The Carotenase AtCCD1 from Arabidopsis thaliana Is a Dioxygenase*

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Apocarotenoids resulting from the oxidative cleavage of carotenoids serve as important signaling and accessory molecules in a variety of biological processes. The enzymes catalyzing these reactions are referred to as carotenases or carotenoid oxygenases. Whether they act according to a monooxygenase mechanism, requiring two oxygens from different sources, or a dioxygenase mechanism is still a topic of controversy. In this study, we utilized the readily available β-apo-8’-carotenal as a substrate for the heterologously expressed AtCCD1 protein from Arabidopsis thaliana to investigate the oxidative cleavage mechanism of the 9,10 double bond of carotenoids. β-Ionone and a C17 dialdehyde were detected as products by gas and liquid chromatography-mass spectrometry as well as NMR analysis. Labeling experiments using H2^{18}O or H^{18}O showed that the oxygen in the keto-group of β-ionone is derived solely from molecular oxygen. When experiments were performed in an H^{18}O_2-enriched atmosphere, a substantial fraction of the C17-dialdehyde contained labeled oxygen. The results unambiguously demonstrate a dioxygenase mechanism for the carotenase AtCCD1 from A. thaliana.

Carotenases or carotenoid oxygenases are non–heme iron oxygenases that cleave carotenoids to apocarotenoids (1–3). These substances are widely distributed in nature and occupy important metabolic and hormonal functions in procaryotes, animals, fungi, and green algae (4–7). Also, in higher plants carotenoid cleavage products serve as signals. Apocarotenoids result from the oxidative cleavage of carotenoids to apocarotenoids (1–3). These substances are non–heme iron oxygenases that cleave carotenoids to apocarotenoids (1–3). These substances are widely distributed in nature and occupy important metabolic and hormonal functions in procaryotes, animals, fungi, and green algae (4–7). Also, in higher plants carotenoid cleavage products serve as signals.

Because the protein showed significant sequence similarity to a bacterial dioxygenase preparation obtained from a Microcystis strain (25). This carotenase cleaves β-carote ne or zeaxanthin at the 7,8 and 7,9’ bonds to form β-cyclocitral (or hydroxy-β-cyclocitral in the case of zeaxanthin) and crocetinal. When β-carote ne cleavage was carried out with this enzyme in an H^{18}O atmosphere, 86% of the β-cyclocitral carried the heavy oxygen isotope. The results strongly pointed toward a dioxygenase mechanism for carotenoid cleavage, but the interpretation of these results was complicated by the fact that the workup of the other reaction products (all aldehydes) had to be performed in an aqueous milieu. Because aldehydes readily exchange their carbonyl oxygen with the oxygen atom of water, this may lead to the loss of an eventual oxygen label. Similar problems were encountered in labeling studies on abscisic acid biosynthesis (26–28). The observed patterns of isotopeologues in these studies and later reports on the precursors of ABA (29) could only be satisfactorily explained with a dioxygenase mechanism for carotenoid cleavage by VP14.

The work on VP14 opened the way for the discovery of a vast number of carotenase genes. Their corresponding proteins cleave the carotenoid backbone at different positions (1–3, 6). One of the first of these was AtCCD1 from Arabidopsis thaliana. It cleaves a variety of carotenoids symmetrically at the 9,10 and/or 9,10’ double bond to form a (di-)aldehyde and one or two C13 products, depending on the carotenoid substrate. Similar enzymes have been identified in a number of other plants (18–21), and to distinguish them from the nine-cis-epoxy-carotenoid dioxygenases within the carotenase family, they were called carotenoid cleavage dioxygenases (CCDs) (18).

The available sequence information on VP14 led to the discovery of carotenases in animals as well. Successful cloning and characterization of a vertebrate enzyme catalyzing the symmetric oxidative cleavage of β-carotene to retinal filled the gap in vitamin A research (22). Being of immense medical interest, the biosynthesis of vitamin A had already been examined in the late 1960s in animal models. In these isotope labeling studies, it turned out that the oxygen in retinol is derived solely from molecular oxygen. As retinol (i.e. vitamin A) is formed in vivo by the immediate reduction of retinal, these results were pointing toward a dioxygenase mechanism for carotenoid cleavage (23).

Recently, the crystal structure of a bacterial retinal-forming enzyme using different apocarotenoids as substrates was established (30). On the basis of this structure, the authors have proposed a monooxygenase-like mechanism for this carotenase, although experimental evidence has not been provided.

With this controversy in mind, we intended to study the oxygen usage of the β-ionone-forming carotenase AtCCD1. Because β-ionone is a
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ketone, it should exchange its carbonyl oxygen with the oxygen of water only at a very low rate (31). We suspected this fact would facilitate the interpretation of the data and help to clarify whether carotenases act according to a monooxygenase or dioxygenase mechanism.

EXPERIMENTAL PROCEDURES

Cloning of AtCCD1 and Expression of the Protein—AtCCD1 from A. thaliana was amplified as described by Schwartz et al. (18) and cloned in the BamHI/EcoRI sites of pGEX-4T-1 (Amersham Biosciences). The construct was transformed and maintained in Escherichia coli JM109 cells (Promega). After sequencing, the plasmid was transformed into an appropriate host for protein expression. Unfortunately, the recombinant AtCCD1 protein formed inclusion bodies and could not be purified to reasonable amounts according to standard techniques. Therefore, we used 4 ml of an E. coli BL21 overnight culture containing the appropriate antibiotics to inoculate 400 ml of LB medium containing 50 µg/ml ampicillin. Cultures were incubated (20 °C, 180 rpm) for 24 h. Uninduced cells were harvested by centrifugation and resuspended in 10 ml of phosphate-buffered saline (32) containing 5 mM sodium ascorbate. Cells were lysed by sonification on ice with a MS 73 sonotrode (Bandelin Electronic, Berlin) three times for 30 s at 10% maximal power. Cell debris was removed by centrifugation (5000 × g, 20 min, 4 °C). The supernatant was frozen at −80 °C until use. BL21 cells containing the empty pGEX-4T-1 vector were treated in the same manner, and the respective extract was used as control in all experiments.

In Vitro Cleavage of β-Apo-8-carotenal and Isotope Labeling—Five hundred µl of ethanol containing 50 µg of β-apo-8-carotenal (Sigma-Aldrich) and 250 µl of an ethanolic β-octylglucoside solution (4% w/v) were combined and evaporated to dryness. One milliliter of the crude cell extract described above was added. The solution was shaken vigorously and incubated at 30 °C for 30 min. Reactions were stopped by the addition of an equal volume of methanol and analyzed by LC-MS or extracted with diethyl ether and analyzed by GC-MS.

For labeling experiments with H2 18O, freeze-dried cell extracts and residues were resuspended in the initial volume of H2 18O. Assays were carried out as described above. Labeling experiments with 18O2 were performed in 8-ml screw-capped glass vessels with a gas-tight Teflon septum. After preparation of the micelles, cell extract was added, and the assay was saturated with 18O2 by aerating the solution on ice for 5 min. The sample was vortexed and incubated as described above.

For the analysis of the C17-dialdehyde, four standard assays were combined and extracted three times with equal volumes of diethyl ether. The combined organic phase was dried with sodium sulfate, evaporated to dryness, and redissolved in 300 µl of methanol. For time course studies, methanol-stopped assays were extracted after defined intervals with an equal volume of chloroform. The extracts were immediately dried with sodium sulfate and stored at −20 °C to prevent oxygen exchange of the dialdehyde. Shortly before analysis the chloroform extracts were transferred to HPLC vials, evaporated to dryness, redissolved in 200 µl of methanol, and subjected to LC-MS analysis.

Preparative Isolation of the C17-Dialdehyde by Thin Layer Chromatography (TLC)—A total of eight standard assays were combined and extracted three times with equal volumes of diethyl ether. The organic phase was concentrated to 2 ml and applied to two silica TLC plates (20 × 20 cm, Nano-SIL-20 UV254, Carl Roth, Karlsruhe, Germany). The plates were developed in n-pentane:ethyl acetate (5:3; v/v). Relevant bands were scraped off and eluted three times with 4 ml of acetone. The fractions were concentrated to 1 ml and applied to TLC plates (20 × 20 cm, Nano-SIL-20 UV254), which were developed this time with n-pentane:diethyl ether (5:3; v/v). Bands were scraped off and eluted as described above. The eluate was evaporated to dryness and redissolved in 750 µl of d6-acetone. This solution contained about 500 µg of the compound. Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich.

NMR Studies—NMR spectra were recorded at 25 °C using an AVANCE 500 spectrometer (Bruker Instruments, Karlsruhe, Germany) at transmitter frequencies of 500.1 and 125.6 MHz for 1H and 13C, respectively. Two-dimensional COSY experiments were performed using standard Bruker software (XWINNMR).

GC-MS Analysis—GC analyses were performed with a Thermo Finnigan Trace DSQ mass spectrometer coupled to a Thermo Finnigan Trace GC with a split injector (1:20) and a 0.25-µm BPX5 20M fused silica capillary column with a 30 m × 0.25 mm inner diameter. The temperature was held at 40 °C for 3 min and then increased to 250 °C at 5 °C/min intervals, with a helium flow rate of 3 ml/min. The electron ionization-MS voltage was 70 eV, and the ion source and interface temperature were both 250 °C. Spectra were recorded and evaluated with Xcalibur software (version 1.4) supplied with the device.

LC-MS Analysis—The HPLC system consisted of a quaternary pump and a variable wavelength detector, all from Agilent 1100 (Bruker Daltonics, Bremen, Germany). The column was a LUNA C18 (2) 100A 150 × 2 mm (Phenomenex, Aschaffenburg, Germany). Coupled to the HPLC was a Bruker esquire 3000plus mass spectrometer with an electrospray ionization interface that was used to record the mass spectra. The ionization voltage of the capillary was 4000 V, and the end plate was set to −500 V. The capillary exit was 106 V, and the octopole radiofrequency amplitude was 112.2 vpp. The temperature of the dry gas (N2) was 300 °C, flowing at 9 ml/min. The full scan mass spectra were measured from m/z 50 to 600 until the ion charge control target reached 20,000 or 200 ms, which ever came first. Tandem mass spectrometry was performed using helium as the collision gas, with collision energy set to 1.20 V. All mass spectra were acquired in the positive ionization mode. The LC parameters went from 100% water (with 0.1% formic acid) to 50% acetonitrile (with 0.1% formic acid)/50% acidic water in 20 min, then in 5 min to 100% acetonitrile, and in 10 min to 70% acetonitrile/30% 2-propanol for 10 min. The concentration was then changed in 3 min back to 100% acetonitrile and in 5 min back to 100% water for 7 min. The detection wavelength was 285 nm.

RESULTS

Cleavage of β-Apo-8-carotenal by AtCCD1 and Characterization of the Products—Experiments revealed that β-apo-8-carotenal is a much better substrate for AtCCD1 than β-carotene or zeaxanthin. The color of the assays changed to yellow within 30 min of incubation, whereas controls (i.e. extracts from BL21 carrying the empty vector) retained...
their original red-orange color. The volatile compound β-ionone, one of the two products of the enzymatic reaction (Fig. 1), was identified by GC-MS and LC-MS analysis by comparison with authentic standards. Its yield was 12–16-fold greater than that of β-carotene experiments using β-carotene at the same molar concentration as substrate. Assays with zeaxanthin or β-carotene always retained their original color (not shown).

In LC-MS analysis of the β-apo-8′-carotenal experiments an UV-absorbing component lacking in the controls appeared at 30.3 min. A pseudomolecular mass [M+H]+ of m/z 257 could be assigned to the unknown compound. When reaction mixtures were extracted with diethyl ether and separated by thin layer chromatography, a prominent bright yellow band lacking in the controls became visible. The enzymatically formed substance corresponded to the newly detected component with a molecular weight of 256.

For NMR analysis an amount of ~0.5 mg of the unknown compound was purified by TLC. The 1H NMR spectrum displayed a doublet at 9.63 ppm with a coupling constant of 7.7 Hz and a singlet signal at 9.50 ppm, respectively. The relative intensities of the two signals were identical. On the basis of this chemical shift region, both signals were tentatively assigned to aldehyde hydrogen atoms. In a two-dimensional COSY experiment, the doublet signal gave a cross-peak to a doublet doublet (coupling constants, 15.3 and 7.7 Hz) at 6.22 ppm. The same doublet was correlated to a doublet (coupling constant, 15.3 Hz) at 7.37 ppm. This pattern suggested a CHO-CH=CH-X spin system with an CH-X spin system with an CH-CH=CH-CH=CH-X spin systems. Signals for methyl atoms could not be detected directly because of the presence of intense signals in the aliphatic region of the spectrum, probably caused by alkane impurities that were coeluted with the putative dialdehyde compound. Taken together with the mass spectrum (Fig. 2), the NMR data were in line with the structure of the C17-dialdehyde as shown in Fig. 1.

The Origin of the Oxygen in the Newly Arisen Carbonyl Groups—For labeling studies with H218O, we freeze-dried the crude enzyme preparation and resuspended the residue in H218O. The mass spectrum of β-ionone formed under this condition did not differ significantly from that of the standard, indicating that the oxygen atom in the keto function of β-ionone did not originate from water. However, product analysis of a reaction performed in an 18O2 atmosphere using GC-MS and LC-MS revealed that the formed β-ionone was almost completely labeled with the heavy oxygen isotope (Fig. 3). Mass spectral analysis of the C17-dialdehyde was only performed by LC-MS because the underivatizated compound turned out to be unamenable to GC-MS analysis. When the enzymatic reaction was performed in H218O, the LC-MS analysis of the dialdehyde product revealed the presence of three isotopologues containing no, one, or two heavy oxygen isotopes in a ratio of ~0.54:0.35:0.11 and 0.32:0.51:0.17 after a 3-h incubation at room temperature (ratio of the integrals of the extracted ion chromatograms [M+H]+ of m/z 257:259:261), pointing to the chemical exchange of the carbonyl oxygen with the oxygen from water. When the cleavage reaction was carried out in an 18O2 atmosphere the isotope containing two 18O atoms was not detectable, and a considerable (27%) portion of the C17-dialdehyde contained one 18O atom (Fig. 4). To estimate the oxygen exchange rates during the catalysis in the buffer medium, in another experiment the reaction was stopped with methanol, and the isotopologue ratio was determined by LC-MS after defined intervals. The ratio changed from 27% at t = 0 min to 17% at t = 20 min and to 14% at t = 40 min (ratio of labeled to the total C17-dialdehyde, i.e. integrals of [M+H]+ of m/z 259 divided by those of 259 + 257). This further dem-
onstrated the high rate of oxygen exchange between water and the dialdehyde.

DISCUSSION

β-Apo-8′-carotenal appears to be a better substrate for AtCCD1 than the described carotenoids β-carotene and zeaxanthin in vitro. We suspect that this is because of its higher solubility in water, which presumably makes it more easily accessible for the non-membrane-bound AtCCD1. A similar observation was described in the work on AtCCD1; xanthophylls, i.e. oxygen-containing carotenoids, appeared to be better substrates for AtCCD1 than β-carotene itself (18).

DIOX1, the retinal-producing enzyme from Synechocystis sp. PCC 6803, also accepts apo-carotenals (together with their corresponding alcohols) as well as apo-lycopenals (33), albeit at very different velocities. The molecular basis for this finding was elucidated by the published structure of the enzyme. The crystal structure shows four essential histidines, involved in the coordination of the iron cofactor (30). It is assumed that the active site as shown for the apocarotenoid-15,15′-oxygenase from Synechocystis sp. PCC 6803 is similar in all carotenases described thus far. The importance of each of the four conserved histidines in the active center has been demonstrated for the retinal-forming 15,15′-carotene-2-oxogenase of mice (34). Consequently, the actual cleavage mechanism is considered to be similar in all carotenases.

Based on sequence similarity to lignostilbene dioxygenases from Pseudomonas paucimobilis, the first described carotenase VP14 was referred to as dioxygenase from the work published by Schwartz et al. (15), and related enzymes identified later were assigned accordingly (35, 36). For the mammalian retinal-forming enzyme, which displays significant sequence similarity to VP14, this mechanism has been a subject of controversy. In the late 1960s, experiments with 18O2 showed that dioxygen, not water, donates the oxygen atom that is incorporated into the terminal alcohol group of retinol, the reduced metabolite of retinal in vivo, evidence that strongly supports the dioxygenase reaction mechanism (23).

In contrast, recent isotope labeling studies provide evidence that β-carotene-15,15′-oxygenase catalyzes the oxidative cleavage by a monooxygenase rather than a dioxygenase mechanism (24). However, During and Harrison (37) have criticized this study. Among other points, the rather long incubation period for an enzymatic reaction of 7.5 h is mentioned. In addition, the horse liver alcohol dehydrogenase used for the in situ reduction of retinal to retinol by Leuenberger et al. (24) is described to display dismutase activity. This occurs especially with increased levels of NAD+ and may lead to the incorporation of a water-derived oxygen atom into retinol. Accordingly, over long incubation times this may falsify the observed ratio of isotopologues (38, 39).

The most convincing evidence for a dioxygenase mechanism of carotenoid cleavage was provided by Jütterner and Hoflacher (25), who performed isotope labeling experiments with a crude carotenase preparation of a cyanobacterium. The observation that 86% of the β-cyclocitrinal was labeled when cleavage was carried out in an 18O2 atmosphere strongly pointed toward a dioxygenase mechanism. In this study (25), GC-MS analysis of β-cyclocitrinal was performed from the head space of the gaseous phase, circumventing the problem of oxygen exchange of the carbinyl group with water.

Here, we observed that virtually all β-ionone (96%) molecules produced by AtCCD1 carried labeled oxygen when the assays were performed in an 18O2 atmosphere, denoting that β-ionone is formed either by a monooxygenase-catalyzed reaction followed by a subsequent regioselective hydrolysis of an epoxycarbinyl intermediate or by a dioxygenase reaction (37) (Fig. 5A).

Additional support for a dioxygenase reaction mechanism was provided by LC-MS analysis of labeled assays carried out in an 18O2 atmosphere. A substantial fraction (27%) of the C15-dialdehyde, one of two products of the recombinant AtCCD1, carried an oxygen atom delivered by molecular oxygen. It should be noted that a considerable fraction of 18O might has been lost, because the dialdehyde oxygens exchange readily with those of water during enzymatic conversion, as illustrated in Fig. 5B. Our time studies confirmed this loss of the label. We observed the decline of labeled dialdehyde from 27 to 14% of the total within 40 min of incubation of the stopped reactions. Additionally,
FIGURE 4. Incorporation of molecular oxygen into the C_{17}-dialdehyde revealed by LC-MS analysis. Mass spectra of the C_{17}-dialdehyde formed by AtCCD1 from β-apo-8'-carotenal under normal conditions (upper spectrum) and in an ¹⁸O₂ atmosphere (lower spectrum) are shown. The ratio of the relative abundance of labeled (m/z = 259) to unlabeled (m/z = 257) mass peaks in the lower spectrum is 36:100.
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FIGURE 5. Proposed reaction mechanisms for the enzymatic cleavage of carotenoids and oxygen exchange of carbonyl groups with water. A, outline of the two postulated mechanisms. B, oxygen exchange of the reaction products with water. Bold, ^18O-oxygen atom originating from the gaseous phase. Gray, oxygen atom originating either from water or from the gaseous phase.

during LC-MS analysis an extra portion of the label might get lost, as the dialdehyde was detected after ~30 min of LC. This is a significant period for the observed oxygen exchange, and the low pH common throughout chromatography is known to accelerate the phenomenon (31, 40).

Oxygen exchange during storage of the dialdehyde in H_2^18O resulted in an increase of the singly or doubly labeled molecules from approx. 46 to 68% of the total within 3 h of incubation, demonstrating the significance of the purely chemical oxygen exchange. This problem has already been discussed in the work on the Microcystis carotenase mentioned earlier (25). These authors observed that cleavage products prepared for analysis in an aqueous system contained only a minor percentage of labeled molecules compared with the β-cyclitol analyzed directly from the gaseous phase (25). They describe and discuss the rapid chemical oxygen exchange of the carbonyl oxygen with water as well (25).

Labeling studies on ABA biosynthesis also had to face the “oxygen exchange problem.” These investigations revealed that one of the two chemically equal oxygen atoms of the carboxyl group of ABA is derived solely from molecular oxygen (26). So, it seemed quite likely that aldehyde intermediates result from the oxidative cleavage of a carotenoid precursor by a dioxygenase. These intermediates are then rapidly converted to ABA. However, in some tissues the labeling oxygen was incorporated to a lesser extent in the carboxyl group of ABA (27, 28). It was already been discussed in the work on the Microcystis carotenase presented here, it that the recombinant AtCCD1 uses a dioxygenase reaction mechanism for carotenoid cleavage. As the reaction center seems to be similar in all carotenases, this may be a common feature of this group of enzymes.

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