Characterization of a Novel Carotenoid Cleavage Dioxygenase from Plants*

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Steven H. Schwartz‡, Xiaqiong Qin‡, and Jan A. D. Zeevaart‡‡¶¶

From the ‡Department of Energy-Plant Research Laboratory and ¶¶Department of Plant Biology, Michigan State University, East Lansing, Michigan 48824

The plant hormone abscisic acid is derived from the oxidative cleavage of a carotenoid precursor. Enzymes that catalyze this carotenoid cleavage reaction, nine-cis-epoxy-carotenoid dioxygenases, have been identified in several plant species. Similar proteins, whose functions are not yet known, are present in diverse organisms. A putative cleavage enzyme from Arabidopsis thaliana contains several highly conserved motifs found in other carotenoid cleavage enzymes. However, the overall homology with known abscisic acid biosynthetic enzymes is low. To determine the biochemical function of this protein, it was expressed in Escherichia coli and used for in vitro assays. The recombinant protein was able to cleave a variety of carotenoids at the 9–10 and 9′-10′ positions. In most instances, the enzyme cleaves the substrate symmetrically to produce a C14 dialdehyde and two C13 products, which vary depending on the carotenoid substrate. Based upon sequence similarity, orthologs of this gene are present throughout the plant kingdom. A similar protein in beans catalyzes the same reaction in vitro. The characterization of these activities offers the potential to synthesize a variety of interesting, natural products and is the first step in determining the function of this gene family in plants.

Apocarotenoids are a class of compounds derived from the oxidative cleavage of carotenoids (1). This is a structurally diverse class of compounds that are widely distributed in nature. The assortment of apocarotenoids results from the large number of carotenoid precursors (more than 600 carotenoids have been identified), variations in the site of cleavage, and modifications subsequent to cleavage. Abscisic acid (ABA)1, which is derived from the oxidative cleavage of a 9-cis-epoxy-carotenoid, is necessary for seed development and adaptation to environmental stresses in plants (2). Although historically referred to as retinoids, vitamin A and related compounds are derived from the cleavage of β-carotene (3).

Plants produce a number of volatile apocarotenoids. These compounds probably serve a function as insect attractants and are valued as flavor/aroma compounds. Apocarotenoid pigments also have some economic value. For example, bixin (annatto) is commonly used as a natural food coloring, and crocin is the major pigment in saffron (4).

The formation of apocarotenoids may result from nonspecific mechanisms, such as lipooxygenase cooxidation or photodestruction. Enzymes capable of cleaving carotenoids at specific sites are believed to be involved in the synthesis of a number of apocarotenoids. The Vp14 gene, which encodes an ABA biosynthetic enzyme in maize (5, 6), was the first carotenoid cleavage enzyme to be cloned from any organism. Since the characterization of VP14, ABA biosynthetic enzymes have been identified in several plants (7–10). Based upon sequence similarity, an enzyme necessary for vitamin A biosynthesis has been identified in Drosophila (11) and vertebrates (12). Another carotenoid cleavage enzyme in animals that reacts with the 9–10 double bond has also been identified (13). The definitive demonstration of both symmetric and asymmetric cleavage pathways appears to resolve a long standing debate concerning vitamin A biosynthesis (14).

A number of hypothetical proteins that are similar to these carotenoid cleavage enzymes have also been identified in the sequence data bases, but their biochemical functions have not yet been demonstrated. In the Arabidopsis genome sequence, nine potential carotenoid cleavage enzymes have been identified. Most of these genes have been annotated as neoxanthin cleavage enzymes or nine-cis-epoxy-carotenoid dioxygenases (NCEDs). Both terms imply an involvement in ABA biosynthesis. Although it is likely that two or more genes encode nine-cis-epoxy-carotenoid dioxygenases, it is doubtful that all nine genes are involved in ABA biosynthesis. Some of these proteins may catalyze carotenoid cleavage reactions not involved in ABA biosynthesis. Others might catalyze a double bond cleavage reaction of substrates other than carotenoids, such as the lignostilbene dioxygenases from prokaryotes (15, 16). In this paper, a putative carotenoid cleavage enzyme from Arabidopsis was expressed in Escherichia coli. It is shown that recombinant protein catalyzes a carotenoid cleavage reaction different from the one in ABA biosynthesis. The gene symbol CCD, Carotenoid Cleavage Dioxygenase, was adopted in this paper to distinguish this gene family from genes that encode ABA biosynthetic enzymes.

EXPERIMENTAL PROCEDURES

Constructs—A truncated cDNA for the AtCCD1 gene was obtained from the Arabidopsis expressed sequence tag collection (N95924). The full-length cDNA sequence (19) (AJ005813) and the genomic sequence (AL163818; gene identification MA212450) have also become available. A full-length clone was obtained by reverse transcription-polymerase chain reaction using RNA from light-grown seedlings. The polymerase chain reaction product was amplified with Pfu polymerase (Stratagene) and the following primer sequences: 5′-CATGCCCAGAACTCGAGT-3′ and 5′-TTATATAGAATTGTTCG-3′. The amplified fragment was cloned into the Smal site of pBluescript SK (Stratagene) and pGEX-2T (Amer sham Pharmacia Biotech) to produce pCZY1 and pCZY2, respectively.

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2 The abbreviations used are: ABA, abscisic acid; NCED, nine-cis-epoxy-carotenoid dioxygenase; CCD, carotenoid cleavage dioxygenase; HPLC, high performance liquid chromatography.

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† To whom correspondence should be sent: Dept. of Energy-Plant Research Laboratory, Michigan State University, East Lansing, MI 48824. Tel.: 517-353-3230; Fax: 517-353-9168; E-mail: zeevaart@msu.edu.

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fragment of the PvNCED2 gene in beans, previously called PaNCED2, was amplified with degenerate primers as previously described (7). The flanking sequences were obtained by polymerase chain reaction from a bean cDNA library with one primer annealing to the initial polymerase chain reaction product and either a T7 or T3 primer annealing to the vector. Once the 5’ and 3’ sequences were determined, a full-length clone was amplified with degenerate primers as previously described (7). The flank- ing sequences were obtained by polymerase chain reaction from a bean cDNA library with one primer annealing to the initial polymerase chain reaction product and either a T7 or T3 primer annealing to the vector. Once the 5’ and 3’ sequences were determined, a full-length clone (AY029525) was amplified from the bean library with the following primer sequences: 5’-TGGATCCATGGGGGATGATGG 3’ and 5’-TG- GATCCCTACAGTTTTGCTTG-3’. The BamHI restriction sites added on to the primer were used to clone into the BamHI site of pGEX-2T (Am- ersham Pharmacia Biotech) to create pXQ1.

Protein Expression and Enzyme Assays—A 5-ml culture of pCZY2 or pXQ1 was used to inoculate a 100-ml culture in 2 × YT medium (per liter: 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl). Cultures were grown at 37 °C until an A(600) of 0.7 was reached. Expression of the protein was induced by the addition of 0.2 mM isopropyl β-D-thiogalacto- pyranoside, and the cultures were grown at 28 °C for an additional 3–5 h. The E. coli cells were harvested by centrifugation, resuspended in Tris-buffered saline, and lysed in a French press. The recombinant protein was purified with glutathione S-transferase-agarose (Sigma) and then released by cleavage with thrombin for 24 h at 37 °C. The recombinant protein with limited sequence similarity to the carotenoid cleavage enzymes in ABA biosynthesis (31% identical and 42% similar to VP14). Although it was suggested that the gene is involved in ABA biosynthesis (19), the biochemical function of the protein has not yet been reported. Hypothetical proteins that share much higher similarity to known ABA biosynthetic enzymes have since been identified in the Arabidopsis genome sequence.

A comparison of the AtCCD1 protein with hypothetical pro- teins from other plant species indicates that it is a member of a highly conserved subfamily of carotenoid cleavage-related proteins (Fig. 1). The AtCCD1 protein is 67% identical and 74% similar to a protein from avocados, PaNCED2 (9). A protein from beans, PvCCD1 (this paper), is 78% identical and 86% similar to AtCCD1. In addition, the expressed sequence tags

RESULTS

In Vitro Characterization of AtCCD1—The AtCCD1 gene, originally called AtNCED1 (19) in Arabidopsis, encodes a protein with limited sequence similarity to the carotenoid cleavage enzymes in ABA biosynthesis (31% identical and 42% similar to VP14). Although it was suggested that the gene is involved in ABA biosynthesis (19), the biochemical function of the protein has not yet been reported. Hypothetical proteins that share much higher similarity to known ABA biosynthetic enzymes have since been identified in the Arabidopsis genome sequence.

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FIG. 1. Alignment of the AtCCD1 protein with related sequences. PaNCED2 was previously identified in avocados (AF224670) (9); PvCCD1 (AY029525) is an apparent ortholog of AtCCD1 from beans; AtNCED5 is a potential ortholog of VP14 that was identified in the Arabidopsis genome sequence (AC074176); AnaCCD1 is an open reading frame from the cyanobacterium Anabaena PCC 7120 (c307: sequence 1467–2894).

FIG. 2. Thin-layer chromatography analysis of assays with the recombinant AtCCD1 protein and various carotenoid substrates. The substrates used were β,β-carotene (β-Car), lutein (Lut), zeaxanthin (Zeax), all-trans-violaxanthin (tViol), 9-cis-violaxanthin (9cViol), and 9′-cis-neoxanthin (9cNeox). Enzyme assay products were separated on a thin-layer silica plate that was developed in hexane, ethyl acetate, and 2-propanol (70:20:10). Following chromatography, the plate was sprayed with 2,4-dinitrophenylhydrazine to detect alde- hydes and ketones. The products were also characterized by a combi- nation of HPLC, UV-visible spectroscopy, and mass spectrometry. Sev- eral of the characterized products are labeled on the chromatogram (see Fig. 3). Residual substrates are indicated by asterisks.
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**Substrates**

| Substrates | Products |
|------------|----------|
| β-Car | I |
| Lut | II |
| Zeax | III |
| tViol | IV |
| 9cViol | V |
| 9cNeox | VI |

**FIG. 3.** Scheme for the reactions catalyzed by the recombinant AtCCD1 protein. The roman numerals correspond to compounds labeled in Fig. 2. I, 4,9-dimethyldec-2,4,6,8,10-pentaene-1,12-dial (C\textsubscript{14} dialdehyde). The absorption spectrum was identical with published data (20). Mass spectrum: 216 [M]\(^+\) (100), 187 (31), 129 (25), 105 (50). II, 5,6-epoxy-3-hydroxy-9-apo-β-caroten-9-one. The chromatography and absorption spectrum were similar to published data (20). Mass spectrum: 224 [M]\(^+\) (31), 191 (2), 137 (3), 125 (11), 124 (13), 123 (100), 109 (5). III, 5,6-epoxy-3-hydroxy-12'-apo-β-caroten-12'-al (C\textsubscript{27} epoxy-apocarotenal). The mass spectrum and absorption spectrum were consistent with published data (31). IV, 3,5-dihydroxy-6,7-didehydro-12'-apo-β-caroten-12'-al (C\textsubscript{27} alenic-apocarotenal). The UV-visible maximum in benzene is 443 nm, with shoulders at 427 and 463 nm. V, (3S,5R,6R)-3,5-dihydroxy-6,7-didehydro-5,6-dihydro-9-apo-β-caroten-9-one (grasshopper ketone). Mass spectrum of trimethylsilyl ether derivative: 368 [M]\(^+\) (28), 353 (100), 311 (98), 278 (32). VI, 3-hydroxy-9-apo-β-caroten-9-one (3-hydroxy-β-ionone). The mass spectrum was consistent with published data (28). β-Car, β,β-carotene; Lut, lutein; Zeax, zeaxanthin; tViol, all-trans-violaxanthin; 9cViol, 9-cis-violaxanthin; 9cNeox, 9'-cis-neoxanthin.

**FIG. 4.** E. coli strains engineered to accumulate zeaxanthin and coexpressing CCD1 proteins. “AtCCD1” is a strain coexpressing pCZY1, and “PvCCD1” is a strain coexpressing pXQ1. The control strain contains the pGEX-2T vector with no insert.

A variety of plants share a high degree of similarity to AtCCD1 at the nucleotide and the deduced amino acid level. Based upon limited sequence data, genes with greater than 80% nucleotide identity are present in the expressed sequence tags of numerous plant species. A hypothetical protein from Anabaena PCC 7120, AnaCCD1, also shares some sequence similarity with AtCCD1 (38% identity and 48% similarity).

To determine the potential function of AtCCD1, the gene was cloned into a glutathione S-transferase fusion vector for expression in E. coli, and the recombinant protein was assayed for cleavage activity with a variety of carotenoid substrates. The assay products were separated by thin-layer chromatography and sprayed with 2,4-dinitrophenylhydrazine to visualize aldehyde and ketone products (Fig. 2). To generate the reaction scheme (Fig. 3), the assay products were also characterized by a combination of HPLC, UV-visible spectroscopy, and mass spectrometry. A variety of C\textsubscript{13} products, resulting from cleavage at the 9–10 and the 9'–10' positions, were identified (e.g., II, V, and VI). In assays containing lutein, zeaxanthin, and all-trans-violaxanthin (Fig. 2, lanes 2, 3, and 4, respectively), the products did not separate from the carotenoid substrates by TLC. It was, however, possible to isolate these products by HPLC for further characterization. The red spot (I) in Fig. 2, lanes 1–6 was the C\textsubscript{14} dialdehyde resulting from symmetrical cleavage at the 9–10 and 9'–10' positions (Fig. 3). This C\textsubscript{14} dialdehyde was a major product when β-carotene, lutein, zeaxanthin, or all-trans-violaxanthin was used as a substrate. The enzyme did not cleave as well adjacent to a 9-cis double bond or an allenic bond found in some carotenoids. In assays containing the 9-cis isomer of violaxanthin (Fig. 2, lane 5), the major product was the C\textsubscript{27} epoxy-apocarotenal (II) that results from a single cleavage distal to the cis double bond. When 9'-cis-neoxanthin was used as a substrate (Fig. 2, lane 6), both a C\textsubscript{27} epoxy-apocarotenal (II) and a C\textsubscript{27} allenic-apocarotenal (IV) were produced.

**Coexpression of AtCCD1 in Carotenoid-accumulating Strains of E. coli**—In the characterization of β,β-carotene-15,15'-dioxygenases, researchers have coexpressed the vitamin A biosynthetic enzymes in E. coli strains engineered to accumulate β-carotene. In these studies, β-carotene did not accumulate, and the bacteria failed to develop color. In the first study, four different products were identified, but less than one-third of the lost β-carotene could be accounted for in E. coli cells (11). In the second study only the presence of all-trans-retinal was reported (12). Expression of AtCCD1 in carotenoid-accumulating strains of E. coli also resulted in colorless colonies (Fig. 4). Analysis of the apocarotenoids produced by these strains is consistent with the reaction scheme for the in vitro assays. A very low level of the C\textsubscript{14} dialdehyde was present in the E. coli cells (data not shown), but additional apocarotenoid products were detected in the medium (Fig. 5A). Analysis of these compounds by UV-visible (Fig. 5B) and fluorescence spectroscopy indicates that peak 1 on the chromatogram is rosafuene, a
serve as insect attractants when produced by plants.

Probable orthologs of the AtCCD1 gene are present in a wide range of plants. An ortholog in beans is able to catalyze the same reaction. The substrate of these enzymes in planta and the function of the apocarotenoid products are not yet known. However, a number of apocarotenoids have been identified in plants that are derived by an AtCCD1-like activity. The C14 dialdehyde is the probable precursor of rosafuene, a highly fluorescent compound found in some varieties of roses (26). The C14 product is also the expected precursor of mycorradicin, a yellow pigment that accumulates in the roots of plants inoculated with arbuscular mycorrhizal fungi (27). Several C14 cleavage products are also abundant in the roots of infected plants. Although Arabidopsis does not appear to form mycorrhizal associations, an AtCCD1-like protein probably catalyzes the synthesis of these apocarotenoids in other plant species.

A C12 product of zeaxanthin or lutein cleavage, 3-hydroxy-β-ionone, accumulates in etiolated bean seedlings on exposure to light, and it has been suggested that this compound may have a function in the light-induced inhibition of hypocotyl elongation (28, 29). The AtCCD1 gene is also induced by light in etiolated seedlings. With the characterization of the AtCCD1 activity in vitro, it is now possible to speculate on the possible function of this gene in plants. By altering the expression of this gene in transgenic plants, it should be possible to characterize the apocarotenoid products and determine their biological roles.

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