

## Review

# Structural and biological aspects of carotenoid cleavage

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**Abstract.** Apo-carotenoid compounds such as retinol (vitamin A) are involved in a variety of cellular processes and are found in all kingdoms of life. Instead of being synthesized from small precursors, they are commonly produced by oxidative cleavage and subsequent modification of larger carotenoid compounds. The cleavage reaction is catalyzed by a family of related enzymes, which convert specific substrate double bonds to the corresponding aldehydes or ketones. The individual family

members differ in their substrate preference and the position of the cleaved double bond, giving rise to a remarkable number of products starting from a limited number of carotenoid substrate molecules. The recent determination of the structure of a member of this family has provided insight into the reaction mechanism, showing how substrate specificity is achieved. This review will focus on the biochemistry of carotenoid oxygenases and the structural determinants of the cleavage reaction.

**Keywords.** Abscisic acid, apo-carotenoid, BCO (beta-carotene cleavage oxygenase), carotenoid cleavage, dioxygenases, monooxygenases, non-heme iron proteins, retinal, RPE65 (retinal pigment epithelium 65 kDa protein).

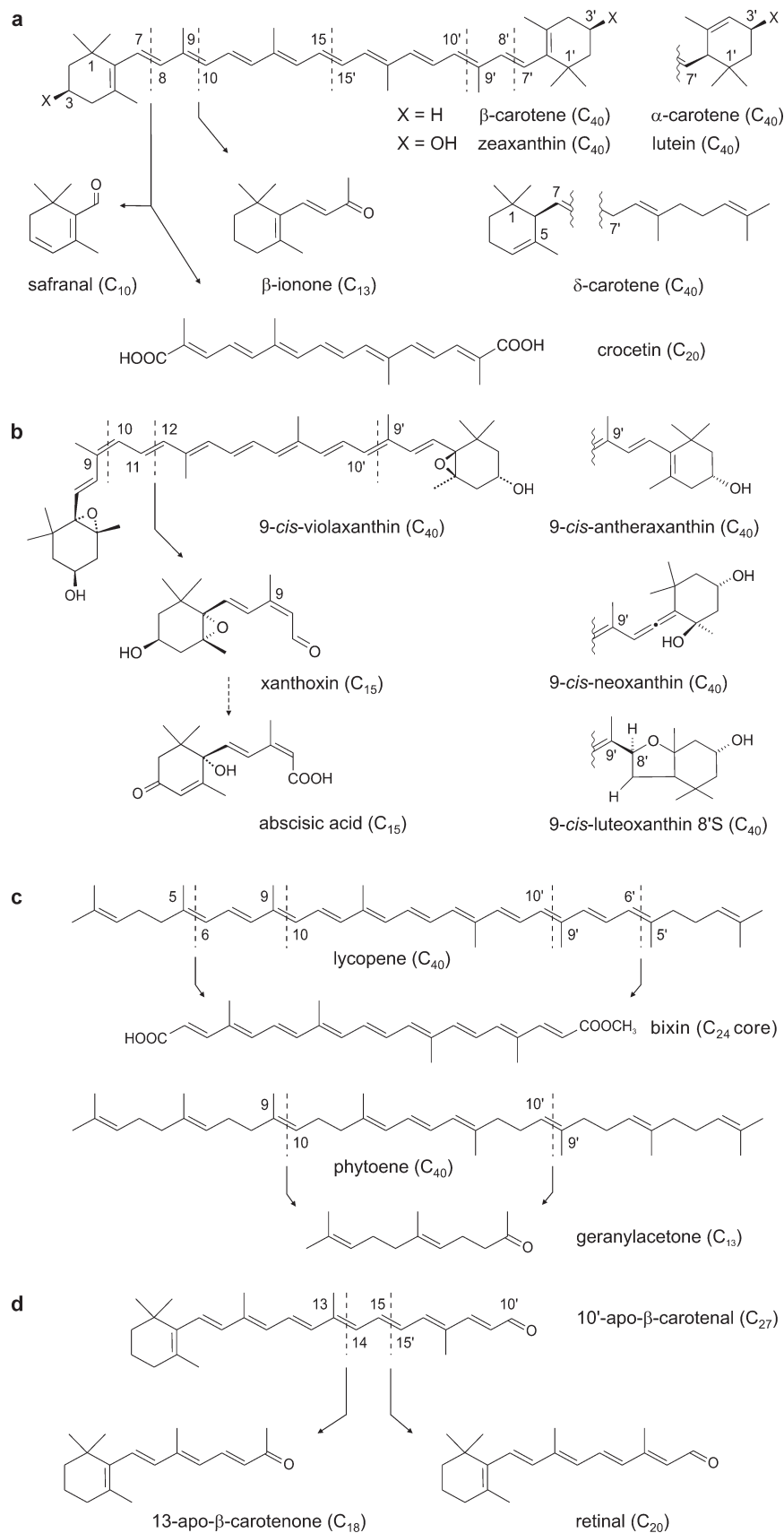
## Introduction

Carotenoids are isoprenoid compounds present in the membranes of all phototrophic as well as many heterotrophic organisms. They are derived from the C<sub>40</sub> compound phytoene by cyclization, isomerization and oxidation [1]. Carotenoids have an extended conjugated double-bond system and absorb light in the UV-vis range. They can be oxidatively cleaved to smaller compounds called apo-carotenoids (Fig. 1). Like their parent molecules, apo-carotenoids are non-polar isoprenoids that possess an extended conjugated double-bond system, and have therefore been intensely studied for their properties as antioxidants and free radical scavengers [2]. Apo-carotenoids with altered spectroscopic and physicochemical properties can be obtained by adding functional groups through oxygenation, isomerization and double-bond cleavage. From a chemical point of view, their distinct and rigid shape combined with a tailorable amphipathic behavior makes these com-

pounds ideally suited as chromophores and signaling molecules, in agreement with their roles observed in all kingdoms of life.

Most apo-carotenoids are not synthesized from smaller molecules, but rather result from the oxidative cleavage of larger isoprenoids and subsequent modification in a process termed ‘oxidative remodeling’ [3–5]. It is now known that the initial step of this remodeling is achieved through members of the carotenoid cleavage oxygenase (CCO) family, which show a high degree of regio- and stereo-specificity giving rise to defined products with distinct biological roles. These enzymes use dioxygen but it has not yet been clarified whether both oxygen atoms end up in the produced apo-carotenoids [6–8], that is, whether the frequently used name ‘dioxygenases’ is correct. As a measure of caution, we therefore use the more general term ‘oxygenases’. The recent determination of the structure of a member of the CCO family has provided for the first time structural insight into this enzyme family [9]. Here, we review current knowledge about the biochemistry of CCO enzymes.

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**Figure 1.** Educts, products and cleavage positions of the substrates of CCO enzymes. (a) Safranal is obtained through cleavage of zeaxanthin at the 7,8 (or 7',8') positions while  $\beta$ -ionone and retinal are directly produced by cleavage of  $\beta$ -carotene at the 9,10 (or 9',10') and 15,15' positions, respectively. In contrast to the symmetric  $\beta$ -carotene with its 5,6 and 5',6' double bonds, the asymmetric substrates  $\alpha$ -carotene and lutein have a 4',5' double bond rendering position 6' chiral. The same double bond is found in  $\delta$ -carotene, which is only cyclic at one end. (b) Cleavage of a number of 9-*cis*-epoxycarotenoids at the 11,12 position results in the C<sub>15</sub> apo-carotenoid xanthoxin, which is further processed to abscisic acid. (c) The linear C<sub>24</sub> core of bixin is synthesized by cleavage of lycopene at both the 5,6 and 5',6' positions. Phytoene can also be cleaved at the 9,10 (9',10') positions. (d) Apo-carotenoids are themselves substrates for further cleavage.

## Biological role of apo-carotenoids

In animals, the most important apo-carotenoid is the  $C_{20}$  compound retinal (15-apo- $\beta$ -carotenal) and its derivatives (Fig. 1). Retinal plays a crucial role as the chromophore of rhodopsin in the vertebrate visual cycle [10]. Four other retinoids are also used as visual chromophores in the animal kingdom: (3,4)-didehydroretinal, (3*R*)-3-hydroxyretinal, (3*S*)-3-hydroxyretinal, and (4*R*)-4-hydroxyretinal [11]. All-*trans*-retinal can be oxidized to all-*trans*-retinoic acid and converted to the 9-*cis* isomer, which play an important role as signaling molecules in the immune system [12], in development [13] and in cancer [14]. They bind to nuclear retinoic acid (RAR) and retinoid X (RXR) receptors. Therefore, the use of natural and synthetic retinoids in cancer therapy has received appreciable attention [15]. Retinal also serves as the chromophore in bacteriorhodopsin as well as in bacteriorhodopsin-like proton pumps in halophilic archaea [16], eubacterial species [17] and in the fungus *Leptosphaeria maculans* [18], which is the first eukaryotic example for such a pump. The same chromophore is also found in sensory rhodopsins of halophilic archaea [19] and eubacteria [20].

In plants and cyanobacteria, apo-carotenoids are found in large amounts in the thylakoid membrane, where they act as accessory and photoprotective pigments [21]. Depending on the size of the chromophore, apo-carotenoids can absorb visible light and are useful as color pigments. Well-known examples are the bright orange-red color of saffron, mainly due to glycosides derived from the  $C_{20}$  apo-carotenoid crocetin (Fig. 1), and the orange-red color of annatto, arising from the  $C_{24}$  apo-carotenoid bixin [22]. When used to color plant tissues, apo-carotenoids are found in large amounts in specialized plastids called chromoplasts. Small apo-carotenoid volatiles are aroma compounds: the  $C_{13}$  volatile  $\beta$ -ionone, for example, is a characteristic component of rose scent.

Several examples of apo-carotenoids acting as chemo-attractants, repellants, growth stimulators and inhibitors have been described [23]. It has long been known that certain apo-carotenoids act as hormones in the regulation of plant growth and architecture. The best characterized example is abscisic acid (ABA), which plays an important role in the regulation of drought tolerance, seed development and sugar sensing [24, 25]. It is derived from the  $C_{15}$  apo-carotenoid xanthoxin (Fig. 1b). The biosynthesis of ABA is mainly regulated at the initial cleavage step leading to xanthoxin [24].

Evidence has been put forward that apo-carotenoids derived from all-*trans* carotenoid precursors play a role as signaling molecules in auxin-mediated apical dominance [26]. Analysis of *Arabidopsis thaliana* (mouse-ear cress) mutants led to the discovery of the two novel carotenoid cleavage oxygenases CCD7 and CCD8, which act in concert to produce an apo-carotenoid that promotes

shoot branching [27]. The exact chemical nature of the produced apo-carotenoid and its mode of action are still unknown. However, the active compound is likely derived from 13-apo- $\beta$ -carotenone, the  $C_{18}$  cleavage product of CCD7 (Table 1), because loss of either CCD7 or CCD8 led to the same bushy phenotype [28]. The various roles of apo-carotenoids in plants have been recently reviewed [5, 23].

## Biosynthesis of apo-carotenoids

The recurrent structural motifs found in the large variety of known apo-carotenoids indicate that they are derived from full-length carotenoids, presumably along common biosynthetic pathways. The first established conversion of a carotenoid to an apo-carotenoid was that of  $\beta,\beta'$ -carotene (provitamin A,  $C_{40}$ ) to retinal (vitamin A,  $C_{20}$ ) in animals. The existence of a specific enzyme catalyzing this reaction was postulated 40 years ago [29]. The isolation and subsequent characterization [30] of the enzyme showed that it cleaves the 15,15'-double bond of  $\beta$ -carotene to yield two molecules of retinal. In animals, the symmetric or asymmetric cleavage of  $C_{40}$  carotenoid compounds is the only known biosynthetic route to retinoids.

All enzymes of the CCO family cleave an internal C-C double bond using dioxygen [3–5]. The reaction gives rise to the corresponding aldehydes or ketones. It is frequently regulated and followed by further biosynthetic steps to form the functional apo-carotenoid [31–33]. However, not all apo-carotenoids in all organisms are synthesized by cleavage of a larger precursor molecule. In the case of ABA, a 'direct' biosynthetic pathway has recently been described in fungi [34]. Here, the  $C_{15}$  isoprenoid farnesyldiphosphate is cyclized and oxidized in several steps to form ABA, eliminating the need for a carotenoid precursor.

## Specificity of CCO enzymes

Of the more than 200 putative CCO enzymes to be found in sequence databases, 26 have been characterized and tested for *in vitro* cleavage activity. They are listed in Table 1, where they are grouped according to the type of cleaved substrate and the position of the scissile bond. All CCO enzymes cleave either one double bond or two such bonds at structurally equivalent positions, and most show a high degree of specificity with respect to the accepted substrate.

The initially characterized CCO enzymes constitute the BCO (beta-carotene cleavage oxygenase) subfamily, all members of which process all-*trans*- $\beta,\beta'$ -carotene. BCO1 catalyzes the cleavage of the 15,15' double bond result-

**Table 1.** Functionally characterized carotenoid cleavage oxygenases.

Name <sup>1</sup>	Source	Established substrate <sup>2</sup>	Cleaved double bond	Product size	Functional product	Reference
BCOI	<i>Drosophila melanogaster</i>	$\beta$ -caro	15,15'	C <sub>20</sub>	retinal	35
BCOI	<i>Gallus gallus</i>	$\beta$ -caro, $\alpha$ -caro	15,15'	C <sub>20</sub>	retinal	36
BCOI	<i>Mus musculus</i>	$\beta$ -caro	15,15'	C <sub>20</sub>	retinal	37
BCOI	<i>Homo sapiens</i>	$\beta$ -caro	15,15'	C <sub>20</sub>	retinal	38
BCOII	<i>Mus musculus</i>	$\beta$ -caro	9',10'	C <sub>27</sub> , C <sub>13</sub>	possibly retinal	40
CCD7	<i>Arabidopsis thaliana</i>	$\beta$ -caro	9',10'	C <sub>27</sub> , C <sub>13</sub>	unknown	28
VP14	<i>Zea mays</i>	9- <i>cis</i> -vio, -neo, -zea, -anth, -luxR, -luxS	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	67
NCED1	<i>Phaseolus vulgaris</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	41
NCED1	<i>Citrus sinensis</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	32
NCED1	<i>Persea americana</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	42
NCED3	<i>Persea americana</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	42
NCED2	<i>Arabidopsis thaliana</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	43
NCED3	<i>Arabidopsis thaliana</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	43
NCED5	<i>Arabidopsis thaliana</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	43
NCED6	<i>Arabidopsis thaliana</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	43
NCED9	<i>Arabidopsis thaliana</i>	9- <i>cis</i> -vio, 9- <i>cis</i> -neo	11,12	C <sub>15</sub> , C <sub>25</sub>	ABA	43
CCD1	<i>Arabidopsis thaliana</i>	$\beta$ -caro, lut, zea, vio, 8'-apo-al, 9- <i>cis</i> -vio, -neo	9,10 (9',10')	C <sub>13</sub> , C <sub>14</sub> <sup>3</sup>	C <sub>13</sub> volatile	45
CCD1	<i>Lycopersicon esculentum</i>	$\beta$ -caro, lut, zea, 9- <i>cis</i> -vio, 9- <i>cis</i> -neo	9,10 (9',10')	C <sub>13</sub> , C <sub>14</sub> <sup>3</sup>	C <sub>13</sub> volatile	48
CCD1	<i>Cucumis melo</i>	$\beta$ -caro, $\delta$ -caro, lyco, phyt	9,10 (9',10')	C <sub>13</sub> , C <sub>14</sub> <sup>3</sup>	C <sub>13</sub> volatile	46
CCD	<i>Crocus sativus</i>	zea	9,10 (9',10')	C <sub>13</sub> , C <sub>14</sub> <sup>3</sup>	C <sub>13</sub> volatile	3
CCD1	<i>Vitis vinifera</i>	zea	9,10 (9',10')	C <sub>13</sub> , C <sub>14</sub> <sup>3</sup>	C <sub>13</sub> volatile	47
CCD1	<i>Petunia hybrida</i>	$\beta$ -caro	9,10 (9',10')	C <sub>13</sub> , C <sub>14</sub> <sup>3</sup>	C <sub>13</sub> volatile	33
ZCD	<i>Crocus sativus</i>	zea	7,8 (7',8')	C <sub>10</sub> , C <sub>20</sub> <sup>3</sup>	safranal + crocetin	3
LCD	<i>Bixa orellana</i>	lyco	5,6 (5',6')	C <sub>8</sub> , C <sub>24</sub> <sup>3</sup>	bixin	22
ACO	<i>Synechocystis</i> PCC 6803	apo-al and -ol, apo-lycopenal and -ol	15,15'	C <sub>20</sub> , C <sub>5</sub> -C <sub>15</sub> <sup>3</sup>	retinal	49
CCD8	<i>Arabidopsis thaliana</i>	10'-apo-al	13,14	C <sub>18</sub> , C <sub>9</sub> <sup>3</sup>	unknown	28

<sup>1</sup> Abbreviations: BCOI, beta-carotene cleavage oxygenase I; CCD1, carotenoid cleavage dioxygenase 1; VP14, viviparous maize protein 14; NCED1, nine-*cis*-epoxycarotenoid dioxygenase 1; ZCD, zeaxanthin cleavage dioxygenase; LCD, lycopene cleavage dioxygenase; ACO, apo-carotene cleavage oxygenase.

<sup>2</sup> All substrates are in all-*trans* configuration unless otherwise indicated. All sizes are C<sub>40</sub> except for ACO and CCD8, which process substrates of up to C<sub>35</sub> and C<sub>27</sub>, respectively. Abbreviations: caro, carotene; vio, violaxanthin; neo, neoxanthin; zea, zeaxanthin; anth, antheraxanthin; luxR, luteoxanthin 8'R; luxS; luteoxanthin 8'S; lut, lutein; lyco, lycopene; apo-al, apo- $\beta$ -carotenal; phyt, phytoene. The molecule structures are shown in Figure 1.

<sup>3</sup> Ring-free dialdehyde product.

ing in two retinal molecules (Fig. 1a). Substrates with *cis*-double bonds are generally not accepted. BCOI enzymes from fruit fly [35], chicken [36], mouse [37] and human [38] sources have been cloned and biochemically characterized. For human BCOI, small variations in the substrate such as an  $\alpha$ -ionone ring on one side are tolerated, but at least one unsubstituted  $\beta$ -ionone ring must be present for efficient cleavage [39].

In contrast to the symmetric cleavage of  $\beta$ -carotene by BCOI, the enzyme BCOII catalyzes the asymmetric cleavage at the 9',10' position (Fig. 1a), producing 10'-apo- $\beta$ -carotenal ( $C_{27}$ ) and  $\beta$ -ionone ( $C_{13}$ ). BCOII enzymes have been cloned from mouse, zebrafish and human sources [40]. In addition to  $\beta$ -carotene, murine BCOII also cleaves lycopene at the 9',10' position. Another enzyme catalyzing the asymmetric cleavage of  $\beta$ -carotene at the 9',10' position is CCD7 from *Arabidopsis* [28]. However, CCD7 has not been classified as a BCOI enzyme because it is only distantly related to the animal BCOI enzymes and because its preferred natural substrate has yet to be determined.

The characterization of a viviparous maize mutant defective in ABA biosynthesis led to the discovery of VP14, an enzyme catalyzing the cleavage of 9-*cis*-epoxycarotenoids at the 11,12 position (Fig. 1b). Several orthologs of VP14 have been cloned from bean [41], orange [32], avocado [42] and *Arabidopsis* [43]. Collectively, these enzymes are grouped into the NCED (nine-*cis*-epoxycarotenoid dioxygenase) subfamily, of which maize VP14 is the best characterized member. It cleaves the 9-*cis* isomers of zeaxanthin, violaxanthin, luteoxanthin (both 8'S and 8'R forms), antheraxanthin and neoxanthin [44]. Except for 9-*cis*-zeaxanthin, all of these substrates have a 5,6-epoxy-3-hydroxy- $\beta$ -ionone ring close to the cleavage site. Moreover, all of them yield xanthoxin as the  $C_{15}$  cleavage product, which in turn is the precursor of ABA. Since the most common 9-*cis*-epoxycarotenoids in plants are violaxanthin and neoxanthin, cleavage assays with members of the NCED family were mostly performed using only these two substrates. The range of 9-*cis*-epoxycarotenoid substrates that are accepted and cleaved is probably larger. As a common feature, none of the NCED family members accept an all-*trans* substrate.

A third group of CCO enzymes cleaves substrates at the 9,10 as well as the 9',10' position and shows a broad substrate specificity. These enzymes constitute the CCD (carotenoid cleavage dioxygenase) subfamily named after CCD1 from *Arabidopsis* [45]. In contrast to murine BCOII, which cleaves at the same position but only accepts all-*trans*- $\beta$ -carotene, CCD1 processes a wide range of all-*trans*- and 9-*cis*-carotenoids as well as epoxycarotenoids, among them the  $C_{30}$  apo-carotenoid all-*trans*-8'-apo- $\beta$ -carotenal [8, 45]. When 9-*cis* substrates are used, CCD1 generally cleaves at the 9',10' site yielding  $C_{27}$  apo-carotenoids. Several other enzymes have been as-

signed to the CCD subfamily based on about 80% sequence identity with CCD1 from *Arabidopsis*. The recently characterized enzyme CCD1 from melon is able to cleave the all-*trans* substrates phytoene and lycopene, as well as  $\beta$ - and  $\delta$ -carotene at the 9,10 (9',10') positions to the respective  $C_{13}$  products [46]. However, CCD from crocus [3] and CCD1 from *Vitis vinifera* (grapevine) [47] were only assayed for cleavage activity towards zeaxanthin, in which case 3-hydroxy- $\beta$ -ionone is obtained as the  $C_{13}$  product. Similarly, CCD1 from petunia was shown to cleave  $\beta$ -carotene yielding  $\beta$ -ionone [33]. Because of the close sequence relationship within the subfamily (Table 1), all of these enzymes are expected to accept a range of substrates similar to that of CCD1 from *Arabidopsis* and tomato [48].

Two enzymes cleaving at positions closer to the ionone rings have been characterized. ZCD (zeaxanthin cleavage dioxygenase) from crocus cleaves at the 7,8 (7',8') positions [3] giving rise to one  $C_{20}$  and two  $C_{10}$  cleavage products that are used for the biosynthesis of safranal and crocetin glycosides, respectively. LCD (lycopene cleavage dioxygenase) from *Bixa orellana* (lipstick tree) cleaves all-*trans*-lycopene at the 5,6 (5',6') positions, which is the first committed step in the biosynthesis of bixin [22]. The resulting  $C_{24}$  dialdehyde is oxidized to the corresponding diacid and esterified at one end (Fig. 1c). LCD is the only CCO enzyme that exclusively cleaves an acyclic substrate. Neither  $\beta$ -carotene nor zeaxanthin are accepted.

Apart from the numerous enzymes acting on  $C_{40}$  carotenoids, some enzymes accept only apo-carotenoids. For example, ACO (apo-carotenoid cleavage oxygenase) from the cyanobacterium *Synechocystis* sp. PCC 6803 cleaves the 15,15' double bond of various all-*trans*-apo-carotenoids (Fig. 1d). It accepts apo-carotenals and apo-carotenols as well as the corresponding 3-hydroxy derivatives, ranging from 12'-apo ( $C_{25}$ ) to 4'-apo ( $C_{35}$ ). The reaction results in all-*trans*-retinal or -3-hydroxy-retinal ( $C_{20}$ ) and a dialdehyde product of variable size. Larger substrates are neither bound nor cleaved [49]. Notably, ACO cleaves acyclic apo-8'-lycopenal at positions other than 15,15' [50]. The *Arabidopsis* enzyme CCD8 cleaves 10'-apo- $\beta$ -carotenal, the cleavage product of CCD7, at the 13,14 position to produce 13-apo- $\beta$ -carotenone ( $C_{18}$ ) and a  $C_9$  dialdehyde [28]. The CCD7 and CCD8 enzymes are thought to act in concert to produce a compound involved in the regulation of shoot branching.

Interestingly, RPE65 (retinal pigment epithelium 65-kDa protein) shows a significant degree of sequence homology to the CCO family. However, its members do not cleave, but rather isomerize a specific double bond in the visual cycle. After photoisomerization of the retinal chromophore in rhodopsin from 11-*cis* to all-*trans*, the retinal is removed from the apo-protein opsin and esterified. RPE65 then binds the retinyl ester and re-isomerizes the

11,12-*trans* double bond to the *cis* configuration in order for the visual cycle to recommence [51–53].

### Carotenoid cleavage by other enzymes

Beside members of the CCO family, a number of rather unspecific carotenoid-degrading enzymes are known to be active during the uptake of foodstuffs. Fatty acid hydroperoxides produced by lipoxygenases, for example, have been shown to mediate  $\beta$ -carotene cleavage in gastric mucosal homogenates [54]. The substrate was cleaved at several positions, resulting in apo-carotenals of sizes from C<sub>18</sub> (13-apo- $\beta$ -carotenone) to C<sub>30</sub> (8'-apo- $\beta$ -carotenal). Similarly, lycopene was cleaved at several positions using rat intestinal homogenates [55]. Cleavage of  $\beta$ -carotene to produce  $\beta$ -ionone was also shown to occur in the presence of radicals generated by xanthine oxidase [56]. Some microorganisms possess membrane-bound or extracellular enzymes for the degradation of environmental carotenoids [57]; for example, a peroxidase from the fungus *Lepista irina* cleaves  $\beta$ -carotene mainly at the 9,10 double bond to form  $\beta$ -ionone [58]. Further characterization of these enzymes is to be expected in the future.

### Subcellular location of CCO enzymes

The substrates of CCO enzymes are generally large, lipophilic molecules that are sparingly water soluble and therefore bound to carrier proteins or dissolved in membranes. In plants, carotenoids are synthesized and stored in plastids. In the thylakoid membrane, they are used as accessory and photoprotective pigments. They are also found in chromoplast membranes and stromata, where they accumulate in large quantities giving rise to brightly colored structures. CCO enzymes involved in the remodeling of carotenoids are therefore often targeted to plastids. The respective signal peptides are present in the sequences of some, but not all plant members.

In the case of VP14 from the NCED subfamily (Table 1), the import of the soluble cytosolic protein into chloroplasts led to two versions of the enzyme, a soluble stromal version and a second version bound to the thylakoid membrane [59]. The enzyme NCED1 from bean is also active in thylakoids [41]. CCD7 and CCD8 as well as their homologues from *Arabidopsis* are targeted to plastids, except for CCD1, which is cytosolic like CCD1 from tomato [48] and CCD from crocus [3].

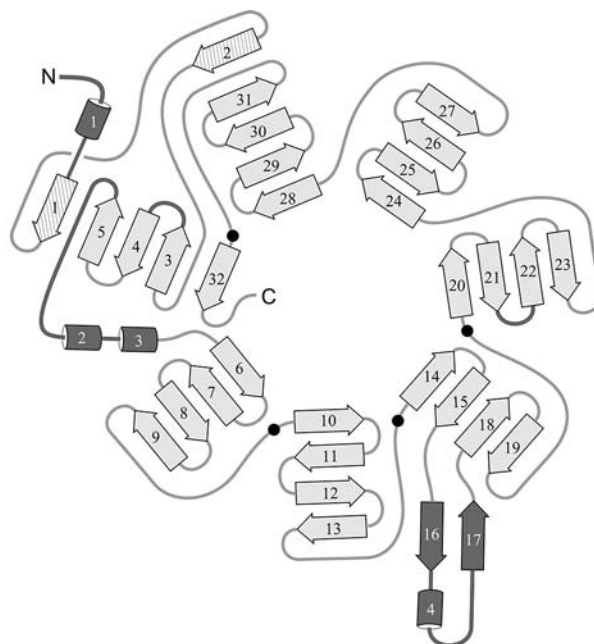
Although most carotenoids are located in the thylakoid membrane, significant amounts of  $\beta$ -carotene have been found in the outer envelope of pea chloroplasts [21] which likely supply the cytosolic CCD1 enzymes. Up to now, little is known about how the cytoplasmic carotenoid oxy-

genases access their substrates. However, the biochemical and structural data of the CCO family member ACO from *Synechocystis* point to a monotopic membrane association mediated by a large non-polar surface patch close to the active center [9]. It remains to be seen whether this also applies to the other CCO enzymes.

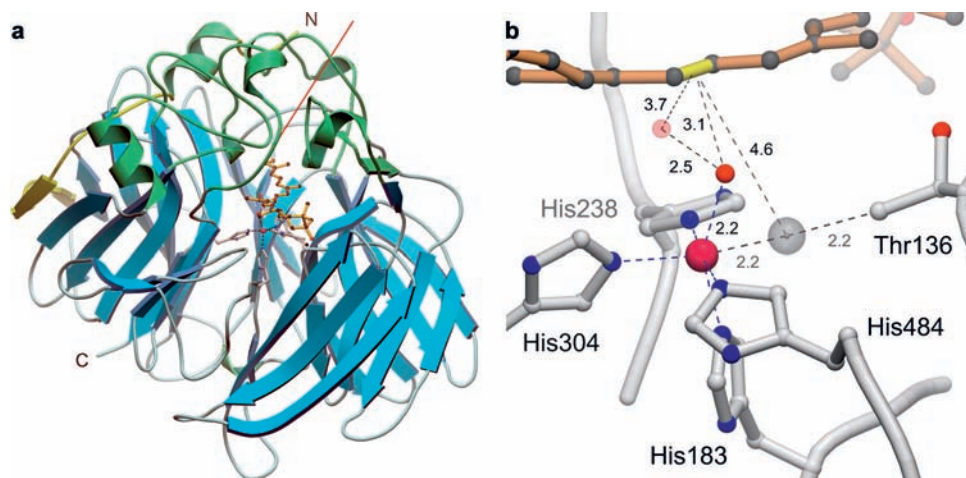
### Structure of CCO enzymes

The only established structure of a CCO family member is that for ACO from *Synechocystis* PCC 6803 [9]. The chainfold of ACO is a seven-bladed  $\beta$  propeller (Fig. 2). Five propeller blades consist of four antiparallel  $\beta$  strands each, while two blades are five-stranded. The bottom side of the propeller is relatively flat and contains short loops connecting the  $\beta$  strands to each other (Fig. 3a). In contrast, the top side of the propeller is crowded where extended loops with short helices form a large dome.

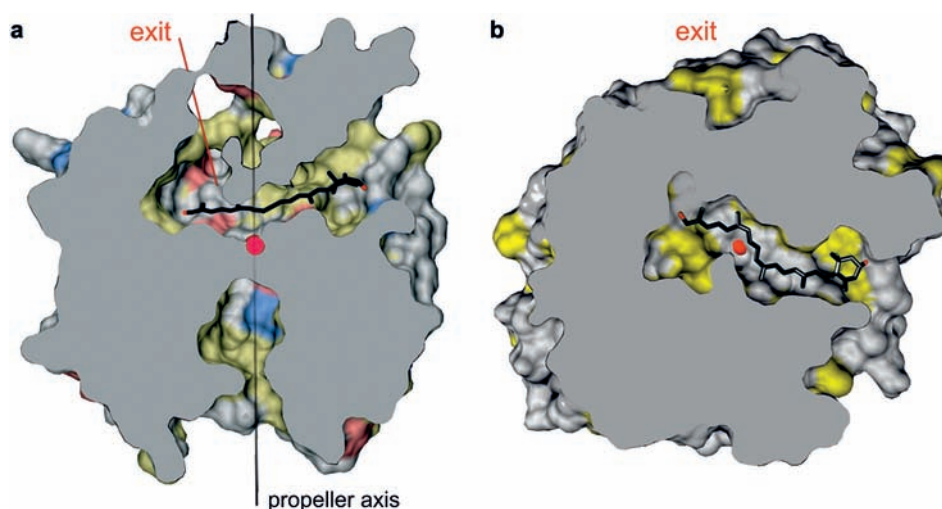
As observed in numerous  $\beta$  propeller structures, the active center of ACO is located near the propeller axis on the top side. The most prominent feature is a large tunnel that enters the protein from one side in a direction perpendicular to the propeller axis (Fig. 4a), passes the active center defined by the iron, and then proceeds to an exit approximately parallel to the propeller axis. The shape of the tunnel is mostly defined by the extended loops of the 'dome' and only slightly by the rigid propel-



**Figure 2.** Sketch of the chainfold of ACO showing the seven-bladed  $\beta$  propeller as the basic motif. The positions of the four histidines coordinating the iron at the center of the propeller are marked by dots. The four loops and the N terminus forming the dome above the active center are dark gray, and the N-terminal extension of the propeller is hatched ( $\beta 1$  to  $\beta 2$ ).



**Figure 3.** The structure of ACO from *Synechocystis* PCC 6803. (a) Ribbon representation of the enzyme showing the  $\beta$  strands of the propeller in blue, the residues of the dome above the active center in green, and the N-terminal extension in yellow. The bound substrate (3*R*)-3-hydroxy-8'- $\beta$ -apo-carotenol, the catalytic iron with its four coordinating histidines as well as two water molecules are shown in a ball-and-stick representation. The view is along the entrance of the active-center tunnel below the dome. The tunnel exit is indicated by a red line. (b) Active-center and substrate-binding geometry. The apo-carotenoid substrate is shown in orange with the scissile 15,15' double bond in yellow. The vacant iron coordination site is indicated by a transparent gray sphere and the weakly defined second water molecule is shown as a transparent red sphere. Some distances are given.



**Figure 4.** Substrate binding in ACO shown in a surface representation. Positively charged, negatively charged and non-polar residues are given in blue, red and yellow, respectively. The catalytic iron atom is indicated by a red circle, the bound substrate is black/red. (a) Cut through the molecule approximately along the propeller axis, revealing a pocket penetrating from the bottom along the propeller axis to the center, where it almost reaches the catalytic iron. Moreover, the cut shows the substrate-binding tunnel that enters the protein from the right side, changes its direction near the center and ends in the tunnel exit at top left. (b) Top view of the substrate (3*R*)-3-hydroxy-8'- $\beta$ -apo-carotenol in the cut enzyme. The 15,15'-double bond of the substrate is at the catalytic iron. On both sides of the cleavage site, the 13,14 and 13',14' double bonds assume a *cis-cis* configuration on binding, giving rise to a doubly kinked chain. Further entry of the substrate into the tunnel is prevented by the narrow entrance. The view is approximately along the propeller axis.

ler scaffold. This allows the individual CCO family members to modulate substrate specificity by changing length and sequence of the connecting loops, while retaining the rigid propeller scaffold for stability. Indeed, sequence alignments within the CCO family clearly show that the most highly conserved regions are within the  $\beta$  strands forming the propeller as demonstrated in Figure 5. This strongly suggests that all members of the CCO family

have the same  $\beta$  propeller chainfold and may therefore be modeled along the lines of the ACO structure.

In ACO, a second cavity enters the protein from the bottom side, along the propeller axis (Fig. 4a). This is an intrinsic feature of the propeller architecture and is therefore not expected to vary greatly within the CCO family. The pocket ends shortly before the active center. Its functional significance is not clear. It may serve as

an auxiliary tunnel for dioxygen, which, however, would have to pass through a short dense area of the protein to reach the iron.

ACO contains a mononuclear iron center consisting of four histidine side chains holding the catalytic iron with their  $N\epsilon$  atoms. The iron-binding site is rigid as it does not change on removal of the metal ion [9]. In the crystal structure of ACO with a ferrous iron and a substrate molecule, the coordination geometry is best described as octahedral with two missing ligands (Fig. 3b). The fifth coordination position is occupied by a water molecule, while the sixth position is empty. The active center location was confirmed by mutations of the iron-ligating histidines, which in mouse BCOI [60] and in human RPE65 [61] led to a total loss of enzymatic activity.

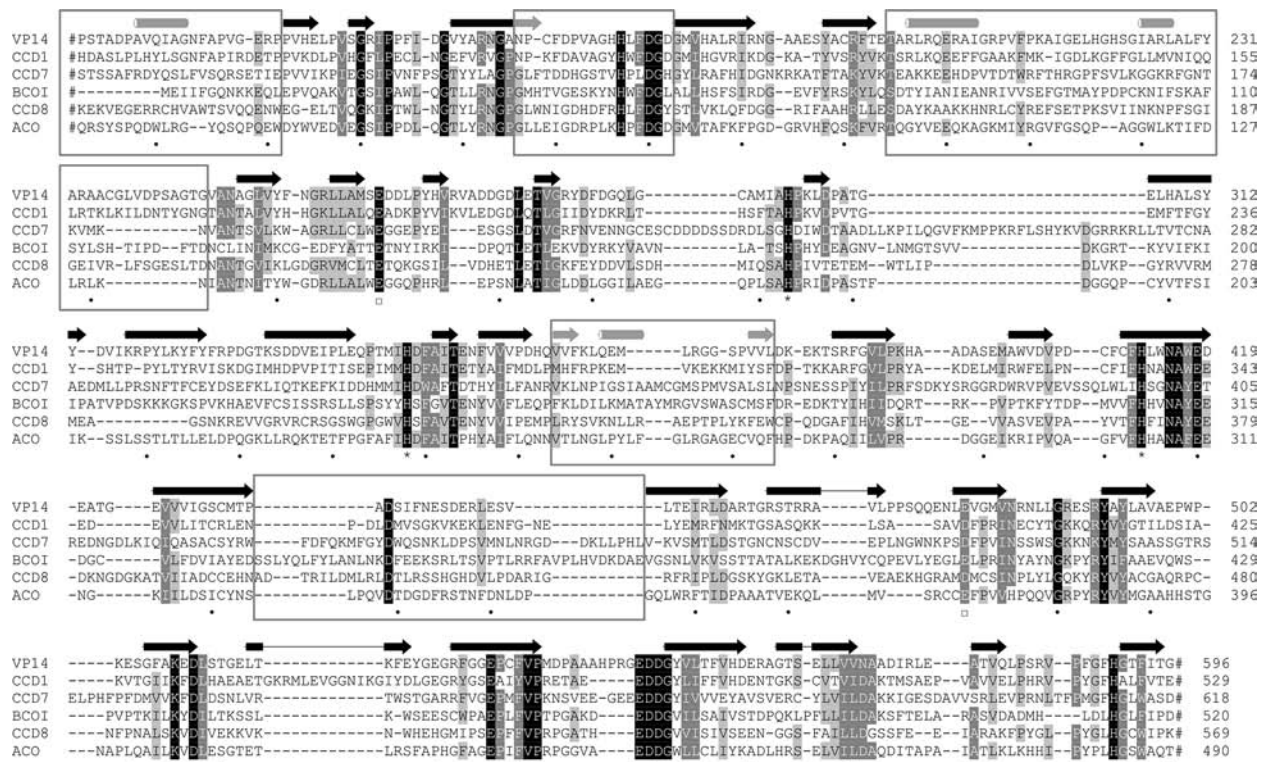
Within the large group of non-heme-iron-containing oxygenases, the iron is usually coordinated by a combination of histidine, aspartate or glutamate and water ligands [62]. Mononuclear iron centers with four histidine ligands are rare, ACO being the fifth example of such a coordination environment. Three of the four histidines are fixed by hydrogen bonds to glutamates. In the entire CCO family as well as in the isomerizing RPE65 group, the histidines and glutamates are strictly and well conserved, espe-

cially (Fig. 5). The nitrogen ligands certainly stabilize the ferrous state of the iron. Unfortunately, no redox potential of any CCO enzyme has yet been determined.

### Structural determinants of substrate specificity

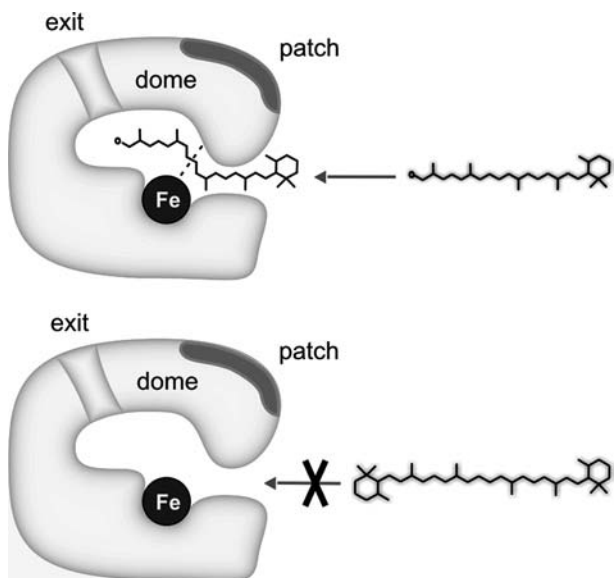
The substrate molecule (3*R*)-3-hydroxy-8'-apo- $\beta$ -carotenol bound to ACO in the crystal provides some clues regarding the recognition of the carotenoids by the CCO enzymes. ACO accepts apo- $\beta$ -carotenals (and -ols) with lengths from  $C_{25}$  (12'-apo) to  $C_{35}$  (4'-apo) and cleaves at the 15,15' position [49], always yielding the same  $C_{20}$  compound retinal (Fig. 1d). Larger substrates such as  $\beta$ -carotene ( $C_{40}$ ) are not cleaved at all, whereas acyclic apocyclopens are cleaved at multiple positions [50].

In the crystal structure, the  $\beta$ -ionone ring of the substrate is invisible, presumably due to its mobility. However, the visible part of the apo-carotenoid indicates clearly that the ionone ring is located on the enzyme surface because it cannot pass through the narrow entrance of the substrate tunnel (Fig. 4). As sketched in Figure 6, the tunnel and its entrance work as a ruler, determining the cleavage position relative to the ionone ring. In addition, the entrance



**Figure 5.** Sequence alignment of six members of the carotenoid cleavage oxygenase family showing the chain segments forming the loops of the dome above the active center (boxes). In pairwise comparisons these enzymes have at most 21% identical residues. Residue conservation is indicated by shading. The four iron-ligating histidines (\*) and the glutamates fixing them (□) are labeled. The dots at the bottom mark every tenth residue of ACO. The enzyme codes are explained in Table 1; CCD1 is from *Arabidopsis*, BCOI is from mouse. The numbers denote the position of the last printed residue of the line. Some sequences are truncated: the N-terminal markers (#) stand for 113, 38, 52, 0, 69 and 11 missing residues (top to bottom), and the C-terminal marks (#) denote 8, 9, 0, 46, 1 and 0 residues.





**Figure 6.** Schematic sketch of apo-carotenoid cleavage in ACO showing the tunnel with entrance, kink and exit. The dome and the non-polar patch near the tunnel entrance are indicated. Top: Apo-carotenoid substrates with only one  $\beta$ -ionone group can enter the enzyme and are held in position by the narrow tunnel entrance. The distance between the catalytic iron and the entrance determines the cleavage position. Bottom: Substrates with  $\beta$ -ionone rings on both sides cannot enter the tunnel and are therefore not cleaved.

prevents  $\beta$ -carotene cleavage because neither of the two substrate ends can enter the tunnel (Fig. 6). This explains the selectivity towards apo-carotenoids. The upper size limit for the apo-carotenoids is dictated by the available linear space between the tunnel entrance and the end of the central cavity. The ruler model is consistent with the observation that substrate binding does not cause an appreciable induced-fit displacement.

It should be noted that besides retinal, ACO produces dialdehydes of different lengths that possess higher solubility. ACO has a large non-polar patch near the entrance (Fig. 6), which is probably used to enter into the interior of a membrane from where it picks up the non-polar educt. After cleavage, the non-polar product retinal is again released to the membrane, whereas the more soluble second product dialdehyde probably dissociates into the cytosol. This is quite possible if the dialdehyde leaves ACO through the exit instead of through the entrance. Accordingly, the observed non-polar entrance-polar exit construction around the active center seems to be explained by the physicochemical properties of educts and products.

An unexpected feature of the ACO crystal structure was the presence of two *cis* double bonds in the bound substrate that were located on either side of the cleaved 15,15' double bond (Figs. 3b, 6), even though the all-*trans* form had been used in the cocrystallization experiment. Obviously, the doubly kinked conformation is enforced by the shape of the active center. However, the isomerization

has to be supported catalytically, because the barrier of methyl-substituted double bonds in an extended conjugated double-bond system is about 100 kJ/mol. Numerous examples of proteins that achieve isomerization of double bonds are known. For example, the activation barrier of the retinal chromophore in rhodopsin is lowered by the surrounding protein to such an extent that thermal isomerization of the 11-*cis* to the all-*trans* form occurs at ambient temperatures [63, 64]. In ACO, the isomerization is most likely catalyzed by the iron atom in conjunction with a ligated oxygen species. Incidentally, the reported isomerization of the ACO ligand is similar to the isomerization activity of RPE65, converting all-*trans* to 11-*cis* retinal [65].

Although the structure of ACO prohibits cleavage of  $\beta$ -carotene (Fig. 6), the homology between ACO and, for example, the carotene-cleaving BCOI (Table 1) indicates that the structural difference should be comparatively small. Some changes of the dome structure above the iron would suffice to convert the tunnel to a cleft, which may then close in an induced fit on a bound  $\beta$ -carotene. The peptide segments forming the dome are boxed in Figure 5. They show large variations, rendering any attempt to model the dome of another CCO family member highly hazardous.

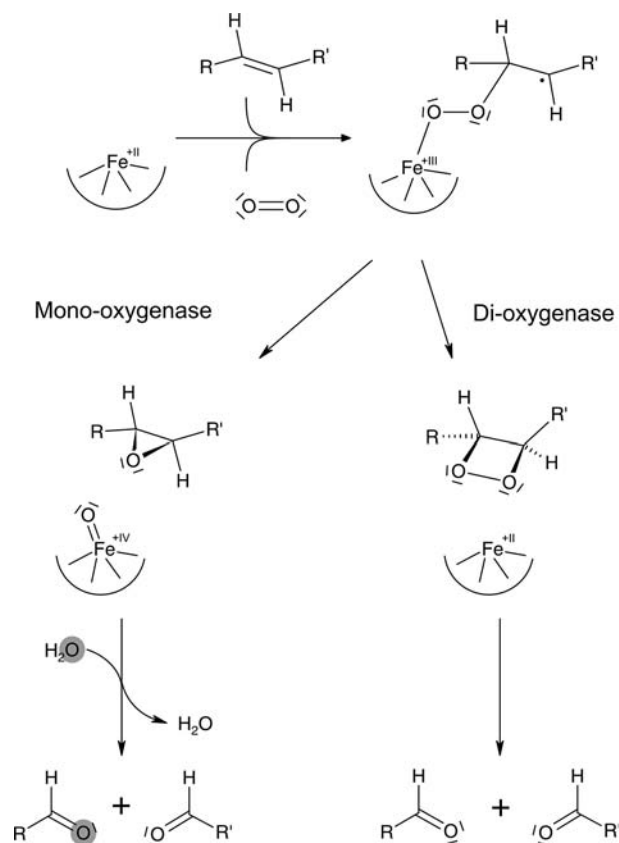
Several CCO enzymes cleave two double bonds at substrate positions related to each other by symmetry or pseudo-symmetry [45, 48]. Examples are the 5,6 and 5',6' bonds in lycopene (symmetric, Fig. 1), the 7,8 and 7',8' bonds in zeaxanthin (symmetric), or the 9,10 and 9',10' bonds in  $\beta$ -carotene (symmetric) or  $\alpha$ -carotene (pseudo-symmetric). Because of the symmetry of substrate and cleavage, CCO enzymes have been proposed to act as dimers [45]. Dimerization is not necessary, however, because an asymmetric polypeptide may well form an almost symmetric binding site. Moreover, if the strongest binding occurs at the symmetric central part of the substrate molecule, pseudo-symmetric substrates with different end structures like lutein or  $\alpha$ -carotene (Fig. 1a) will also be accepted in both orientations. A similar case of binding the pseudo-symmetric molecule  $\text{NAD}^+$  in both orientations is known [66]. Presumably, these enzymes cleave their  $\text{C}_{40}$  substrate once, release the products, bind the primary apo-carotenoid product again in reverse orientation, and cleave it a second time on the other side. Therefore, this subgroup of the CCO family should be able to cleave apo-carotenoids. This conclusion was confirmed for CCD1 from *Arabidopsis*, which accepted an apo-carotenoid *in vitro* [8].

### Reaction mechanism

In general, enzymatic carotenoid cleavage reactions *in vitro* are performed in the presence of a reductant such

as ascorbate [67], DTT [68], TCEP [39] or excess  $\text{Fe}^{2+}$  [35] in order to keep the iron in its ferrous state. However, it is unlikely and has never been demonstrated that the reductant participates stoichiometrically in the reaction itself. All electrons in the products can be derived exclusively from the carotenoid and dioxygen. Nevertheless, it is clear that all enzymes of the CCO family are iron dependent. BCOI enzymes isolated from various animal sources have long been known to be inhibited by iron-chelating agents such as 1,10-phenanthroline and 2,2'-dipyridine [30, 69]. Moreover, the cleavage activity of VP14 was lost on the removal of iron with EDTA and restored by reconstitution with  $\text{Fe}^{2+}$ , but not with  $\text{Fe}^{3+}$  [67]. Also, BCOI activity in the small intestine of rats was shown to increase with rising iron levels [70]. Dependence on ferrous iron was also shown for RPE65, which was inactivated by iron chelation, but recovered on addition of  $\text{Fe}^{2+}$ , though not on addition of  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$  or  $\text{Fe}^{3+}$  [65]. An inductively coupled plasma (ICP) emission analysis revealed one bound iron atom per protomer in chicken BCOI [7] and bovine RPE65 [65]. This is consistent with the crystal structure of ACO [9], and we conclude that the reaction mechanism requires one ferrous iron atom near the scissile double bond.

While the requirement of  $\text{Fe}^{2+}$  and  $\text{O}_2$  for the reaction is clear, the nature of the involved oxygen species has not yet been clarified. Due to their conjugated double bonds, carotenoids are highly reactive, so that unspecific oxygenation and cleavage can occur in a number of chemical, thermal or enzymatic processes. In contrast, the reactions catalyzed by the CCO enzymes are well defined. The mechanism of these reactions, however, remains controversial. It is clear that the C-C double bond is cleaved to the corresponding aldehydes or ketones using dioxygen as reactant and ferrous iron as a cofactor, but whether the reaction follows a monooxygenase or a dioxygenase pathway has not been established. In the first case, the scissile double bond is first epoxidized by activated dioxygen and the epoxide is then opened by a water molecule (Fig. 7). Subsequently, the resulting vicinal diol is cleaved, leading to the incorporation of one oxygen atom from dioxygen and another one from a water molecule into the products. According to the alternative dioxygenase mechanism, the double bond accepts dioxygen to form a dioxetane intermediate that decays to two aldehydes or ketones (Fig. 7). To discriminate between these two possibilities, several isotope-labeling experiments have been performed *in vitro* and *in vivo*. Experiments using leaves from a water-stressed *Xanthium strumarium* (cocklebur) in an  $^{18}\text{O}_2$  atmosphere showed an  $^{18}\text{O}$  incorporation into the carboxyl group of ABA [71]. As the  $^{18}\text{O}$  label was probably introduced by the oxidative cleavage of the 11,12-double bond of a 9-*cis*-epoxycarotenoid to form xanthoxin (Fig. 1b), the presumed full substitution with  $^{18}\text{O}$  was interpreted in terms of a dioxygenase mechanism. This may have been



**Figure 7.** Discussed reaction mechanisms of CCO enzymes. The oxygen species at the iron is putative. The essentials of the monooxygenase mechanism passing through an epoxide and of the dioxygenase mechanism with a dioxetane intermediate are given as alternatives. The putative oxygen derived from water is shaded.

a premature conclusion, because the second product of the cleavage reaction had not been analyzed. By itself, the label at ABA is not conclusive because it may arise if the epoxide were opened regio-specifically in a monooxygenase mechanism, and regio-specificity is a common feature of enzymes.

For an avian  $\beta$ -carotene-15,15'-oxygenase BCOI (Table 1), the reaction mechanism was analyzed using  $^{18}\text{O}$ -labeled water and  $^{17}\text{O}$ -labeled dioxygen during *in vitro* cleavage assays with recombinant protein [6]. The asymmetric  $\alpha$ -carotene (Fig. 1a) instead of the symmetric  $\beta$ -carotene was used, in order to obtain two separable reaction products. In this experiment,  $^{17}\text{O}$  and  $^{18}\text{O}$  were found in equal amounts in both products, clearly supporting a monooxygenase mechanism, because a dioxygenase mechanism would have led to the incorporation of  $^{17}\text{O}$  alone [7]. The observed labeling symmetry can be explained by  $\alpha$ -carotene binding in both orientations to the enzyme, which is consistent with the structure of ACO, where the ring of the substrate is only loosely bound [9]. Alternatively, the opening of the epoxide by water may not occur regio-specifically which, however, seems unlikely for an enzyme.

The experiment was criticized as being inconclusive, because the reductase used for converting the produced aldehydes to alcohols may have scrambled the oxygens [8]. The reductase was required to fix the oxygen in a stable alcohol in order to avoid an oxygen exchange at the aldehyde stage. This criticism awaits experimental testing.

Recently, a further labeling experiment was performed with recombinant CCD1 from *Arabidopsis* [8] using 8'-apo- $\beta$ -carotenal as substrate in an  $^{18}\text{O}_2$  atmosphere. Cleavage at the 9,10 position resulted in  $\beta$ -ionone ( $\text{C}_{13}$ ) and a  $\text{C}_{17}$  dialdehyde. Almost all of the produced  $\beta$ -ionone and some dialdehyde carried the  $^{18}\text{O}$  label, which was suggested to prove the dioxygenase mechanism [8]. While the  $\beta$ -ionone ketone is stable, the  $\text{C}_{17}$  dialdehyde is known to exchange rapidly the oxygen in water. Unfortunately, the required 100% labeling of one of the two aldehyde groups could not be demonstrated with certainty, so this experiment cannot be considered conclusive. In summary, the BCOI experiment [6] would be conclusive for a mono-oxygenase mechanism if the applied reductase were proven to keep the aldehyde oxygen during the reaction with NADH, while the CCD1 [8] experiment requires a more direct proof of the full label at the dialdehyde.

## Conclusions

Since the discovery of carotenoid cleavage by specific oxygenases, much insight has been gained into this fascinating family of enzymes. Although the structure determination of a member of the CCO family in complex with a bound substrate has revealed the architecture of the active center, the details of the reaction and the nature of the involved oxygen species have yet to be clarified. It seems likely that the question whether these enzymes are monooxygenases or dioxygenases, i.e. whether one or both atoms of dioxygen are incorporated into the products, will soon be answered by conclusive isotope labeling experiments.

In the ACO crystal structure, the isomerization of two specific substrate C-C double bonds upon binding has been observed. At present, it is unclear whether this is a speciality of ACO, or a general feature of substrate binding in CCO enzymes, or is even required for the reaction mechanism. The RPE65 group functioning as non-cleaving double-bond isomerases points to the latter possibility.

Understanding substrate specificity in the CCO family remains a fascinating endeavor. The structure of ACO shows how the correct substrate double bond is selected for a series of apo-carotenoids. However, a different selection process should exist in enzymes cleaving all-*trans*- or 9-*cis*- $\text{C}_{40}$  carotenoids, or in enzymes cleaving a

broad range of both all-*trans* and 9-*cis* substrates such as CCD1 from *Arabidopsis*. The structure of ACO showed that all these enzymes have a different dome structure on top of a stable  $\beta$  propeller scaffold and, furthermore, that the loops forming the dome can easily vary during evolution. Consequently, novel substrate specificities should arise frequently during evolution. Unfortunately, the structure of ACO does not permit the modeling of the dome of homologous enzymes at a reasonable confidence level, because the variations are too extensive. Further structures of the CCO family members are needed to explain the great variety of substrate specificities.

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