Identification and Characterization of a Mammalian Enzyme Catalyzing the Asymmetric Oxidative Cleavage of Provitamin A*

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In vertebrates, symmetric versus asymmetric cleavage of \( \beta \)-carotene in the biosynthesis of vitamin A and its derivatives has been controversially discussed. Recently we have been able to identify a cDNA encoding a metazoan \( \beta,\beta\)-carotene-15,15'-dioxygenase from the fruit fly Drosophila melanogaster. This enzyme catalyzes the key step in vitamin A biosynthesis, symmetrically cleaving \( \beta \)-carotene to give two molecules of retinal. Mutations in the corresponding gene are known to lead to a blind, vitamin A-deficient phenotype. Orthologs of this enzyme have very recently been found also in vertebrates and molecularly characterized. Here we report the identification of a cDNA from mouse encoding a second type of molecularly characterized. Here we report the identification of a cDNA from mouse encoding a second type of mammalian enzyme as the chromophores of various visual pigments (rhodopsins) throughout the animal kingdom (1, 2). In vertebrates both symmetric and asymmetric cleavage pathways exist for carotenoids, revealing a greater complexity of carotene metabolism.

Vitamin A and its analogs have a variety of physiological functions. Retinal or related compounds such as 3-hydroxyretinal serve as the chromophores of various visual pigments (rhodopsins) throughout the animal kingdom (1, 2). In vertebrates, the vitamin A derivative retinoic acid (RA)\(^1\) additionally exerts effects in development and cell differentiation by binding to specific nuclear receptors involved in the regulation of gene transcription (3–5). The key step in the formation of vitamin A is the oxidative cleavage of \( \beta \)-carotene by enzymes encoded in the genome of the animal. The properties of these enzymes have been a subject of controversy because both symmetric and asymmetric cleavage of \( \beta \)-carotene was reported in crude extracts (6–8) (Fig. 1). Thanks to its sequence similarity to a plant carotenoid-cleaving enzyme, VP14 (9-cis neoxanthin cleavage enzyme from Zea mais) (9), we have been able to clone from a metazoan (the fruit fly Drosophila melanogaster) a cDNA encoding a \( \beta,\beta\)-carotene-15,15'-dioxygenase (\( \beta\)-diox) catalyzing exclusively the symmetric cleavage of \( \beta \)-carotene to give two molecules of retinal (10). Orthologs of this enzyme have since been cloned and characterized from the chicken and the mouse by others (11, 12). The enzymes belong to a widespread and diverse class of polyene chain dioxygenases previously described in bacteria and plants (10, 13). Besides the \( \beta\)-diox, in vertebrates another putative polyene chain dioxygenase, RPE65, is found (14, 15). A function in retinoid metabolism for this protein was proposed by mutant analysis, but its biochemical function is still unknown (16).

In contrast, in Drosophila only one representative of this class of enzymes is found, and mutations in the corresponding gene (\( \text{ninaB} \)) cause a blind, vitamin A-deficient phenotype (17), demonstrating that vitamin A is formed exclusively by the symmetric cleavage of the provitamin. Besides this blindness, no other defect becomes manifest; so here vitamin A functions are restricted to the visual system. In vertebrates, vitamin A formation and metabolism are probably more complex, considering the multiple vitamin A effects in development and cell differentiation exerted by its metabolite, RA. This inference is strengthened by the fact that besides the formation of RA from retinal, as the initial product of symmetric \( \beta \)-carotene cleavage, direct formation of RA from \( \beta \)-carotene has been described in vertebrates (18). In these investigations retinal was not found to be an intermediate in RA formation, indicating that an alternative pathway for RA formation is present in vertebrates. Biochemical evidence for this alternative pathway in RA formation comes from the observation that, besides symmetric cleavage of \( \beta \)-carotene, asymmetric cleavage occurs (7, 19, 20).

\(^1\) The abbreviations used are: RA, retinoic acid; \( \beta\)-diox, \( \beta,\beta\)-carotene-15,15'-dioxygenase; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; \( \beta\)-diox-II, \( \beta,\beta\)-carotene-9,10'-dioxygenase; Tricine, \( \beta\)-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; GC, gas chromatography; APCl, atmospheric pressure chemical ionization; RPE, retinal pigment epithelium.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{TM} / EBI Data Bank with accession number(s) AJ290392, AJ290393, AJ290390, and AJ290391.

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**Fig. 1. Schematic overview of mammalian β-carotene/retinoid metabolism. Solid arrows, vitamin A formation by the symmetric cleavage pathway. The retinal formed can be further metabolized to give retinol and retinylesters (storage) or can be oxidized to give retinoic acid. Broken arrows, β-apo-carotene (8’, 10’, 12’) formation by the asymmetric cleavage of β-carotene. For retinoic acid formation the β-apo-carotenals have to be shortened by a mechanism similar to β-oxidation of fatty acids (for further details see the Introduction).**

This asymmetric cleavage leads to the formation of two molecules of β-apo-carotenal with different chain lengths. For RA formation, the β-apo-carotenal with the longer chain length must be shortened, yielding one molecule of RA. For this, a mechanism similar to the β-oxidation of fatty acids has been proposed (21). Furthermore, in vertebrates several physiological effects are caused by β-carotene (22). β-carotene itself or metabolites derived from it by alternative oxidative cleavage reactions are most likely responsible. Therefore, vertebrate β-carotene metabolism and especially RA formation could well be more complex and not just a matter of producing retinal and converting it to retinoic acid.

Taking the mouse as a model for vertebrate β-carotene metabolism, we searched EST libraries for additional putative polypeptide chain dioxygenases. Here we report on the cloning and biochemical characterization of a carotene dioxygenase catalyzing the asymmetric cleavage of β-carotene. We were able to identify the cleavage products as β-apo-10’-carotenal (C_{27}) and β-ionone (C_{13}) from β-carotene (C_{40}). In addition, this new type of metazoan polypeptide chain dioxygenase catalyzes the oxidative cleavage of lycopene, resulting in the formation of apolycopene. The existence of two different types of carotene dioxygenases in vertebrates was verified by cloning the corresponding cDNAs from man and the zebrafish. Thus, asymmetric cleavage of β-carotene exists in vertebrates and may provide a precursor for RA formation and/or may exert until now unknown physiological functions.

**EXPERIMENTAL PROCEDURES**

Sequence Comparison and Phylogenetic Tree Analysis—Vector NTI suite 6.0 (InforMax Inc., Oxford, UK) was used.

Chemicals—The following chemicals were used: β-ionone (Roth, Karlsruhe, Germany), β-apo-12’-carotene (BASF, Ludwigshafen, Germany), and β-apo-8’-carotenal (Sigma).

Preparation of Total RNA from Different Tissues of Mice—For the experiments 7-week-old BALB/c mice (male and female) were sacrificed, and different tissues (colon, small intestine, stomach, spleen, brain, liver, heart, kidney, lung, and testis) were dissected by hand and frozen immediately in liquid nitrogen. 50—100 mg of each tissue was homogenized with a pestle in a mortar with liquid nitrogen, and total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). The concentrations of the isolated total RNA were determined spectrophotometrically.

Cloning of cDNAs Encoding β-Diox-homologous Proteins from the Mouse—For cloning of full-length cDNAs encoding putative mouse β-carotene dioxygenases, RACE-PCRs were performed using a 5’/3’ RACE kit (Roche Molecular Biochemicals). Reverse transcription was carried out using 500 ng of total RNA isolated from liver, an oligod(T) anchor primer, and Superscript reverse transcriptase (Life Technologies, Inc.). For PCR the Expand PCR system (Roche Molecular Biochemicals), an anchor primer, and a specific primer and a specific primer were used (23). The following sets of primers were used: β-diox, up, 5’-ATGGAGAATATTTTGCGCAC-3’, and down, 5’-AACCTGACACCCAGATTT-3’, β-diox-II, up, 5’-ATGTTGGACGAAAGC-3’, and down, 5’-TGGCTCAGTATGATACTACC-3’. As a control for the intactness of the individual RNA samples, the mRNA of β-actin was analyzed using the following primers: up, 5’-CACAACCGTTGAAAAGATGACC-3’, down, 5’-CAGCACTGTCCTGGGATCAGG-3’.

**RESULTS**

Cloning of a cDNA Encoding a New Type of Carotene Dioxygenase—For the cloning of the cDNA encoding the new type of carotene dioxygenase, we searched mouse EST data bases and found an EST fragment (AW044715) with significant peptide sequence similarity to the RPE65 and the recently characterized β-dioxes from Dro sophila, the chicken, and the mouse (10—12). However, it was not identical with the mouse RPE65 and β-diox and thus represented a new heretofore unknown
representative of this class of polyene chain dioxygenases. To obtain a full-length cDNA, we designed upstream primers deduced from the EST fragment. Then we performed RACE-PCR on a total RNA preparation derived from the liver of a 7-week-old BALB/c male mouse. The PCR product was cloned into the vector pBAD-TOPO, and sequence analyses were carried out. The cDNA encoded a protein of 532 amino acids. Sequence comparison revealed that the deduced amino acid sequence shared 39% sequence identity with the mouse \( \beta \)-\( \beta \)-carotene-15,15'-dioxygenase (Fig. 2). Several highly conserved stretches of amino acids and six conserved histidines probably involved in binding the cofactor \( \mathrm{Fe}^{2+} \) were found (10), indicating that the encoded proteins belong to the same class of enzymes. Thus, in the mouse, besides the \( \beta \)-diox and RPE65, a third type of polyene chain dioxygenase (\( \beta \)-diox-II) exists.

The New Type of Carotene Dioxygenase Catalyzes the Asymmetric Cleavage of \( \beta \)-Carotene, Resulting in the Formation of \( \beta \)-Apo-10'-carotenal and \( \beta \)-Ionone—For functional characterization of \( \beta \)-diox-II, we expressed it as a recombinant protein in \( E. \ coli \) and performed an in vitro test for enzymatic activity under the conditions described for \( \beta \)-diox (24). HPLC analysis revealed that no retinoids were formed from \( \beta \)-carotene. However, a compound with a retention time of 4.6 min was detected (Fig. 3A). In the presence of hydroxylamine during extraction, the retention time of this compound shifted from 4.6 to 16 min, indicating that the compound has an aldehyde group from which the corresponding oxime can be formed (Fig. 3B). The increase of the putative \( \beta \)-carotene cleavage product catalyzed by the new type of \( \beta \)-carotene dioxygenase was linear up to 2 h of incubation time. The UV-visible absorbance spectra of the compounds resembled those of \( \beta \)-apocarotenals and \( \beta \)-apocarotenoloximes, respectively (Fig. 3C). However, they were not identical with \( \beta \)-apo-8'-carotenoal/oxime and \( \beta \)-apo-12'-carotenoal/oxime, as judged by comparing the spectra of reference substances in stock in our laboratory. The spectra resembled the spectra of \( \beta \)-apo-10'-carotenal (424 nm) and \( \beta \)-apo-10'-carotenoloxime (435 nm) as found in the literature (25). A definite identification of the compounds would require further investigations. However, the turnover rates and, therefore, the amounts of cleavage product formed were quite low in vitro, as already observed for the \( \beta \)-dioxes (10–12). To obtain large amounts of this substance for further chemical analysis, we decided to take advantage of an \( E. \ coli \) test system already successfully used to characterize the \( \beta \)-diox from \( Drosophila \) (10). This test system offered the advantage of combining \( \beta \)-carotene biosynthesis and further metabolism by carotene dioxygenases in one organism. In the case of retinoid formation catalyzed by \( \beta \)-diox, \( \beta \)-carotene cleavage became visible by a color shift of the bacteria. As a control we used the \( \beta \)-diox from the mouse. Whereas the \( E. \ coli \) strain expressing the \( \beta \)-diox from the mouse became white, in the \( E. \ coli \) strain expressing the \( \beta \)-diox-II no such pronounced color shift occurred (Fig. 4). However, the \( \beta \)-carotene content of the \( E. \ coli \) strain expressing the \( \beta \)-diox-II was significantly reduced compared with a control strain (22.8 pmol/mg of dry weight versus 60.9 pmol/mg of dry weight of the \( E. \ coli \) control strain). To identify the putative cleavage products, we extracted and subjected them to HPLC analyses as has been described (10). Besides \( \beta \)-carotene, six peaks were detected, which were assigned to two classes of compounds by their UV-visible absorbance spectra (Fig. 5). The first class (peaks 2, 5, and 6) showed an absorbance maximum at 424 nm, identical with the putative \( \beta \)-apo-10'-carotenoloxime already found in the in vitro tests (Figs. 3C and 5, B and C). The second class of compounds (peaks 1, 3, and 4) had an absorbance maximum at 386 nm and a UV-visible spectrum resembling that of \( \beta \)-apo-10'-carotenol (25) (Fig. 5, A and D). The occurrence of compounds with the same absorbance spectra but different retention times could be due to the stereoisomeric composition of the products formed and/or, in the case of the aldehydes, due to the syn or anti configuration of the oximes formed. Depending on the induction time, first the putative \( \beta \)-apo-10'-carotenol and then the putative \( \beta \)-apo-10'-
carotenol became detectable, indicating that the aldehyde is converted to the corresponding alcohol in *E. coli* (data not shown). The conversion of retinal to the corresponding alcohol retinol in *E. coli* was already found by expressing the \( \beta \)-diox from *Drosophila* or from the mouse as shown here (Fig. 5A). To positively identify the putative \( \beta \)-apo-10'-carotenal formed, we converted it to the corresponding \( \beta \)-apo-10'-carotenaloxime and subjected it to LC-MS analyses. Because the system was operated in the APcI mode, quasimolecular ions generally appeared as [M + H] \(^+\) signals. \( \beta \)-apo-10'-carotenaloxime was identified by its quasimolecular ion at \( m/z \) 392 [M + H] \(^+\), which is the base peak of the spectrum. The even-numbered [M + H] \(^+\) mass signal clearly proved the presence of a nitrogen in the compound and thus established the transformation of the aldehyde group into the corresponding oxime. Fragmentation of the polyene chain, yielding characteristic daughter ions, was not observed. Additionally, the characteristic UV-visible spectrum, showing maxima at 405 (shoulder), 424, and 446 nm, is in accordance with the chromophoric system of \( \beta \)-apo-10'-carotenaloxime and consistent with spectroscopic data reported previously (25).

Thus, from \( \beta \)-carotene \( \beta \)-apo-10'-carotenal is formed. However, the second compound that should result from the oxidative cleavage of \( \beta \)-carotene at the 9',10' double bond of \( \beta \)-carotene, \( \beta \)-ionone, was not detectable by HPLC. This could be explained by its volatility and/or its partitioning to the

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**Fig. 3.** Analyses of the products formed in *in vitro* tests for enzymatic activity conducted with \( \beta \)-diox-II. Crude extracts from *E. coli* expressing \( \beta \)-diox-II were incubated in the presence of \( \beta \)-carotene for 2 h. Then the compounds formed were extracted, and HPLC analyses were carried out. A, formaldehyde/chloroform extract; B, hydroxylamine/methanol extract. After extraction in the presence of formaldehyde/chloroform, a compound with a retention time of 4.6 min was detected, whereas in the presence of hydroxylamine/chloroform its retention time shifted to 16 min. C, UV-visible spectrum of peak 1. D, UV-visible spectrum of peak 2.

**Fig. 4.** Colors of \( \beta \)-carotene- and lycopene-synthesizing and -accumulating *E. coli* strains after expressing either the \( \beta \)-diox or \( \beta \)-diox-II. A, \( \beta \)-carotene-accumulating *E. coli* control strain; B, \( \beta \)-carotene-accumulating strain expressing \( \beta \)-diox from the mouse; C, \( \beta \)-carotene-accumulating strain expressing \( \beta \)-diox-II; D, lycopene-accumulating strain expressing \( \beta \)-diox-II; E, lycopene-accumulating control strain.

**Fig. 5.** HPLC analyses of the \( \beta \)-carotene cleavage products formed in \( \beta \)-carotene-producing and -accumulating *E. coli* expressing \( \beta \)-diox or \( \beta \)-diox-II. Bacteria pellets were extracted with the hydroxylamine/methanol method as has been described (10). A, HPLC profile at 360 nm of an extract derived from an *E. coli* strain expressing \( \beta \)-diox (upper trace) from the mouse compared with a control extract (lower trace). The composition of the retinoids found is indicated in the figure. B, HPLC profile at 400 nm of an extract derived from an *E. coli* strain expressing \( \beta \)-diox-II (upper trace) from the mouse compared with a control extract (lower trace). Six substances were detected and assigned to two different classes of compounds, \( \beta \)-apo-10'-carotenoloxime (C) (peaks 2, 5, and 6) and \( \beta \)-apo-10'-carotenol (D) (peaks 1, 3, and 4) based on their UV-visible spectra and LC-MS analysis.
medium. Therefore, we analyzed the bacterial growth medium after solid phase extraction of lipophilic compounds by GC-MS. In the medium of the E. coli strain expressing β-diox-II, significant amounts of β-ionone (identical in its retention time and mass spectra to a β-ionone standard) was detected, and these were not found in the medium of the E. coli control strain. Taken together, the analyses demonstrated that β-diox-II catalyzes the asymmetric cleavage of β-carotene at the 9\(\sim\)10 carbon double bond, resulting in the formation of β-apo-10-carotenal and β-ionone. Therefore, we have termed this enzyme β,β-carotene-9\(\sim\)10′-dioxygenase.

To test whether the enzyme catalyzes the oxidative cleavage of carotenoids different from β-carotene, we transformed it into an E. coli strain able to synthesize and accumulate lycopene (Fig. 4). The experiment was performed as described above. In this strain significant amounts of putative apolycopenals became detectable. This was shown by converting the aldehydes to the corresponding oximes (data not shown). Therefore, the new type of carotene dioxygenase catalyzes the oxidative cleavage of lycopene in the E. coli test system as well, resulting in the formation of apolycopenals tentatively identified by their UV-visible spectra.

**Cloning of cDNAs Encoding the New Type of Carotene Dioxygenase from Man and the Zebrafish**—To verify the existence of the second type of dioxygenase, β-diox-II, in other metazoan organisms, we searched for EST fragments with sequence identity in the data base. We found EST fragments from man and the zebrafish. Then we cloned and sequenced the corresponding full-length cDNAs. The cDNA cloned from total RNA derived from human liver encoded a protein of 556 amino acids, whereas the cDNA isolated from the zebrafish encoded a protein of 549 amino acids. The deduced amino acid sequences shared 72 and 49% sequence identity, respectively, to the protein of 549 amino acids. The phylogenetic tree calculation based on a sequence distance method and utilized the neighbor-joining algorithm (26) with the deduced amino acid sequences of metazoan polyene chain dioxygenases and the plant VP14. As shown in Fig. 6, in vertebrates three groups of polyene chain dioxygenases are found: the two different types of vertebrate carotene dioxygenases and RPE65. The sequence analysis revealed that the three vertebrate polyene chain dioxygenases most likely emerged from a common ancestor. In contrast, in Drosophila and Caenorhabditis elegans, only one type of dioxygenase was found in the entire genome. As judged by the E. coli test system, the C. elegans dioxygenase catalyzes the symmetric cleavage of β-carotene to form retinal. Therefore, the occurrence of additional genes encoding this class of enzymes, the β-diox-II and the RPE65, is apparently related to vertebrate carotene/retinoid metabolism.

**Tissue-specific Expression of the New Type of Carotene Dioxygenase**—We analyzed total RNA from several tissues of 7-week-old BALB/c mice (male and female) and estimated the steady-state mRNA levels of the two types of carotene dioxygenases by RT-PCR analyses. RT-PCR products of both types of carotene dioxygenase mRNAs became detectable in small intestine, liver, kidney, and testis, whereas low abundance steady-state mRNA of the new type of carotene dioxygenase was additionally present in spleen, brain, lung, and heart (Fig. 7). The intactness of the RNA preparations was verified by analyzing the β-actin mRNA. By omitting the reverse transcriptase in the assays, it was shown that the RT-PCR products derived from mRNA and not from DNA contaminations. By using a multiple tissue mRNA blot (CLONTECH), analyzed with a riboprobe of the human cDNA, we were able to find a 2.2-kilobase pair message in heart and liver for the new type of carotene dioxygenase, whereas a transcript of 2.4 kilobase pairs for the β-diox was found mainly in kidney (data not shown).

**DISCUSSION**

Here, we report on the cloning, characterization, and tissue-specific expression of a new type of carotene dioxygenase from mouse catalyzing the asymmetric cleavage of β-carotene. By expressing the enzyme in a β-carotene-synthesizing E. coli
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strain, β-apocarotenal formation at the expense of β-carotene was shown. The cleavage products formed were identified by their absorbance spectra, by the conversion of the aldehyde to the corresponding oxime, and by LC-MS or GC-MS as being β-apo-10'-carotenal and β-ionone. In vitro, the enzyme catalyzed the same reaction as in the E. coli test system. Thus, the characterized enzyme catalyzed the oxidative cleavage at the 9'-10' double bond in the polyene backbone of its substrate, β-carotene.

Besides the overall sequence identity to the β-diox previously described (10–12), there is a distinct conserved pattern of histidine residues, which has been proposed to be involved in the binding of the cofactor Fe²⁺ (12). Thus, including RPE65, three different representatives of the polyene chain dioxygenase family are found in vertebrates. Although the biochemical function of the RPE65 protein remains to be elucidated, we show that besides symmetrical cleavage of β-carotene, asymmetric cleavage also occurs, positively resolving the controversial debate on the significance of this reaction. The analysis of the tissuespecific expression showed that mRNAs for both enzymes are found together in several tissues, e.g. small intestine and liver. These findings verify biochemical results on the molecular level that both symmetric and asymmetric cleavage of β-carotene can be found in the same tissue. The expression patterns in the mouse and man were not consistent. This could be either because of interspecies differences in carotene metabolism or reflect differences in the age and nutritional status of the individuals investigated, thus possibly presenting an additional factor to explain the conflicting results obtained in several investigations. In earlier studies conducted with tissue homogenates, a variety of β-apocarotenals of different chain length resulting from asymmetric β-carotene cleavage were found. Therefore, the term random cleavage was used for this reaction by several authors. Here we show that the characterized enzyme does not catalyze such side reactions; instead it is specific for the 9'-10' double bond. The formation of β-apocarotenals different from β-apo-10'-carotenal found in vitro may be caused by further metabolism of the primary cleavage product or by additional, yet unknown, carotene dioxygenases. However, the in vitro activity of the metazoan polyene chain dioxygenases is difficult to obtain, and β-apocarotenal formation from β-carotene by enzymatic degradation has been reported in an aqueous environment (27).

After the molecular identification of a cDNA encoding this new type of carotene dioxygenase, the question arose as to the physiological relevance in vertebrate carotene metabolism. Sharma et al. (28) showed in rats and chickens that β-apocarotenals can be bioactive precursors for RA formation. After absorption of these compounds, the corresponding acid is first formed and then shortened to yield retinoic acid. The same study also showed that only small proportions of β-apocarotenals are attacked by the β-diox to give retinal. This possibility could be of importance considering the co-expression of both dioxygenases in several tissues shown here. Napoli and Race (18) showed that several tissues are able to synthesize RA and that retinal, the primary product of the symmetric cleavage of β-carotene, was not detected as an intermediate. By analyzing RA formation from β-apocarotenals, a mechanism similar to β-oxidation of fatty acids was proposed by Wang et al. (21). In their study, RA formation from β-apocarotenals was ensured by giving citral, a potent inhibitor of retinalaldehyde dehydrogenases that catalyze the oxidation of retinal to RA. Therefore, the asymmetric cleavage reaction most likely represents the first step in an alternative pathway in the formation of RA and may contribute to RA homeostasis of the body, certain tissues, or cells. The second product resulting from asymmetric cleavage, β-ionone, is known as a scent compound in plants. This short chain compound is volatile, and a putative physiological role in animals remains to be investigated.

In Drosophila vitamin A is exclusively formed by the symmetric cleavage reaction (17). In vertebrates the two different carotene dioxygenases and the RPE65 protein are found. Sequence comparison indicated that the vertebrate dioxygenases arose from a common ancestor. In contrast to Drosophila, in vertebrates RA plays an important role in development and cell differentiation. Thus, the existence of different β-carotene dioxygenases could be related to the emergence of RA effects. By in situ hybridization in zebrafish embryos, high steady-state mRNA levels of the zebrafish homologue of the β-diox were found before gastrulation. The zebrafish homologue to the β-carotene-9',10'-dioxygenase could only be detected after organogenesis. The finding of high steady-state mRNA levels of the β-diox at early times in development has been reported recently for the mouse (12). This indicates that retinoid formation from β-carotene catalyzed by the symmetric oxidative cleavage reaction may contribute to the retinoid homeostasis of the embryo. Therefore, besides maternal, preformed vitamin A, de novo biosynthesis from the provitamin seems to be an important source for retinoids during development. However, the asymmetric cleavage reaction may contribute to RA formation in certain tissues during later stages of development. In this context, the observed expression of the β-diox-II in brain and lung could be of relevance. In cell differentiation processes in the nervous system, RA plays an important role (29). In a ferret model, under certain conditions such as exposure to cigarette smoke, β-carotene toxicity on lung has been reported (22).

Asymmetric cleavage of β-carotene was discussed as being involved in these toxic effects (for review see Ref. 30). Furthermore, RA formation from β-carotene has been found in vitro in the testis, small intestine, liver, and kidney (18). Here, we show that in all these tissues mRNAs encoding the two different types of carotene dioxygenases are found. This indicates that besides the small intestine and liver, several tissues may contribute to their own RA homeostasis by endogenous retinoid formation from β-carotene, until now an underestimated, unappreciated feature in retinoid homeostasis.

As judged in an E. coli test system, the enzyme was also able to catalyze the oxidative cleavage of lycopene. This indicates with respect to substrate specificity that the polyene chain backbone of carotenes plays an important role, whereas the ionone ring structures of β-carotene seem to be of marginal relevance. This result was also obtained upon analyzing the mouse β-diox (12). Favorable effects of lycopene on human health have been reported (31). Lycopene is accumulated primarily in liver but also in intestine, prostate, and testis, tissues in which both β-diox and β-diox-II mRNAs are expressed. The cleavage of lycopene and the formation of apolycoprenals are indicative of a putative role in vertebrate physiology. In vertebrates, several nuclear receptors with unknown ligands exist, i.e. orphan receptors. Besides being a putative precursor for RA formation in the case of β-carotene cleavage, the compounds formed by the asymmetric cleavage reaction of β-carotene and/or lycopene may represent putative ligands for these receptors.

Taken together, the data presented here led to the molecular identification of an enzyme, ββ-β-carotene-9',10'-dioxygenase, catalyzing the asymmetric cleavage of β-carotene. Thus, besides the symmetric cleavage of β-carotene, a second enzymatic activity is present in vertebrates. The molecular identification of enzymes involved in the cleavage of β-carotene will open new

2 J. M. Lampert and J. v. Lintig, unpublished results.
avenues of research on the impact of metabolites derived from carotenoids in animal physiology and human health.

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REFERENCES
1. Wald, G. (1968) Nature 219, 800–807
2. Vogt, K. (1984) Z. Naturforsch. 39, 196–197
3. Dowling, J. E., and Wald, G. (1960) Proc. Natl. Acad. Sci. U. S. A. 46, 587–608
4. Giguere, V., Ong, E. S., Segui, P., and Evans, R. M. (1987) Nature 330, 624–629
5. Chambon, P. (1996) FASEB J. 10, 940–954
6. Goodman, D. S., and Huang, H. S. (1965) Science 149, 879–880
7. Glover, J. (1960) Vitam. Horm. 18, 371–386
8. Wolf, G. (1995) Nutr. Rev. 53, 134–137
9. von Lintig, J., Welsch, R., Bonk, M., Giuliano, G., Batschauer, A., and Kleinig, H. (1997) Plant J. 12, 625–634
10. Wyss, A., Wirtz, G., Woggon, W., Brugger, R., Wyss, M., Friedlein, A., and Hunkeler, W. (2000) Biochem. Biophys. Res. Commun. 271, 334–336
11. Redmond, T. M., Gentleman, S., Duncan, T., Yu, S., Wiggert, B., Gant, E., and Cunningham, F. X., Jr. (2001) J. Biol. Chem. 276, 6560–6565
12. Tan, B. C., Schwartz, S. H., Zeevaart, J. A. D., and McCarty, D. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12235–12240
13. Bavik, C. O., Eriksson, U., Allen, R. A., and Peterson, P. A. (1991) J. Biol. Chem. 266, 14978–14985
14. Hamel, C. P., Tailou, E., Pfeffer, B. A., Hooks, J. J., Detrick, B., and Redmond, T. M. (1999) J. Biol. Chem. 274, 15751–15757
15. von Lintig, J., Dreher, A., Kiefer, C., Wernet, M. F., and Vogt, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1130–1135
16. Napoli, J. L., and Race, K. R. (1988) J. Biol. Chem. 263, 17372–17377
17. Wang, X. D., Tang, G. W., Fox, J. G., Krinsky, N. I., and Russell, R. M. (1991) Arch. Biochem. Biophys. 285, 6–16
18. Tang, G. W., Wang, X. D., Russell, R. M., and Krinsky, N. I. (1991) Biochemistry 30, 9829–9834
19. Wang, X. D., Russell, R. M., Liu, C., Stickel, F., Smith, D. E., and Krinsky, N. I. (1996) J. Biol. Chem. 271, 26490–26498
20. Wang, X. D., Liu, C., Bronson, R. T., Smith, D. E., Krinsky, N. I., and Russel, R. M. (1999) J. Natl. Cancer Inst. 91, 60–68
21. Henry, L. K., Puspitasari-Nienaber, N. L., van Breemen, R. B., Catignani, G. L., and Schwartz, S. J. (2000) J. Agric. Food Chem. 48, 5008–5013
22. Sharma, R. V., Mathur, S. N., and Ganguly, J. (1976) Biochem. J. 158, 377–383
23. McCaffery, P., and Drager, U. C. (2000) Cytokine Growth Factor Rev. 11, 233–249
24. Barua, A. B., and Olson, J. A. (2000) J. Nutr. 130, 1996–2001
25. Henry, L. K., and Rasmussen, N. L. (1999) J. Nutr. 129, 1345–1350
26. Russell, R. M. (2000) Am. J. Clin. Nutr. 71, 878–884
27. Clinton, S. K. (1998) Nutr. Rev. 56, 35–51