtures to serve as internal standards for the HPLC analysis.

HPLC Analyses—For reverse phase HPLC analysis, retinoids were separated on a 4.6 × 250-mm Ultralight C₁₈ column (Beckman, Fullerton, CA) preceded by a C₅ guard column (Supelco Inc., Bellefonte, PA), using 10% water in acetonitrile containing 0.1% ammonium acetate as the running solvent at 1 ml/min. For normal phase HPLC analysis, retinal and retinol isomers and β-carotene were separated on a 4.6 × 150-mm Supelcosil LC-Si column (Supelco Inc.) preceded by a silica guard column (Supelco Inc.) using hexane:ethyl acetate:butanol (9:6:3:0.1, v/v) as the mobile phase flowing at a rate of 0.8 ml/min. Isomers of retinol and retinal, and β-carotene were detected by absorbance of 325, 365, and 450 nm, respectively, using a Waters 996 photo-diode array detector (Waters Associates, Milford, MA). Retinol and retinal peaks were identified by comparing retention times and spectral data of experimental compounds with those of authentic standards. Each retinol and retinal isomer was quantitated by comparing its integrated area under the peak with those of known amounts of purified standards. The loss during extraction was accounted for by adjusting the recovery to that of the internal standards, either 13-cis-retinol or TMMP-ROH.

Complete digestion of Recombinant Cellular Retinol-binding Proteins—Recombinant cellular retinol-binding proteins, types I, II, and III (CRBP I, CRBP II, and CRBP III) were expressed in E. coli. For expression of CRBP II, we employed a cDNA encoding rat CRBP II (26) which was cloned in a pMON vector. The rat CRBP II cDNA was provided as a generous gift by Dr. Ellen Li of Washington University, St. Louis. The conditions we employed for induction of CRBP II expression were identical to those described previously in the literature (27). Bacteria expressing the CRBP II were pelleted by centrifugation at 6,000 × g for 15 min, and the pellet was resuspended in 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 10 mM β-mercaptoethanol, 15% glycerol, 0.05% sodium azide, and 0.05 mM phenylmethanesulfonyl fluoride. Bacteria were then sonicated and centrifuged again at 10,000 × g for 15 min, and the supernatant was used for pull-down assays as described below. Both mouse CRBP I and CRBP III clones in pET11a vector (Novagen, Madison, WI) were provided as a generous gift by Dr. Silke Vogel of Columbia University. Both clones were induced to express the proteins as described by Vogel et al. (28). Expression of the CRBP I, II, and III was verified by SD-SAGE followed by Coomassie staining of the gel.
sequence alignments of the deduced amino acid sequences for carotene 15,15'-dioxygenase for mouse, human, and chicken are shown. Conserved sequences for the three species are highlighted in black. The overall sequence identity shared among the three species is 65%.

Characterization of Carotene 15,15'-Dioxygenase Expression by RT-PCR—Total RNA (3 μg) from liver, testes, intestine, and kidney (for isolation details, see above) was first treated with DNase I to remove any genomic DNA contamination, and reverse transcription (RT) was performed using a preamplification kit (Life Technologies, Inc.) and random hexamers according to the manufacturer's recommendations. The RT reaction (2 μl) was subsequently used for the PCR. The primers used for carotene 15,15'-dioxygenase expression analysis were 5'-AGACATGGGAGGTCTTCTAC-3' (forward) and 5'-TCCGGGCTGGCAATGACTGT-3' (reverse), which should give rise to a 1,006-base pair product. PCR reactions were in a total reaction volume of 25 μl with 3 pmol of each primer, 3 mM MgCl₂, 1.5 units of Taq-polymerase (Life Technologies, Inc.). Thermal cycling parameters were 94 °C for 5 min (94 °C for 30 s, 55 °C for 30 s, 72 °C for 5 min) × 35 cycles and 72 °C for 5 min. As negative controls, PCR incubations containing no cDNA template or without reverse transcription were employed. As a positive control, CCEpcDNA3 plasmid was used. The amplicons (20 μl) were loaded onto a 1.2% agarose gel, separated by electrophoresis, and visualized on a UV-transilluminator upon staining with ethidium bromide.

In Situ Hybridization—In situ hybridization studies of expression of carotene cleavage enzyme mRNA in the embryonic mouse were performed using digoxigenin-labeled riboprobes essentially as described by Mendelsohn et al. (29). Similarly, the procedures we employed to stage the mouse embryos for their fixation and the sectioning of embryos were also described in Mendelsohn et al. (29). For antisense probes, the mouse carotene cleavage enzyme cDNA in pcDNA3 was linearized with KpnI, and antisense transcripts were generated with SP6 polymerase. For preparation of sense probes, the same cDNA was linearized with XhoI, and sense transcripts were generated with T7 polymerase.

RESULTS

Identification of a cDNA Clone for Mouse Kidney Carotene 15,15'-Dioxygenase—By homology search of the mouse expressed sequence tag data base, we identified a clone (accession number AW044715) generated from a mouse kidney cDNA library which showed 48% amino acid sequence identity to a sequence reported previously for a putative carotene 15,15'-dioxygenase from D. melanogaster (22). This cDNA clone was obtained from Research Genetics and sequenced fully in both directions. The cDNA clone appeared to represent a full-length cDNA including polyadenylation sites. An open reading frame consisting of 566 amino acids was encoded by the cDNA. The protein deduced from this sequence has a molecular mass of ~64,000. At the time we sequenced the cDNA, the mammalian protein that shared the most sequence identity with the deduced protein was the rat and human retinal pigment epithelium protein, RPE65, which showed 41% identity. Since then, annotated expressed sequence tags encoding carotene 15,15'-dioxygenases from various species have also appeared in GenBank™ (accession numbers AJ721386, chicken; AJ728064, mouse; AF294900, NM_017429, human). The primary sequence alignment of chicken (AJ721386), mouse, and human (NM_017429) carotene cleavage enzymes are shown in Fig. 1. The identity among these sequences is 65% and between mouse and human is 65%. We note a discrepancy between our cDNA sequence and the mouse sequence (AF294900) deposited in GenBank™ which would be manifested at the C terminus of the protein. Because of a one-nucleotide difference in the open reading frame toward the 3’-end of the two sequences, our deduced protein has additional 17 amino acids at the C terminus compared with the sequence deposited as AF294900. The primary sequence for mouse carotene 15,15'-dioxygenase provided in Fig. 1 reflects our sequencing results for the cDNA clone obtained from Research Genetics (clone 2192191).

Biochemical Characteristics of Recombinant Mouse Kidney Carotene 15,15'-Dioxygenase—To determine whether the mouse cDNA exhibits carotene 15,15'-dioxygenase activity, we subcloned the open reading frame of the cDNA into a bacterial expression vector, pGEX-3X. E. coli containing this expression plasmid were treated with isopropyl-β-D-thiogalactopyranoside (Roche) to induce recombinant protein expression. Homogenates prepared from induced bacterial cultures by sonication were subjected to SDS-PAGE, and a prominent protein band at ~90 kDa was observed (data not shown). This size corresponds to the expected size of bacterial GST fused to mouse carotene 15,15'-dioxygenase. Immunoblot analysis using commercial

Fig. 1. Comparison of the deduced amino acid sequences of mouse, human, and chicken carotene 15,15'-dioxygenase. Primary sequence alignments of the deduced amino acid sequences for carotene 15,15'-dioxygenase for mouse, human, and chicken are shown. Conserved sequences for the three species are highlighted in black. The overall sequence identity shared among the three species is 65%.
antiserum against bacterial GST indicated that GST was present as part of this 90 kDa band (data not shown). To identify whether the GST fusion protein possessed the ability to cleave \( \beta \)-carotene, bacterial homogenates containing the dioxygenase-GST protein or GST protein alone were incubated for 1 h with 15 \( \mu \)M \( \beta \)-carotene in our standard assay condition (see "Experimental Procedures"). After incubation, the reaction mixture was analyzed for retinoid and \( \beta \)-carotene content by normal phase HPLC. A representative HPLC chromatogram for these assays is provided in Fig. 2. A peak that coelutes with authentic all-trans-retinal was detected for the reaction mixture containing dioxygenase-GST fusion protein but not for the reaction mixture containing GST protein alone (Fig. 2). To confirm this result further, we repeated this analysis using reverse phase HPLC. As expected, we were able to identify a peak with a retention time that corresponded to that of authentic all-trans-retinal. In addition, spectral analysis of the peaks obtained for reaction mixtures that were terminated immediately after the addition of \( \beta \)-carotene, no all-trans-retinal was produced (Fig. 2). Aside from a small amount of 13-cis-retinal (always less than 10% of the amount of all-trans-retinal produced) that was probably formed through isomerization of all-trans-retinal, no other compounds were detected upon either normal or reverse phase HPLC analysis. Taken together, these data are consistent with the suggestion that the cDNA encodes a protein that catalyzes the cleavage of \( \beta \)-carotene, primarily at the central 15,15'-double bond.

Combination of \( \beta \)-carotene by the recombinant GST fusion protein was protein-dependent over the entire protein concentration range examined (0–550 \( \mu \)g of bacterial protein/assay) (Fig. 3A). Similarly, the rate of \( \beta \)-carotene cleavage was time-dependent for incubation periods ranging from 0 to 120 min (Fig. 3B). The rate of \( \beta \)-carotene cleavage to all-trans-retinal showed saturation with increasing concentrations of \( \beta \)-carotene (Fig. 3C). However, the rates of product formation were not linear with respect to either protein concentration or time, and the relationship between \( \beta \)-carotene concentration and the reaction velocity was sigmoidal in nature. Computer analysis of data relating the dependence of reaction velocity on substrate concentration best fit the Hill equation, yielding a Hill coefficient of 0.659, a \( K_{m} \) for \( \beta \)-carotene of 0.96 \( \mu \)M, and a \( V_{max} \) of 368 pmol/mg/h. Taken together, these kinetic data suggest that the carotene cleavage reaction involves complex interactions between an insoluble substrate dispersed in mixed micelles with cholate and Tween 40, carotene 15,15'-dioxygenase, and prob-
ably other proteins present within the homogenates employed for these assays.

The early literature indicates that intestinal carotene 15,15'-dioxygenase requires ferrous iron for activity and that this enzyme is extremely sensitive to the presence of metal chelating agents. Sonicates prepared from bacteria expressing mouse carotene 15,15'-dioxygenase-GST fusion protein (≈260 μg) were incubated with 15 μM β-carotene in the presence of various amounts of chelators at 37 °C for 1 h. Production of all-trans-retinal was measured by normal phase HPLC. These results are expressed as a percentage of the all-trans-retinal formed when no chelator was added to the assay mixture. □, α,α'-bipyridyl; ●, o-phenanthroline; ♦, EDTA.

**Tissue Distribution of Carotene 15,15'-Dioxygenase in the Mouse**—The expression pattern of carotene 15,15'-dioxygenase mRNA in different mouse tissues was investigated by Northern blot analysis using a full-length 32P-labeled cDNA as probe. For this purpose, total RNA was prepared from mouse small intestine, kidney, liver, heart, muscle, and testes. Surprisingly, Northern blot analysis revealed that carotene 15,15'-dioxygenase mRNA is expressed most highly in the testis followed by liver and kidney, and with a much lower level in small intestine. Also consistent with our Northern blot analysis, the testes showed two differently sized amplification products when primers spanning the 5'-portion of the open reading frame were employed for the RT-PCR analysis (data not shown), adding support to the notion that the smaller sized transcript is missing a portion of 5'-region of the cDNA.

We also investigated the expression pattern of carotene 15,15'-dioxygenase during early stages of mouse embryogenesis. For this purpose, we examined by in situ hybridization the tissue sites of expression of mRNA for carotene 15,15'-dioxygenase in mouse embryos in utero at embryonic days 7.5 (E7.5) and 8.5 (E8.5). As seen in Fig. 5B, for both E7.5 and E8.5, mRNA for this enzyme is highly expressed in maternal tissue surrounding the embryo but is not present at detectable levels in embryonic tissues.

**Partial Purification and Characterization of Carotene 15,15'-Di-oxygenase**—Because bacterial homologues containing dioxygenase-GST fusion protein possessed substantial amounts of β-carotene cleavage activity that was not present in homogenates prepared from bacteria transformed with the empty vector, we attempted to purify the mouse carotene 15,15'-dioxygenase from
several mammalian species using classic column chromatography techniques have been reported in the literature (13, 16). These reports indicate that purification of the enzyme to homogeneity could not be achieved. Because the recombinant dioxygenase-GST protein could be purified rapidly through its interaction with glutathione-Sepharose and the GST portion of the protein subsequently cleaved by factor Xa, potentially giving rise to a purified preparation of mouse carotene 15,15'-dioxygenase, we undertook such an affinity purification. When purified protein preparation was examined on SDS-PAGE followed by Coomassie staining, only the 90-kDa dioxygenase-GST fusion protein and one other 60-kDa protein were detected. However, the specific activity of this purified dioxygenase-GST fusion protein was only 30% of that observed in the unfractionated bacterial homogenate employed as the source of the fusion protein. This suggested that to maintain the activity of the dioxygenase-GST fusion protein another component(s) present in the crude homogenate is required for maintaining its optimal activity. Upon testing this possibility, we observed that the activity of the purified dioxygenase-GST fusion protein could be reconstituted through addition of either the 12,000 × g supernatant prepared from homogenates of E. coli transformed with empty vector pGEX-3X or addition of the 10,000 × g supernatant from homogenate of CHO cells transfected with empty vector pcDNA3. Because neither the bacterial supernatant nor the CHO cell supernatant possessed any detectable carotene 15,15'-dioxygenase activity, this indicated that the purified recombinant dioxygenase-GST fusion protein must be missing some essential component(s) that is needed either for catalysis of carotene cleavage or to maintain the stability of the enzyme.

To explore this observation in more detail, we investigated first whether the factor(s) present in the 12,000 × g CHO cell supernatant was dialyzable through a membrane with a molecular weight cutoff of 3,500. Even after exhaustive dialysis against PBS, addition of the dialyzed bacterial supernatant to the purified recombinant dioxygenase-GST fusion protein was still able to restore carotene cleavage activity to the purified fusion protein. Thus, it would appear that the component(s) responsible for restoring the dioxygenase activity is not a small dialyzable molecule. We next asked whether the restoration of carotene cleavage activity came about through a nonspecific process that was dependent solely on protein concentration. We did not observe any restoration of enzymatic activity when the purified fusion protein was incubated with bovine serum albumin at a protein concentration that was similar to those of the 12,000 × g supernatant used to restore activity. This suggested that a specific interaction between a component(s) in the bacterial supernatant and the CHO cell homogenates is needed to restore β-carotene cleavage activity to the recombinant dioxygenase-GST fusion protein. We asked further whether the factor(s) responsible for reactivation was heat-sensitive. When either boiled (5 min) or native CHO cell supernatant was added to the purified recombinant dioxygenase-GST preparation, only the un-denatured supernatant restored enzymatic activity. Moreover, this reactivation of the fusion protein by CHO cell supernatant was protein-dependent (Fig. 6).

The recombinant dioxygenase-GST fusion protein was stable when stored at −20 °C for over a month if kept as bacterial homogenate and in the absence of repeated freezing and thawing. Dioxygenase activity, however, decreased rapidly when 1% Triton X-100 was added to bacterial homogenates to improve the lysis of bacterial wall. For Triton X-100-containing homogenates, most of the dioxygenase activity was lost within 2–3 days even when the homogenates were kept in −20 °C.

Interactions of Recombinant Mouse Carotene 15,15'-

![Fig. 6. The activity of purified mouse carotene 15,15'-dioxygenase is restored in a protein-dependent manner upon addition of protein fraction from sham transfected CHO cells. Glutathione-Sepharose affinity-purified mouse carotene 15,15'-dioxygenase-GST fusion protein (−40 μg of total protein) was incubated with increasing amounts of the 12,000 × g supernatant protein prepared from sham transfected CHO cell homogenates, and the resulting mixture was assayed in the presence of 15 μM β-carotene for 1 h at 37 °C. The production of all-trans-retinal was monitored by normal phase HPLC at 365 nm. The 12,000 × g supernatant was added to the reaction mixture either as native protein or after 5 min of denaturation at 100 °C. All-trans-retinal production was increased upon addition of increasing amounts of native CHO cell homogenate. This effect saturated when ~280 μg (40 μl) of protein was added to the affinity-purified carotene 15,15'-dioxygenase. ○, native CHO cell homogenate; □, boiled CHO cell homogenate.](http://www.jbc.org/fig6.jpg)
proteins with the purified dioxygenase-GST fusion protein. PAGE analysis of the glutathione-Sepharose pull-down products detected between the two recombinant proteins upon SDS-PAGE. The major protein band, indicated by the arrow, pulled down upon incubation with the glutathione-Sepharose-bound dioxygenase-GST fusion protein with the testis supernatant, was analyzed by MALDI-MS. The asterisk (*) indicates the position of the carotene 15,15'-dioxygenase-GST fusion protein. Lane 1, molecular mass markers are labeled to the left in kDa. Lane 2, proteins pulled down after incubation of the glutathione-Sepharose-bound carotene 15,15'-dioxygenase-GST fusion protein with the 12,000 × g supernatant prepared from a mouse testis homogenate. The arrow points to the pulled down testis protein band taken for study by MALDI-MS. Lane 3, proteins pulled down after incubation of glutathione-Sepharose-bound GST with the same 12,000 × g testis supernatant employed in lane 2. Lane 4, proteins pulled down after incubation of glutathione-Sepharose-bound carotene 15,15'-dioxygenase-GST fusion protein with the 12,000 × g supernatant prepared from a mouse testis homogenate different from the one employed in lanes 2 and 3. The gap in the SDS-polyacrylamide gel represents the site where the testis protein was excised for MALDI-MS analysis (see Fig. 8). Lane 5, proteins pulled down after incubation of glutathione-Sepharose-bound GST with the 12,000 × g testis supernatant employed for the pull-down shown in lane 4. Lane 6, proteins pulled down after incubation of the glutathione-Sepharose-bound carotene 15,15'-dioxygenase-GST fusion protein with homogenate buffer alone. Lane 7, proteins pulled down after incubation of glutathione-Sepharose-bound GST with homogenate buffer alone.

Dedro protein-protein interactions with the purified dioxygenase-GST fusion protein. Thus, we incubated the 12,000 × g supernatant of bacteria homogenates containing recombinant rat CRBP II with purified dioxygenase-GST fusion protein bound to glutathione-Sepharose. No significant binding was detected between the two recombinant proteins upon SDS-PAGE analysis of the glutathione-Sepharose pull-down product. Similarly, we were unable to pull down either mouse CRBP I or mouse CRBP III upon incubation of these recombinant proteins with the purified dioxygenase-GST fusion protein.

DISCUSSION

Because animals are incapable of the de novo synthesis of retinoids, the first biochemical event needed to facilitate retinoid actions within the body is the enzymatic conversion of provitamin A carotenoids, like β-carotene, to retinal. Through studies carried out in the 1960s, it was established that this conversion, termed carotene cleavage, is catalyzed by the enzyme carotene 15,15'-dioxygenase (11, 32, 33). This early work demonstrated convincingly that this enzyme is a soluble ferrous iron-dependent protein that utilizes molecular O₂ as a substrate and incorporates both O₂ atoms into the product retinal (11, 13, 16, 32, 33). Carotene cleavage activity has been demonstrated in cytosol preparations from small intestine, liver, lung, and kidney for a variety of mammalian species including man, rats, hogs, guinea pigs, and rabbits (10, 14–21). Because carotene 15,15'-dioxygenase lost enzymatic activity when attempts were made to purify it from cytosol fractions, details regarding the biochemical properties of this enzyme(s) have remained elusive. The identification and cloning of a carotene cleavage enzyme from maize mutants (34) several years ago facilitated the recent cloning of a cDNA from Drosophila melanogaster that, upon expression in bacteria, catalyzed β-carotene cleavage to retinal (22). By sequence homology analysis employing the published sequence for this Drosophila cDNA, we have identified a cDNA that is expressed in adult mouse intestine, liver, kidney, and testis and maternal tissues during embryogenesis and which encodes a 64-kDa protein possessing carotene 15,15’-dioxygenase activity.

When this mouse cDNA was expressed in either bacteria or CHO cells that lack endogenous carotene cleavage activity, expression of the cDNA conferred the activity on the bacterial and CHO cell homogenates (Fig. 2). All-trans-retinal along with some 13-cis-retinal (always <10% of all-trans-retinal) were the sole products that could be detected upon incubation of all-trans-β-carotene with the recombinant protein. The 13-cis-retinal likely arises as an isomerization product of all-trans-retinal produced upon all-trans-β-carotene cleavage because the concentration of 13-cis-retinal detected was directly dependent on the amount of all-trans-retinal produced upon β-carotene cleavage. HPLC peaks suggesting the presence of apocarotenals in extracts from incubation mixtures were not observed. These data indicate that cleavage of the β-carotene occurs primarily at the central 15,15’-carbon-carbon double bond. Unexpectedly, the dependence of reaction velocity for the recombinant dioxygenase showed a sigmoidal relationship with β-carotene concentration. Our kinetic data best fit the Hill equation. Analysis of the data gave rise to a K_m of 0.95 µM, a V_\text{max} of 368 pmol/mg/h, and a Hill coefficient of 0.659. As far as we are aware, the early literature on the kinetic properties of carotene 15,15’-dioxygenase indicates that the enzyme displays a hyperbolic relationship between reaction velocity and substrate concentration, with reported apparent K_m values ranging from 0.52 to 9.5 µM and reported V_\text{max} values ranging from 23.8 to 1,300 pmol/mg protein/h (13, 35–39). We do not understand the basis for this discrepancy between our kinetic data obtained using recombinant enzyme and data obtained by early investigators employing partially purified enzyme preparations. However, in keeping with this early literature, the recombinant mouse dioxygenase was markedly inhibited by the chelating agents o-phenanthroline and α,α’-bipyridyl, but to a much lesser degree by EDTA (Fig. 4). This observation is consistent with the reported properties of partially purified intestinal carotene cleavage enzyme obtained from hog, rabbit, and guinea pig (13, 39). In this regard, the
mammalian carotene 15,15'-dioxygenase may be different from the Drosophila enzyme, which was strongly inhibited by 10 mM EDTA (22). Taken together, these data support the conclusion that we have identified a murine cDNA that encodes an enzyme that possesses catalytic properties that are very similar to those originally reported for mammalian carotene 15,15'-dioxygenase.

Our attempts to purify catalytically active recombinant mouse carotene 15,15'-dioxygenase-GST fusion protein to homogeneity from bacterial homogenates were unsuccessful. As proved to be the case for early attempts to purify carotene 15,15'-dioxygenase from tissue sources, the purified dioxygenase rapidly lost catalytic activity. However, the activity of the purified recombinant dioxygenase was quickly restored upon addition of either the 12,000 g supernatant from homogenates of bacteria transformed with empty pGEX-3X vector or the 12,000 g supernatant from sham transfected CHO cell homogenates that do not express carotene 15,15'-dioxygenase mRNA. Thus, it appears that a protein(s) present in these supernatants is responsible for this effect and that the activating factor(s) was both nondialyzable and heat-labile. It is possible that such interactions are significant for maintaining or facilitating the physiologic actions of carotene 15,15'-dioxygenase, but this remains to be established conclusively by further investigations.

Based on the observations regarding the need of the recombinant carotene cleavage enzyme for other protein factors to maintain its activity and on the kinetic data that suggest complex interactions between the β-carotene and the protein species that catalyze its cleavage to retinal, we investigated by pull-down assays employing the 12,000 g supernatant from mouse testis, the tissue with the highest level of carotene 15,15'-dioxygenase expression (see Fig. 5A), showed a major protein species that copurified with the dioxygenase-GST fusion protein bound to glutathione-Sepharose. MALDI-MS analysis of the protein excised from the SDS-PAGE gel identified the protein as an atypical testis-specific LDH-C. LDH-C was originally identified in the 1960s and demonstrated to catalyze the reversible oxidation of lactate and other secondary alcohols (45, 46). However, the physiologic role of this enzyme has often been questioned because the other widely distributed lactate dehydrogenase species (LDH-A and LDH-B) are both highly expressed in the testis (45, 46). We are unable to find in the literature any information as to whether LDH-C will catalyze either the oxidation or the reduction of aldehydes like retinal, the product of β-carotene cleavage. If LDH-C can catalyze either retinal oxidation to retinoic acid, this would suggest that carotene 15,15'-dioxygenase acts physiologically in concert with other proteins involved in retinoid metabolism. We are presently investigating this possibility.

The tissue pattern of carotene 15,15'-dioxygenase mRNA expression in the mouse is somewhat surprising (Fig. 5). This expression pattern does not overlap fully with carotene 15,15'-dioxygenase activity levels reported in early studies, which indicate that the highest specific activity level of carotene 15,15'-dioxygenase is present in small intestine followed by the liver, brain, lung, and kidney (20). As seen in Fig. 5, dioxygenase mRNA expression is relatively low in the mouse small intestine compared with testis, liver, and kidney. This may be attributed to species differences between the mouse and the rat (and other mammalian species) because the published studies of tissue activity levels have not employed mouse small intestine as a source of enzyme activity. Moreover, the literature indicates that carotene 15,15'-dioxygenase activity is limited to the proximal portion of the small intestine, including the duodenum and part of jejunum (8, 15, 40) and is present only in mature functional enterocytes within these anatomic regions (18). Because we were concerned about the possible rapid degradation of intestinal RNA, as a source of intestinal total RNA we employed the entire small intestine without first separating the mucosal lining. This may also have contributed to the apparent relatively low levels of dioxygenase mRNA in the mouse small intestine having the effect of diluting the dioxygenase mRNA in the total RNA pool. Alternatively, the enzyme encoded by the cDNA isolated from a kidney library may be
distinct from the one studied earlier in intestinal homogenates. However, this possibility seems unlikely because the biochemical characteristics of the recombinant enzyme strongly resemble those reported for carotene 15,15'-dioxygenase preparations from intestine. The relatively high level of dioxygenase mRNA expression in testes was also unexpected. There are reports in the literature that β-carotene accumulates in the testes in humans and ferrets (41, 42). It is possible that this testicular β-carotene may serve as a previously unrecognized source of retinoid for maintaining testis function.

The presence of carotene 15,15'-dioxygenase mRNA, at days 7.5 and 8.5 of mouse embryogenesis, in maternal tissue at the site of dioxygenase expression appears to coincide with the region of the uterus which may be important in nutrient exchange. Thus, the presence of carotene cleavage activity in this maternal tissue would be consistent with the notion that maternal cleavage of this enzyme and the biochemical processes that are important for carotenoid processing and retinoid formation in vivo.

In summary, we have identified a mouse kidney cDNA that encodes a 64-kDa protein that catalyzes the cleavage of β-carotene to retinal in vitro. The recombinant enzyme possesses most of the biochemical properties that have been reported for mammalian intestinal carotene 15,15'-dioxygenases. Based on our characterization, we suggest that the protein encoded by this cDNA is indeed the same enzyme that was first described in the 1960s as carotene 15,15'-dioxygenase. This identification raises new possibilities for extending our understanding of both the biochemistry of this enzyme and the biochemical processes that are important for carotenoid processing and retinoid formation in vivo.

Acknowledgments—We thank Dr. Silke Vogel (Columbia University) for the gift of the CRBP I and CRBP III expression constructs and Dr. Ellen Li (Washington University, St. Louis) for the CRBP II construct. We acknowledge gratefully the assistance of Roseann Piantedosi in preparing total RNA from mouse tissues and Ekatherina Batourina for technical help in carrying out in situ hybridizations. We thank the late Prof. James A. Olson (Iowa State University, Ames) for helpful conversations about this work. All of those who knew him will miss his collegiality and wise advice.

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Expression and Characterization of a Murine Enzyme Able to Cleave β-Carotene: THE FORMATION OF RETINOIDS
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J. Biol. Chem. 2001, 276:32160-32168. doi: 10.1074/jbc.M010086200 originally published online June 19, 2001

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