



Published in final edited form as:

Chembiochem. 2008 June 16; 9(9): 1450–1461. doi:10.1002/cbic.200700724.

Identification of bacterial carotenoid cleavage dioxygenase homologs that cleave the interphenyl α,β double bond of stilbene derivatives via a monooxygenase reaction

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Abstract

Carotenoid cleavage oxygenases (CCOs, also referred to as carotenoid cleavage dioxygenases (CCDs) in the literature) are a new class of non-heme iron-type enzymes that oxidatively cleave double bonds in the conjugated carbon chain of carotenoids. The oxidative cleavage mechanism of these enzymes is not clear and both monooxygenase and dioxygenase mechanisms have been proposed for different carotenoid cleavage enzymes. CCOs have been described from plants, animals, fungi and cyanobacteria but little is known about their distribution and activities in bacteria other than cyanobacteria. We surveyed bacterial genome sequences for CCO homologs and report the characterization of CCO homologs identified in *Novosphingobium aromaticivorans* DSM 12444 (*NOV1* and *NOV2*) and in *Bradyrhizobium* sp. (BRA-J and BRA-S). In vitro and in vivo assays with carotenoid and stilbene compounds were used to investigate cleavage activities of the recombinant enzymes. The NOV enzymes cleaved the interphenyl α,β double bond of stilbenes with an oxygen functional group at the 4' carbon (e.g. resveratrol, piceatannol, and rhaponticin) to the corresponding aldehyde products. Carotenoids and apocarotenoids were not substrates for these enzymes. The two homologous enzymes from *Bradyrhizobium* sp. did not possess carotenoid or stilbene cleavage oxygenase activities, but showed activity with farnesol. To investigate whether oxidative cleavage of stilbenes proceeds via a monooxygenase or dioxygenase reaction, oxygen labeling studies were conducted with NOV2. Our labeling studies show that double-bond cleavage of stilbenes occurs via a monooxygenase reaction mechanism.

Keywords

carotenoids; enzymes; microbiology; phytohormones; oxygen

Introduction

Mono- and dioxygenases play important roles in the oxidative modification and cleavage of metabolic compounds. Recently, a new non-heme iron family of oxygenases has been described that catalyze cleavage of double bonds in the conjugated carbon chain of

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Supporting information for this article is available on the WWW under <http://www.chembiochem.org> or from the author.

carotenoids to produce apocarotenoids.^[1,2] Carotenoid cleavage products have important biological functions as signal molecules, hormones, and attractants for pollinators (reviewed in ^[1]) and are also of considerable interest for medical and agricultural applications (reviewed in ^[1-3]).

Carotenoid cleavage oxygenases (CCOs, also referred to as carotenoid cleavage dioxygenases (CCDs) in the literature, see below) have now been identified in all taxa. ^[4-9] In higher plants, cleavage enzymes have been identified that produce signaling molecules to regulate growth and development, influence fruit color, and affect aroma (reviewed in ^[8]). Carotenoid oxygenases also play important metabolic and signaling roles in metazoans. ^[7,9] The symmetric cleavage of β,β -carotene to retinal via carotenoid oxygenase activity, for example, was an important medical discovery (reviewed in ^[3]). The function of the oxygenase enzymes in microorganisms is far less clear, although recent studies on cloning and characterization of CCOs from cyanobacteria begin to address these questions. ^[4, 10, 11]

Cloning and characterization of a number of mostly plant derived CCOs showed that these enzymes exhibit different cleavage site and substrate specificities. ^[6, 12-18] However, very little is known about the mechanism by which these enzymes catalyze oxidative cleavage of double bonds to form two aldehyde cleavage products. Currently only one crystal structure is available for an apocarotenoid specific CCO from the cyanobacteria *Synechocystis sp.* PCC6803. ^[19] The structure shows that the enzyme contains a Fe^{2+} coordinated to four His residues in the active site, which is embedded in a seven-bladed β -propeller chain arrangement topped by a dome comprised of six large loops. However, whether this enzyme catalyzes oxidative cleavage via a mono- or dioxygenase mechanism cannot be deduced from the structure. Labeling studies from plants producing abscisic acid suggested a dioxygenase mechanism. ^[20] These data were supported by labeling studies examining the production of the aroma compound β -ionone by *Arabidopsis thaliana* CCD1 (*AtCCD1*).^[21] However, researchers studying vitamin A biosynthesis have suggested at different times with different enzyme examples both a dioxygenase mechanism and a monooxygenase-like mechanism through a postulated epoxy intermediate.^[22] Consequently, we chose to use the term CCO (for carotenoid cleavage oxygenase) to describe this class of enzymes.

We have recently begun to characterize putative CCO homologs identified in genome sequences of cyanobacterial strains in order to gain a better understanding of their functions in photosynthetic bacteria.^[4] Our analysis of bacterial genome sequences for new members of the CCO family also identified several putative CCO homologs in carotenogenic and noncarotenogenic bacteria, indicating that at least some of these enzymes probably cleave substrates other than carotenoids. In the early 1990's enzymes that cleave the interphenyl α,β double bond of trans-stilbenes have been described from the soil bacterium *Sphingomonas paucimobilis* TMY1009 (four isoforms SPA1-4).^[23,24] These enzymes are believed to have a catabolic function by cleaving stilbene-type compounds derived from lignin degradation and have therefore been named lignostilbene-alpha,beta-dioxygenases (LSD, EC 1.13.11.43); although dioxygen incorporation has never been experimentally established for these enzymes. The identification of CCOs several years later showed that the *Sphingomonas* enzymes are CCO homologs that are most closely related to 9-*cis*-

epoxycarotenoid dioxygenases (NCEDs) that generate the precursor of the plant hormone abscisic acid.^[16,25]

In this study we survey bacterial genomes for other CCO homologs and describe the characterization of two CCO paralogs NOV1 and NOV2 identified in the non-carotenogenic *Novosphingobium aromaticivorans* DSM12444 and two CCO homologs from the non-carotenogenic *Bradyrhizobium japonicum* USDA110 and carotenogenic *Bradyrhizobium* sp. BTAi1 (BRA-J and BRA-S respectively). In addition, isotopic oxygen labeling experiments show that NOV2 is a monooxygenase, which is in contrast to a recent study suggesting a dioxygenase mechanism for the *Arabidopsis thaliana* CCD1 enzyme.^[21]

Results and Discussion

Bacterial CCO homologs

We previously surveyed cyanobacterial genome sequences for putative CCO enzymes and characterized cleavage activities of several recombinant enzymes.^[4] Like in plants, we expected to find in cyanobacteria mostly CCO enzymes that cleave (apo)carotenoids as based on their presumed function in general carotenoid breakdown and synthesis of apocarotenoids for light-sensing (retinal, rhodopsin) and/or other signaling functions. In fact, cloned putative cyanobacterial CCOs cleaved (apo)carotenoids with different selectivities and cleavage specificities (9, 10, 9'10'; 15,15'; apo-9,10), although several of these CCO sequences, e.g. from *Nostoc punctiforme* and *Nostoc* sp. PCC7120, were annotated as lignostilbene α , β dioxygenases.^[4] A BLAST analysis of published bacterial genome sequences with sequences of experimentally characterized CCOs identifies a number of CCO homologs in bacteria with and without annotated carotenoid pathways in their genomes (Figure 1 and Supplementary Table 1). The absence of carotenoid biosynthetic pathways in some bacteria suggests alternative catalytic activities other than carotenoid cleavage. We selected four sequences from carotenogenic and non-carotenogenic proteobacteria for further characterization: two paralogs from *Novosphingobium aromaticivorans* DSM12444 (NOV1 and NOV2), one each from *Bradyrhizobium japonicum* USDA110 (BRA-J) and *Bradyrhizobium* sp. BTAi1 (BRA-S). *Novosphingobium* does not have characterized carotenoid biosynthetic pathways, although there are several carotenoid associated genes in the genome according to the KEGG database.^[26] *Novosphingobium* is well known for its ability to degrade phenolic structures.^[27] *Bradyrhizobium* strains are nitrogen-fixing symbionts of legumes.^[28] While *B. japonicum* USDA110 is non-photosynthetic and does not synthesize carotenoids, *B. sp. BTAi1* is photosynthetic and therefore makes carotenoids.

The phylogenetic tree in Figure 1 shows that BRA protein sequences are most closely related to putative CCO homologs in *Ralstonia* and *Rhodopseudomonas*, while NOV sequences cluster with two known LSDs from *Sphingomonas paucimobilis* SPA1 (accession number AAC60447) and SPA3 (accession number AAB35856).^[29] All four putative CCO sequences (NOV and BRA) are more closely related to cyanobacterial enzymes than to plant CCOs. Alignments show that NOV1 and the SPA1 and SPA3 proteins from *Sphingomonas paucimobilis* share the highest degree of amino acid sequence identity (55% and 56% respectively) between genera (Supplemental Figure 1). This is considerably different than

the similarity between SPA enzymes and NOV2, which are 37-38%. The SPA enzymes are more alike one another (68% identity) than are the NOV enzymes (36%). The two BRA proteins share 32-37% identity to both the SPA enzymes and the NOV enzymes (with 80% similarity between BRA-J and BRA-S).

Survey of cleavage activities in carotenoid or stilbene synthesizing *E. coli* strains

A CCO enzyme from *Synechocystis* has been shown to be membrane associated;^[11] membrane association is one contributing factor to the difficulty of developing optimal in vitro assay conditions for CCO enzymes (other difficulties are described in ^[30]). As a result, in vivo detection of carotenoid cleavage activity through co-expression of the CCO enzyme in question with carotenoid biosynthetic pathways has become the standard approach to identifying active enzymes. To determine the cleavage activity of NOV and BRA enzymes, genes were amplified from genomic DNA and cloned into the constitutive *E. coli* expression vector pUCmod.^[31] For a survey of stilbene or carotenoid cleavage activities, genes were expressed in recombinant *E. coli* producing β -carotene, zeaxanthin, torulene or different stilbene compounds.

In vivo cleavage of carotenoid structures produced in *E. coli* was investigated essentially as described previously for the characterization of cyanobacterial CCOs.^[4] Briefly, CCO homologs on pUCmod were co-expressed with a carotenoid pathway expressed from pACmod. Based on previous results showing that the bicyclic carotenoid β -carotene is a substrate for many CCOs ^[6, 9, 22], β -carotene produced by genes encoded on plasmid pAC-crtE-crtB-crtI14-crtY ^[31] was chosen as the model carotenoid for this study. Two additional carotenoids with other structural features were also tested: torulene as a monocyclic carotenoid with one β -ionone end group and a linear end, and zeaxanthin as a bicyclic carotenoid with hydroxylated β -ionone end-groups. Cleavage of carotenoids in *E. coli* destroys the chromophore, causing a loss of cell color (also referred to as bleaching) that can be visually detected when compared to control cells. However, none of the tested four enzymes caused bleaching of the cell color of carotenoid producing *E. coli* suggesting that carotenoids are not likely a substrate of NOV and BRA enzymes.

We applied the same in vivo experimental approach to test the activity of NOV and BRA enzymes against stilbene substrates. In previous research, we created recombinant *E. coli* cells that co-express stilbene synthase (STS) and 4-coumaroyl CoA-ligase (4CL), enabling the synthesis of stilbene compounds from phenylpropionic acid precursor compounds fed to the recombinant cells.^[32] Biotransformation of phenylpropionic acid precursors coumaric acid, cinnamic acid, or caffeic acid results in the synthesis of resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), pinosylvin (5-(2-phenylvinyl)-1,3-benzenediol), and piceatannol (3,3',4,5-tetrahydroxy-*trans*-stilbene), respectively (Figure 2). *E. coli* cells expressing the stilbene pathway were co-transformed with NOV and BRA genes and enzymatic cleavage of resveratrol, pinosylvin and piceatannol produced by the recombinant cultures was investigated. In addition, *E. coli* cells co-expressing only 4CL and putative CCO enzymes were fed phenylpropionic acid precursors and the resulting compounds analyzed to rule out cleavage of CoA-activated phenylpropionic acids by NOV and BRA enzymes.

NOV1 and NOV2 both efficiently cleaved resveratrol resulting in complete degradation of resveratrol by recombinant *E. coli* strains after 16 hrs of cultivation and in the accumulation of two new products (Figure 2). The two new compounds were structurally identified as 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde by comparison of retention time and mass spectra with those from authentic compounds. The dihydroxy product, 3,5-dihydroxybenzaldehyde, was not extracted from the medium in stoichiometric amounts suggesting it was further degraded by *E. coli* enzymes or formed Schiff's base adducts. Piceatannol, the stilbene compound produced from caffeic acid, was also cleaved by NOV1 and NOV2 into the corresponding aldehyde products 3,4-dihydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde (data not shown). However, since conversion of fed caffeic acid to the corresponding stilbene piceatannol by the recombinant *E. coli* cells is much slower compared to the production of resveratrol from 4-coumaric acid [32], the amounts of piceatannol cleavage products detected in the cultures was correspondingly lower. Cleavage of pinosylvin, the stilbene product from cinnamic acid, by NOV1 or NOV2 was not observed. Both NOV1 and NOV2 were specific for stilbene cleavage and no cleavage products of fed phenylpropionic acids or CoA activated phenylpropionic acids were detected in culture extracts (data not shown).

Surprisingly, no stilbene cleavage products were detected in recombinant *E. coli* cultures expressing BRA-J or BRA-S despite their sequence similarity to the NOV and SPA enzymes. Previously characterized cyanobacterial CCOs such as NSC1, NSC2, and SYC2 [4, 10] were also expressed in stilbene producing *E. coli* cells and found to not cleave stilbenes.

Characterization of in vitro cleavage activities

Assays with purified protein and/or whole cell protein extracts were conducted to confirm the cleavage results obtained in recombinant *E. coli* and to test additional substrates. NOV and BRA genes were overexpressed from a pET expression vector in a recombinant *E. coli* strain that also expressed the GroEL and GroES chaperones to aid in the production of soluble protein. Overexpressed histidine-tagged proteins were purified by immobilized metal affinity chromatography and used in in vitro assays. However, as with other reports from this family of enzymes ([21,30]), purified enzymes were far less active than enzymes in protein extracts from whole cell lysates (losing more than 75% of their activity during purification). As a consequence, protein extracts from whole cell lysates are frequently used in assays with CCO enzymes [21]. We used both purified proteins and protein extracts from whole cell lysates for in vitro assays with NOV and BRA enzymes and observed similar cleavage specificities for both preparations; but ~ 5-fold lower cleavage rates were obtained with purified proteins. A series of cofactors and reducing agents tested with the enzyme (NAD, FAD, NADH, FADH, ascorbate, excess Fe²⁺, glutathione, dithiothreitol) did not improve enzyme activity. Protein extracts from whole cell lysates were prepared from *E. coli* cells overexpressing NOV and BRA genes from the constitutive expression vector pUCmod (Supplementary Figure 2).

A series of stilbene substrates (rhapontigenin, resveratrol, rhaponticin, piceatannol and pinosylvin) with different hydroxyl and methoxy functional groups were tested in in vitro

assays with NOV and BRA enzymes (Figure 3). NOV1 and NOV2 cleaved stilbene compounds that have a hydroxy or methoxy group at the 4' position at the central double bond. As observed in the *in vivo* cleavage survey, pinosylvin which carries no substitution at the 4' position, was not a substrate for NOV enzymes. Resveratrol was the preferred substrate for NOV1 and NOV2, followed by piceatannol and the 4' methoxy group bearing stilbenes rhapontigenin and rhaponticin. *In vitro* assays with equal amounts of protein lysates resulted in complete cleavage of 1 mM (68.5 μ g) resveratrol in 20 min, while complete cleavage of 1 mM (73.3 μ g) piceatannol required 60 min of incubation (Figure 3). Cleavage of 1 mM (77.4 μ g) rhapontigenin and its glucosylated derivative rhaponticin by NOV1 and NOV2 was much slower and only 20% of the substrates were cleaved after 60 min (data not shown).

To confirm the *in vivo* results showing that none of the four tested enzymes cleaved carotenoids, NOV and BRA enzymes were tested against the apocarotenoid substrate β -apo-8'-carotenal. β -apo-8'-carotenal was chosen as a substrate instead of β , β -carotene because of its greater solubility, resulting in higher activities of carotenoid cleaving enzymes with apocarotenoid substrates compared to full-length carotenoids.^[21] Moreover, some carotenoid cleavage enzymes are specific for apocarotenoids.^[10] However, no cleavage products of β -apo-8'-carotenal were detected in *in vitro* assays with NOV and BRA enzymes.

BRA-J and BRA-S enzymes were also assayed with several stilbene compounds (Figure 3). However, no cleavage activity of stilbene compounds was detected confirming the results obtained with stilbene producing *E. coli* cells. *Bradyrhizobium* strain USDA 110 has been shown to produce the plant phytohormone abscisic acid via an unknown pathway.^[50] We confirmed abscisic acid biosynthesis for strain *B. japonicum* USDA 110 and *Bradyrhizobium* sp. BTAi1 (Supplemental Figure 4). Because these *Bradyrhizobium* strains produce either no carotenoids at all (USDA 110) or no epoxy-carotenoids (BTAi1), abscisic acid biosynthesis in *Bradyrhizobium* must occur via a different route than the plant pathway, which involves oxidative cleavage of epoxy-carotenoids by a CCO (NCEDs).^[16] In filamentous fungi, abscisic acid is synthesized from farnesol via a partially described pathway. The recent identification of an abscisic acid gene cluster in *Botrytis cinerea* suggests the involvement of several oxidative steps in the conversion of farnesol to abscisic acid.^[34] Therefore we tested whether the BRA CCO homologs have activity against farnesol. GC-MS analysis of *in vitro* assays with farnesol as substrate showed conversion of farnesol into a new compound by BRA-J and BRA-S, but not by the NOV enzymes or in the control reaction (Supplemental Figure 5). The parent ion detected for this new product is *m/z* 290, which is consistent with it being a methanol adduct [*M* + 32] of a farnesol derivative containing two additional oxygen groups *m/z* 258 (*M*⁺). However, the structure of this compound can not be deduced from the MS data alone.

Oxygen labeling studies with NOV2

Cleavage of the central double bond of stilbenes catalyzed by CCO homologs from *Novosphingobium* identified in this study and *Sphingomonas* LSDs reported previously^[35], is similar to the carotenoid cleavage reaction observed with NCED enzymes in the

production of abscisic acid [16] and the central double bond cleavage observed by CCOs responsible for the production of retinal from β -carotene.[36] The enzyme mechanism using molecular oxygen and ferrous iron is thought to be similar among the different types of carotenoid or stilbene cleaving oxygenases.[25] Incorporation of one or two molecules of oxygen from atmospheric oxygen during catalysis by these enzymes is still controversial and both mono- and dioxygenase mechanisms have been suggested for carotenoid cleaving oxygenases (Scheme 1).[20-22] Poor activities of CCOs in *in vitro* assays and cleavage of water insoluble substrates may largely be responsible for the lack of rigorous mechanistic studies of this new class of non-heme iron oxygenases. Despite a few reports of characterized purified recombinant CCOs ([10, 36]), many studies rely on protein lysates.[21] However, compared to previously studied CCOs [4, 21], cleavage reactions with NOV enzymes were fast and stilbene substrates are much more soluble than carotenoids and can be analyzed by GC-MS. Both properties allow short assay times followed by a quick analysis of reaction products, thereby minimizing unspecific label exchange during oxygen labeling studies. We therefore sought to perform oxygen labeling studies with NOV2 to determine whether this class of oxygenases uses a mono- or dioxygenase mechanism.

First, resveratrol cleavage by NOV2 was assayed in an atmosphere of labeled oxygen $^{18}\text{O}_2$. The reaction was stopped after 15 min and labeled cleavage products analyzed by GC-MS. Only one of the expected cleavage products, 4-hydroxybenzaldehyde, was found to carry significant amount of the ^{18}O label (over 69% labeled) (Figure 4). In contrast, the 3,5-dihydroxybenzaldehyde product did not contain an equivalent amount of the heavy oxygen label, suggesting that its aldehyde oxygen must come from unlabeled water in the reaction.

To confirm the oxygen labeling results, a second assay was performed with labeled H_2^{18}O . As expected, a reverse labeling pattern of the cleavage products was observed (Table 1). Now, the 3,5-dihydroxybenzaldehyde carried the ^{18}O label (7.1 (m/z 282) : 92.9 (m/z 284)) and the 4-hydroxybenzaldehyde cleavage product was predominately unlabeled (93.3 (m/z 194): 6.7 (m/z 196)) (Figure 4). The ratio is determined by the ratio of the extracted ion chromatograms [M+H] ratio of heavy to total 4-hydroxybenzaldehyde (i.e. 196/194+196) as in Schmidt et al. 2006 [21]. The ratio of labeled to unlabeled aldehyde cleavage products were higher in this H_2^{18}O labeling experiment compared to the corresponding atmospheric oxygen labeling experiment. One reason for the apparent imbalance may be due to nonenzymatic oxygen exchange of the hydroxyl groups in the labeled water leading to an over representation of the labeled 3,5-dihydroxybenzaldehyde product. A time course (1, 5, 15, 30 minute) was performed to monitor oxygen exchange during the enzyme assay and in control reactions containing authentic 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde without NOV2 protein (Supplemental Figure 3 and Supplemental Table 2). Exchange of oxygen label in the enzyme assays does occur more rapidly with the di-hydroxy cleavage product, 3,5-dihydroxybenzaldehyde, than with the mono-substituted 4-hydroxybenzaldehyde (Supplemental Figure 3). After 15 minutes the labeling pattern in the enzyme reaction clearly showed heavy label mainly on one product, 3,5-dihydroxybenzaldehyde and only a small fraction of the 4-hydroxybenzaldehyde was labeled. Control reactions show that some non-enzymatic label exchange occurs predominantly with the di-hydroxy cleavage product. Together these results suggest that

NOV2 cleaves resveratrol via a monooxygenase mechanism and may have a stereo-preference for oxygen attack.

Conclusion

In this work we describe the cloning and partial characterization of four bacterial enzymes belonging to a recently described class of non-heme iron oxygenases that so far mostly includes carotenoid cleaving enzymes from plants, mammals and cyanobacteria. Two enzymes (SPA1 and SPA3) from *Sphingomonas paucimobilis*, previously known to cleave substrates other than carotenoids, have been named lignostilbene dioxygenases (LSDs) (for reviews see [3]). This study expands the number of non-carotenoid cleaving family members with the finding that two enzymes from *Novosphingomonas aromaticivorans* DSM12444 are stilbene cleaving oxygenases. We show *in vivo* and *in vitro* that the enzymes NOV1 and NOV2 cleave the central double bond of *trans*-stilbene derivatives but do not cleave bicyclic, monocyclic, or hydroxylated model carotenoid substrates. In contrast, the putative CCOs from *Bradyrhizobium japonicum* USDA110 and *Bradyrhizobium* sp. BTai1 were not active against stilbenes or carotenoids but showed activity with farnesol. In the course of these studies, we also tested known carotenoid cleavage enzymes for their ability to cleave stilbenes and we did not find stilbene cleavage activity with enzymes from the cyanobacteria *Nostoc punctiforme*, *Nostoc* sp. PCC7120, or *Synechocystis* sp. PCC6803.[4, 10, 11]

NOV1 and NOV2 are, to our knowledge, the second reported examples of stilbene cleaving oxygenases. Studies of LSD isoforms from *Sphingomonas paucimobilis* differ from this report in a few significant respects.[23, 24, 29, 35, 37] In our assays, the recombinant enzymes were tested *in vivo* and *in vitro* against natural substrates from plants such as resveratrol and piceatannol. Previous reports identified the 4'-hydroxyl group of stilbenes as a key structural feature for cleavage.[35] We found that the recombinant *Novosphingobium* enzymes cleaved compounds with 4' hydroxyl groups or 4' methoxy functional groups *in vitro* suggesting that an oxygen functional group (not specifically a hydroxyl) in that position may be important for substrate binding. NOV1 and NOV2 did not cleave pinosylvin, a substrate lacking oxygen on the 4' carbon. The NOV enzymes in this study displayed similar substrate preferences (cleaving resveratrol better than the other substrates) as opposed to the *Sphingomonas* enzymes which all had different substrate specificities.[29] Finally, we performed labeling studies to determine the oxygenase cleavage mechanism of these enzymes.

The current controversy over the oxygenase mechanism of this family of non-heme iron enzymes stems from contradictory findings from previous labeling studies and a lack of rigorous biophysical studies as the result of the difficulties associated with assays using purified carotenoid cleavage enzymes.[21,22] The stilbene cleavage reaction has several advantages over carotenoid cleavage, making it a good candidate for mechanistic studies. First, the reaction, when carried out with protein extracts from whole cell lysates, is fast (complete resveratrol cleavage under 20 minutes with 5 μ g of protein). Second, the stilbene substrate resveratrol is more soluble in aqueous systems than the lipophilic carotenoids and the cleavage products can be easily worked up in organic solvents limiting water exchange. Third, stilbene cleavage products can be readily identified by GC-MS after derivatization,

limiting the amount of oxygen exchange of the carbonyl group. In contrast, the aqueous and acidic HPLC conditions used for the analysis of carotenoid cleavage products in previous labeling studies lead to oxygen exchange rendering the interpretation of results difficult.^[20,21]

Assays performed in an $^{18}\text{O}_2$ environment with NOV2 and resveratrol as substrate resulted in predominant labeling (~ 69%) of one product, 4-hydroxybenzaldehyde. In the converse experiment with H_2^{18}O in an unlabeled O_2 environment, the aldehyde group of the other cleavage product, 3,5-dihydroxybenzaldehyde, is almost completely labeled. Control experiments with authentic aldehyde cleavage products and no enzyme added (Supplemental Table 2 and Supplemental Figure 3) show that some unspecific label exchange is observed with the more reactive 3,5-dihydroxybenzaldehyde product and H_2^{18}O . Despite the observed label exchange with the 3,5-dihydroxybenzaldehyde product, the data from these assays indicate that the recombinant NOV2 stilbene oxygenase uses a monooxygenase reaction mechanism and that the atmospheric oxygen is preferentially added to the 4-hydroxybenzaldehyde cleavage product.

Our results contradict findings from an abscisic acid labeling study in plants as well as a recent in vitro oxygen labeling study conducted with the recombinant CCO from *Arabidopsis* (*AtCCD1*) which suggest a dioxygenase mechanism for this enzyme family.^[20, 21] The abscisic acid study has been criticized for examining only one cleavage product and prematurely describing the reaction as a dioxygenase mechanism; we examined both cleavage products and found regio-selectivity, which could explain the abundant label found on the one cleavage product analyzed in the abscisic acid study. In vitro labeling studies with a 15,15' carotenoid cleavage oxygenase from chicken also suggest a monooxygenase mechanism, which is similar to our findings.^[22] However, the Leuenberger et al. study used a coupled enzyme reaction in which the formed aldehyde cleavage products are converted in situ to the corresponding less reactive alcohol products in order to reduce label exchange and facilitate GS-MS analysis of derivatized alcohols. It is possible that different members of this enzyme family catalyze similar reactions by a different oxygenase mechanism, which may even vary depending on the cleaved substrate. Rieske oxygenase family members, for example, have been shown to have mono- or di-oxygenase activity based on different substrates and enzymes.^[43] Examinations of Rieske-type oxygenases show that subtle changes in the active site can alter the enzyme mechanism.^[43] Perturbations in the active site may create an environment advantageous for a monooxygenase-like mechanism rather than a dioxygenase mechanism or vice versa.

Exact mechanistic details have not been determined for this new class of non-heme iron oxygenases because of the poor activity of in vitro assays. Assays frequently use a reductant such as ascorbate, DTT, TCEP, or excess Fe^{2+} to preserve the ferrous iron, but no other cofactors, iron-sulfur proteins or reductases have been identified as required to balance the electron flow. It has been suggested that all the electrons in the product can come exclusively from the substrate and oxygen.^[2] Unfortunately, there is only one crystal structure of a carotenoid cleavage enzyme (*Synechocystis* sp. PCC6803)^[19] available and the stilbene oxygenases from *Novosphingobium* model poorly onto the solved structure (Supplemental Figure 6). The β -strands forming the propellers are conserved but there is

large variation in the amino acid residues forming the dome and entrance loop. Descriptions of the *Synechocystis* protein structure state that ring structures will not fit through the active site tunnel. [29] Cleavage of stilbene structures, however, requires positioning of at least one phenol ring in the active site of the NOV enzyme, illustrating that there may be important differences in structure and function among the members of this enzyme family. NOV2 residues surrounding the tunnel entrance loops (residues Leu239-Lys243 and Phe106-Pro110) are structurally different in the model than in the *Synechocystis* structure. More labeling and mechanistic studies along with structural investigations are required to begin to understand catalysis of these enzymes.

This study also showed that analysis of sequence information is not sufficient to predict carotenoid or stilbene activity and substrate specificity need to be determined empirically for all new examples of these oxygenases. The two *Bradyrhizobium* enzymes share a similar degree of sequence identity to *Sphingomonas* enzymes as the NOV2 enzyme does (~35%). However, the two enzymes from *Bradyrhizobium* did not cleave stilbenes or carotenoids and instead showed activity with farnesol. Although the mass fragmentation pattern of the farnesol reaction product does not allow structural assignment, the fragment at m/z 259 [M^+] arising from the loss of methanol from the methanol adduct parent (m/z 290) suggests the addition of two oxygen groups to farnesol (and likely bond rearrangement in order to arrive at m/z 258) rather than oxidative cleavage of farnesol by BRA enzymes which would result in products with lower molecular weight and shorter retention times. Farnesol has been identified as the precursor of abscisic acid in filamentous fungi. [34] It is postulated that conversion of farnesol to abscisic acid involves several oxidative steps. [34] Knockout studies in *Botrytis cinerea* have identified two P450 monooxygenases that likely catalyze two of the postulated oxidation reactions. [38] Biosynthesis of the phytohormone abscisic acid in the *Bradyrhizobium* must also occur through a different route than the epoxy-carotenoid cleavage pathway in plants as these bacteria are either non-carotenogenic or do not produce epoxy-carotenoids. Additional studies involving the creation of gene knockouts will be necessary to investigate whether BRA-J and BRA-S are involved in abscisic acid biosynthesis in the plant symbiont *Bradyrhizobium*. Interestingly, a BLAST search of the *Botrytis cinerea* genome sequence (Broad Institute) with the BRA protein sequences identifies two putative CCO homologs. Deletion of these putative CCO genes could establish whether one of them catalyzes yet unknown steps in abscisic acid biosynthesis in this ascomycete.

The activity of the BRA enzymes indicates that there may be many new activities to be discovered for other putative microbial CCO homologs (Figure 1). Of equal interest are investigations aimed at identifying the biological functions of these enzymes in bacteria and fungi. Carotenoid cleavage in plants and mammals has functions that extend beyond pigment degradation and synthesis of visual pigments as more and more roles of carotenoid cleavage compounds in signaling are being discovered. [8] It can be assumed that the bacterial and fungal representatives of the CCO family have similarly diverse functions beyond simple degradation.

Experimental Section

Chemicals and materials

Caffeic acid, ferulic acid, piceatannol, rhaponticin, β -apo-8'-carotenal and bistrimethylsilyltrifluoroacetamide (BSTFA) were purchased from Sigma Aldrich (St. Louis, MO). 4-coumaric acid was purchased from ICN (Aurora, OH) and resveratrol was from Calbiochem (San Diego, CA). The 95% heavy water $H_2^{18}O$ was from Cambridge Isotope Laboratories (Andover, MA). All solvents were of HPLC grade and purchased through Fisher Scientific (Pittsburgh, PA). HPLC grade water was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Vent DNA polymerase, T4 DNA ligase and restriction enzymes were from New England Biolabs (NEB, Boston, MA). Restriction buffers were SuRE/Cut buffers from Roche (Indianapolis, IN).

Gene cloning

Homology searches were performed using NCBI BLAST software based on the *Sphingomonas paucimobilis* lignostilbene oxygenase proteins (SPA1 (isoform I) AAC60447; SPA3 (isoform III) AAB35856 [24, 29] and previously characterized cyanobacterial CCO's [4, 10]. CCO homologs were identified in published complete genome sequences obtained from NCBI and Joint Genome Institute (Figure 1 and Supplementary Table 1). Putative CCO homologs from *Novosphingobium aromaticivorans* DSM 12444 (NOV1 (YP_496081); NOV2 (YP_498079)), *Bradyrhizobium japonicum* USDA110 (BRA-J (NP_772430) and *Bradyrhizobium* sp. BTAi (BRA-S) were selected for cloning and functional characterization. The putative CCO genes were amplified from genomic DNA by PCR with Vent polymerase using gene specific primers with added restriction sites. NOV1 and NOV2 were cloned into the *Bgl*III and *Not*I sites of the constitutive expression vector pUCmod [31] to give pUCmod-NOV1 and pUCmod-NOV2. BRA-J and BRA-S were cloned into the *Nde*I and *Xho*I sites of pUCmod yielding pUCmod-BRA-J and pUCmod-BRA-S.

For expression of higher protein amounts for protein purification of NOV1 and NOV2, genes were subcloned into the *Nde*I and *Xho*I sites of the inducible expression vector pET28b+ (Invitrogen, Carlsbad, CA) giving plasmids pET-NOV1 and pET-NOV2. The stop codon was eliminated from the sequences for in frame fusion with a C-terminal 6X histidine tag encoded on the pET28b+ vector to facilitate protein purification. BRA-J and BRA-S were subcloned in a similar fashion into the inducible expression vector pET24b+ (Invitrogen, Carlsbad, CA) to yield plasmids pET-BRA-J and pET-BRA-S.

For co-expression of CCO homologs NOV1, NOV2, BRA-J and BRA-S (in the following collectively referred to as CCO's) with stilbene biosynthetic genes in *E. coli*, the entire CCO expression cassettes (including the constitutive *lac*-promoter and gene coding region as described in [31]) in pUCmod-CCO were amplified by PCR using sequence specific primers with added restriction sites and subcloned into the *Xba*I of plasmid pAC-4CL [32] that contains the gene for 4-coumaroyl ligase (4CL) from *Arabidopsis thaliana* under the control of a constitutive *lac*-promoter. The resulting plasmid was called pAC-4CL-CCO.

All cloning and DNA manipulation were carried out in *E. coli* strain JM109 by following standard techniques described elsewhere [57]. Cloned gene sequences were verified by sequencing.

Culture conditions and strains

Unless otherwise indicated, *E. coli* cultures were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotics ampicillin (100 $\mu\text{g ml}^{-1}$) and chloramphenicol (50 $\mu\text{g ml}^{-1}$) at 30°C. *E. coli* strains JM109 and BL21(DE3) were used for gene expression from pUCmod and pET-plasmids, respectively.

E. coli strain BW27784 [40] was used for *in vivo* analysis of stilbene cleavage by CCO homologs. A modified M9 medium containing yeast extract (1.25 g/L), glycerol (0.5% v/v) and appropriate antibiotics was used for stilbene biosynthesis as described previously.[32]

Protein expression and purification

Both pET-CCO and pUCmod-CCO plasmids were used for protein overexpression. For expression of genes from pUCmod-CCO, recombinant *E. coli* JM109 overnight cultures (4 ml) were used to inoculate 1:100 LB medium (400 ml) containing the appropriate antibiotics. Cultures were grown for 16 hrs at 30°C and cells were harvested by centrifugation and stored at -20 °C until used. Cells collected from the culture (50 ml) were lysed with BugBuster® protein extraction reagent (2 ml) (Novagen, Madison, WI). Cell debris was cleared by centrifugation (14,000 rpm, 5 minutes, 4°C) and the cleared protein extract used in *in vitro* activity assays. Protein levels were estimated by SDS gel electrophoresis and concentrations adjusted so that comparable levels were added to assays.

The pET-CCO plasmids were used for the overexpression of CCO proteins for protein purification. As previously observed with other CCO's [4, 21], these proteins are prone to inclusion body formation when expressed at high levels (e.g. from the strong T₇-promoter present in pET plasmids). To facilitate the expression of soluble proteins, pET-CCO plasmids were transformed into *E. coli* BL21(DE3) harboring plasmid pGRO7 (Takara, Madison, Wisconsin) that expresses the GroEL and GroES chaperones (see also [4]). *E. coli* BL21 co-transformed with putative CCO homolog and groES-groEL were grown overnight at 30°C in LB media (4 mL). This culture was used to inoculate (1:100) LB (400 mL) and chaperone expression was induced with arabinose (0.5 mg/mL). The cells were grown at 30°C until an OD₆₀₀ of 0.6. Then the cultures were cooled on ice and induced with isopropyl- β -D-thiogalactopyranoside (IPTG, 1 mM) before cultivation was continued overnight at 18°C and cells were harvested by centrifugation and stored at -20 °C until used. Cells collected from culture (50 ml) were lysed with BugBuster® protein extraction reagent (2 ml) (Novagen, Madison, WI). Cell debris was cleared by centrifugation (14,000 rpm, 5 minutes, 4°C). Aliquots of the cleared protein extract were saved for *in vitro* assays and the remainder used to purify CCO proteins by metal affinity chromatography. Soluble protein was loaded onto a Talon Resin immobilized metal affinity chromatography (IMAC) (Invitrogen, Carlsbad, CA) column and eluted in 50 mM phosphate buffer pH 7.2 with imidazole (300 mM) following three washing steps. The CCO proteins eluted in a fraction (4 mL) and was concentrated using an Amicon ultracentrifuge concentrator with a 10 kDa

molecular weight cut off. The Amicon concentrator was used to desalt the protein by exchanging the buffer 4 times against phosphate buffer (50 mM pH 7.2). The protein was subjected to iron center reconstitution by incubation with FeSO₄ (100 mM) under argon gas for 30 minutes to insure incorporation of Fe²⁺ into the active site. Protein concentrations were determined using Bradford reagent (BioRad, Hercules, CA).

In vivo analysis of carotenoid cleavage activity

To investigate in vivo carotenoid cleavage, CCO enzymes on pUCmod were co-expressed with β , β -carotene biosynthetic genes expressed from pAC-crtE-crtB-crtI14-crtY^[31], pAC-crtE-crtB-crtI14-crtY-crtX, pAC-crtE-crtB-crtI14-crtY2 in *E. coli* JM109 as described previously for cyanobacterial CCO.^[4] Briefly, single colonies of *E. coli* JM109 transformants harboring the carotenoid and CCO plasmids to be tested were grown overnight and for 48 hrs in LB media (50 ml) supplemented with ampicillin and chloramphenicol at 30°C. The color intensities of the resulting cell pellets from six replicate cultures were then compared by visible inspection to control cells harboring the corresponding carotenoid plasmid and empty pUCmod plasmid.

In vivo analysis of stilbene cleavage activity

CCO enzymes on plasmid pAC-4CL-CCO also containing 4-coumaroyl CoA-ligase 4CL were co-expressed with stilbene synthase (STS) from *Arachis hypogaea* (peanut) on the constitutive expression vector pUC-STS. The construction of stilbene and flavonoid biosynthetic pathways has been described previously^[31,32].

To investigate in vivo stilbene cleavage by CCO's, single colonies of *E. coli* BW27784 transformants harboring plasmids pUC-STS and pAC-4CL-CCO or only pAC-4CL-CCO (control for cleavage of CoA-activated phenylpropionic acids) were grown overnight in modified M9 medium (4 ml) at 30°C and used to inoculate 1:100 modified M9 medium (50 ml). Cultures were grown to an OD of 0.1 at 30°C when phenyl propionic acid precursor compounds (1 mM, 200 μ l of 4-coumaric acid, caffeic acid or ferulic acid in DMSO) were added to the cultures to initiate their biotransformation into stilbene compounds by the recombinant *E. coli* pathway (STS and 4CL). Following an additional 16 hrs of cultivation at 30°C, the culture supernatant was extracted and analyzed for product formation essentially as described in Watts et al. 2006^[32]. In brief, culture (1 ml) was centrifuged at maximum speed to pellet cells. The media was decanted to a fresh 1.5 ml microfuge tube and the pH was adjusted by addition of 1N hydrochloric acid (50 μ l). Then the media was extracted twice with ethyl acetate (500 μ l) and the extracts were combined and dried under nitrogen. The dried residue was resuspended in methanol (100 μ L) and all samples were stored at -20°C prior to HPLC and LC-MS analysis (see below).

In vitro assays

Assays were performed with purified proteins (100-250 μ g) or protein extracts (50 μ L) in 300 μ l reactions containing phosphate buffer (50 mM, pH 7.2), NaCl (300 mM), sodium ascorbate (10 mM) and FeSO₄ (0.5 mM). After 5 min of equilibration, stilbene substrates (1 mM from 1M resveratrol, piceatannol or rhapotinigenin dissolved in DMSO) or carotenoid substrate (0.27 mM from 2 mM β -apo-8'-carotenal dissolved in 1% Tween 40^[4]). *In vitro*

reactions were carried out at 30°C for the prescribed amount of time (5 min to 12 hrs) before being stopped with HCL (1N, 50 µL) and extracted three times with ethyl acetate (500 µl). β-apo-8'-carotenal assays were extracted with diethyl ether. The organic fractions were combined, dried under nitrogen and stored until HPLC or GC analysis.

Isotope labeling

For labeling experiments with H₂¹⁸O, the protein extracts were freeze-dried to remove all water and residues were resuspended in of H₂¹⁸O (100 µl). No buffer, NaCl or FeSO₄ was added to avoid contamination with unlabeled H₂O. Reactions were started by adding resveratrol (1 mM in DMSO). After 15 min of incubation at 30°C, assays were extracted two times with ethyl acetate (500 µl), dried under nitrogen and immediately derivatized to silyl ethers for GC-MS analysis as described below.

Labeling experiments with ¹⁸O₂ were performed in screw-capped glass vessels (2 ml) with a gas-tight Teflon septum using reaction conditions described above for standard assays with resveratrol. The vials were flushed three times with ¹⁸O₂ and protein extract was added with an airtight Hamilton syringe. The reaction mixture was allowed to equilibrate for 5 minutes before resveratrol (1 mM) was injected into the vial. The reaction was stirred for 15 minutes before being extracted two times with ethyl acetate (500 µl). Samples were dried and immediately derivatized to silyl ethers for GC-MS analysis as described below.

HPLC and LC/MS analysis

HPLC analysis was performed using an Agilent 1100 HP system with a quaternary pump and a photodiode array detector (Palo Alto, CA). Several HPLC conditions were utilized to analyze possible β-apo-8'-carotenal cleavage products as described in Marasco et al. [4]. Briefly, cleavage of β-apo-8'-carotenal at the 15,15' position to retinal was analyzed by applying sample (50 µL) to an Adsorbosil C-18 column (4.6 × 250 mm, 5 µm; Alltech, Deerfield, IL). The gradient program was modified from Ruch et al. [10] using a solvent system of MeOH : *tert*-butylmethyl ether : water (120:4:40, v/v/v) (B) and MeOH : *tert*-butylmethyl ether (500:500 v/v) (A). The gradient conditions were solvent B (100%) to solvent B (43%) over 45 min, solvent B (43%) to solvent B (0%) for 11 min, solvent B (0%) for 14 min with a flow rate of 1 mL min⁻¹. Dialdehyde cleavage products were determined by applying sample (100 µL) to a Zorbax RX-C18 column (4.6 × 250 mm, 5 µm; Agilent Technologies, Palo Alto, CA). The solvent system was MeOH : water (70:30, v/v) with 0.1% ammonium acetate (B) and MeOH (A). The gradient conditions were solvent B (100%) to solvent B (0%) over 16 min, solvent B (0%) until 26 min, and then return to A (100%) with a flow rate of 0.8 mL min⁻¹.

Stilbene cleavage products were detected using conditions modified from HPLC methods described by Watts et al. [32] for the analysis of stilbene compounds. 20 µL of sample was applied to a reverse phase Eclipse XDB-C8 column (4.6 × 150 mm, 5 µm; Alltech, Deerfield, IL) and analyzed with an isocratic program using a solvent system of water:trifluoroacetic acid (99.9:0.1, v/v) (A) and methanol:trifluoroacetic acid (99.9:0.1, v/v) (B) in a ratio of 73:27 with a flow rate of 0.8 ml min⁻¹. To achieve better resolution of piceatannol cleavage products, a gradient program was used with a flow rate of 0.5 mL/min

and the following conditions: from 0-10 min 75:25 A:B, followed by a gradient from 75:25 A:B to 50:50 A:B in 15 minutes, followed by 5 min 50:50 A:B. Stilbenes and cleavage compounds were identified by comparisons of retention times and UV/Vis spectra of standard compounds (resveratrol, piceatannol, rhaptonin, ferulic acid, coumaric acid, caffeic acid, 4-hydroxybenzaldehyde, 3,5-dihydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde) and mass spectrometry. For quantification of products, standard curves were constructed by plotting peak areas of known quantities of standards.

Mass fragmentation spectra were monitored in a mass range of m/z 50–500 on a LCQ mass spectrophotometer equipped with electrospray chemical ionization interface (Thermo Finnigan, USA). Mass fragmentation spectra of standard compounds and the extracted compounds were monitored with a negative electron spray ionization (ESI) interface. Negative ion values for standard compounds were as follows: 4-coumaric acid (m/z 163.1), caffeic acid (m/z 179.1), ferulic acid (m/z 193.1), resveratrol (m/z 227.1), piceatannol (m/z 243.1), 4-hydroxybenzaldehyde (m/z 121.0), 3,5-dihydroxybenzaldehyde (m/z 137.0). The chromatography conditions were identical to the HPLC conditions described above excluding the addition of trifluoroacetic acid.

GC-MS analysis

For GC-MS analysis, dried samples were derivatized to silyl ethers by addition of BSTFA (50 μ l) (N,O-Bis(trimethylsilyl)acetamide) reagent. GC-MS analyses were performed with a HP6890 Series GC coupled to a HP5973 mass selective detector. GC conditions consisted of an HP-5 column (30 m by 0.25 mm ID by 1.5 μ m coated with 5% phenyl methyl silicone) and a split injector (1:20) set to a temperature of 250 °C. The temperature started at 60 °C and increased to 280 °C at 8°C/minute intervals with a helium flow rate of 1 ml/min. The EI-MS ionization voltage was 70 eV (electron impact ionization) and the ion source and interface temperature were both 250 °C. Mass spectra were scanned in a range of m/z 40-500 at 1 sec intervals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Science Foundation (#0332478). E. Marasco is supported by the National Institute of General Medical Sciences / National Institutes of Health (NIGMS/NIH) Biotechnology Training Grant (#T32 GM08347). We thank Tom Markis (Lipscomb Laboratory, University of Minnesota) for his help in the labeling experiments, and the Wilmot laboratory (University of Minnesota) for their generous gift of labeled water.

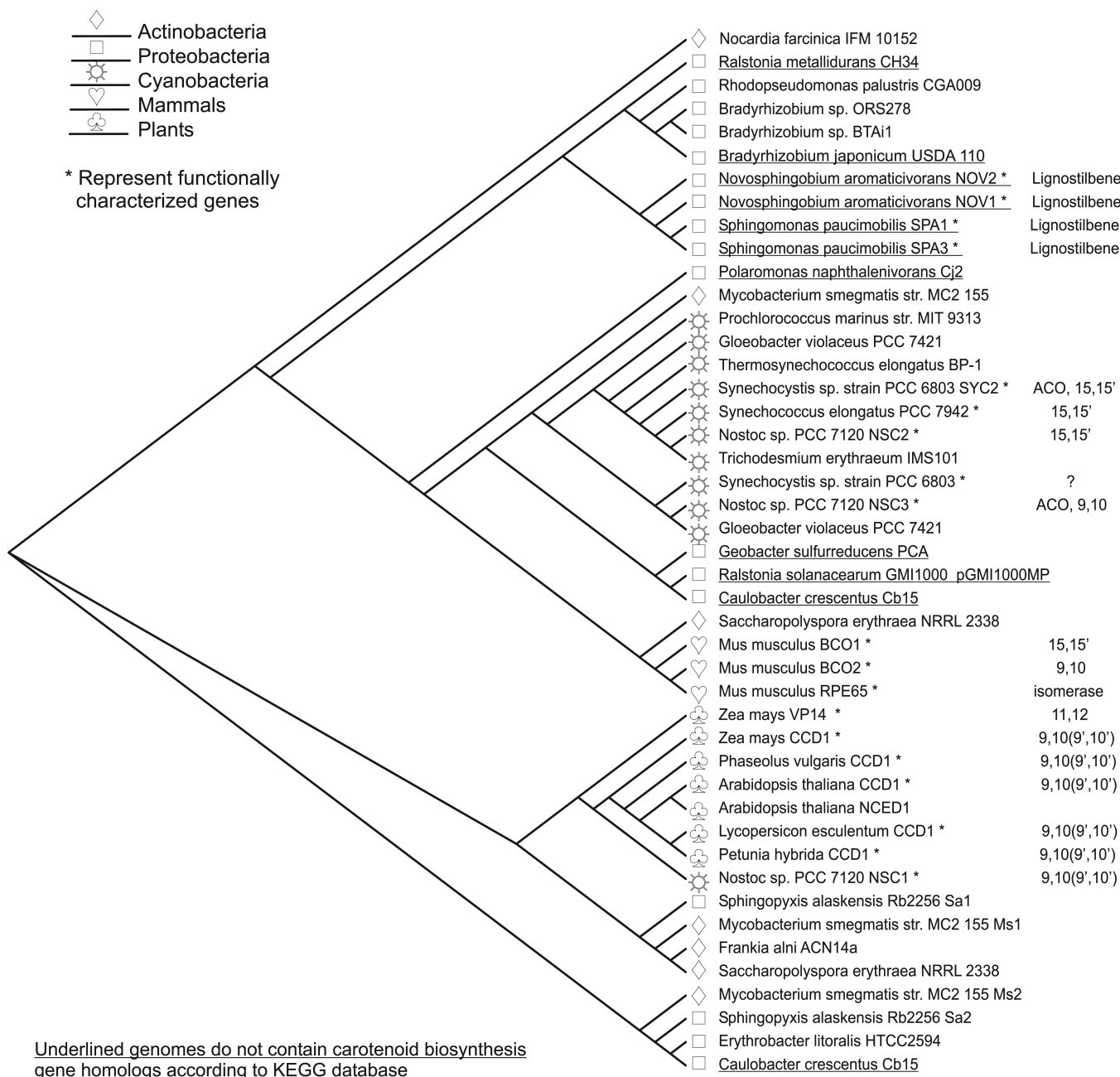
The abbreviations used are

CCO	carotenoid cleavage oxygenase
NCED	9-cis-epoxydioxygenase
LSD	lignostilbene-alpha,beta-dioxygenases
STS	stilbene synthase

4CL 4-coumaroyl ligase**References**

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**Figure 1.**

Phylogenetic tree [average distance by percent identity] from NOV1 and NOV2 amino acid sequences with additional representatives from sequenced microbial genomes (BLAST hits with high identity) and other characterized carotenoid oxygenase family representatives.

Lignostilbene oxygenase activity: NOV1 (accession no. YP_496081), NOV2 (accession no. YP_498079) (this study), *Sphingomonas paucimobilis* isoform 1 (SPA1, accession no. AAC60447) [23] and isoform 3 (SPA3, accession no. AAB35856). [24] **Apocarotenoid cleavage activity:** *Synechocystis PCC6803* (SYC2, accession no. S76169), [10] *Nostoc* sp. PCC7120 9,10 (NSC3, accession no. ZP_00112423). [4] **15, 15' carotenoid cleavage**

activity: *Synechococcus elongates* PCC 7942 (SYO, accession no. ZP_00351210) (unpublished), *Nostoc* sp. PCC7120 (NSC2, accession no. accession no. AE2341),^[4] mouse 15,15'-dioxygenase (MmBCO1, accession no. Q9JJS6).^[41] **Unknown activity:** *Bradyrhizobium japonicum* USDA110 (BRA-J, accession no. NP_772430) (this study), *Bradyrhizobium* sp. Btai1 (BRA-S, accession no. ZP_008636652) (this study), *Synechocystis* PCC6803 (SYC1, accession no. S76206).^[10] **9,10-9'10' carotenoid cleavage activity:** mouse 9,10-9'10'-dioxygenase (MmBCO2, accession no. Q99NF1),^[9] *Zea mays* (*ZmCCD1*, *ABF8565B*), *Phaseolus vulgaris* (*PvCCD1*, *Q94IR2*)^[6], *Arabidopsis thaliana* (*AtCCD1*, accession no. NP_191911.1)^[6], *Lycopersicon esculentum* (*LeCCD1*, accession no. AAT68187),^[17] *Petunia × hybrida* (*PhCCD1*, accession no. AAT68189),^[18] *Nostoc* sp. PCC7120 (NSC1, accession no. BAB73063).^[4] **Isomerase activity:** mouse RPE protein (MmRPE65, accession no. Q91ZQ5);^[42] **9-cis-epoxycarotenoid 11,12 cleavage activity:** *Zea mays* (*VP14*, accession no. AAB621811.1),^[16] *Arabidopsis thaliana* (*AtNCED1*, accession no. AAN17413). Additional enzymes and accession numbers for putative oxygenases can be found in Supplemental Table 1.

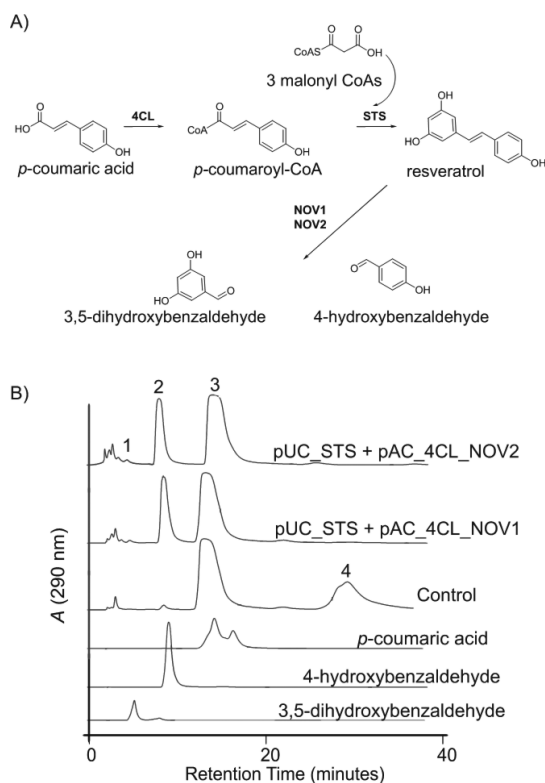


Figure 2.

In vivo cleavage of resveratrol by NOV1 and NOV2. A) Engineered pathway in *E. coli* for resveratrol biosynthesis from fed coumaric acid and cleavage of synthesized resveratrol in *E. coli* by co-expressed NOV enzymes. Enzymes shown are 4-coumaroyl:CoA ligase (4CL; EC 6.2.1.12), stilbene synthase (STS; EC 2.3.1.95), and NOV oxygenases (NOV1, NOV2). B) HPLC analysis of extracts from coumaric acid fed recombinant *E. coli* cultures co-expressing stilbene biosynthetic genes and NOV1 (pUC_STS + pAC_4CL+NOV1) or NOV2 (pUC_STS + pAC_4CL+NOV2). The control culture contained only stilbene biosynthesis genes (pUC_STS + pAC_4CL). Shown are HPLC traces of culture extracts and of authentic standard compounds. Peaks are (1) 3,5-dihydroxybenzaldehyde (m/z 137.0), (2) 4-hydroxybenzaldehyde (m/z 121.0), (3) *p*-coumaric acid (m/z 163.2), (4) resveratrol (m/z 227.1). Control cultures convert coumaric acid to resveratrol. Addition of the oxygenase enzymes NOV1 and NOV1 results in a decrease in resveratrol and the appearance of 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde. The products were confirmed by standards and mass spectral analysis. 3,5-dihydroxybenzaldehyde is not extracted from the medium in stoichiometric amounts.

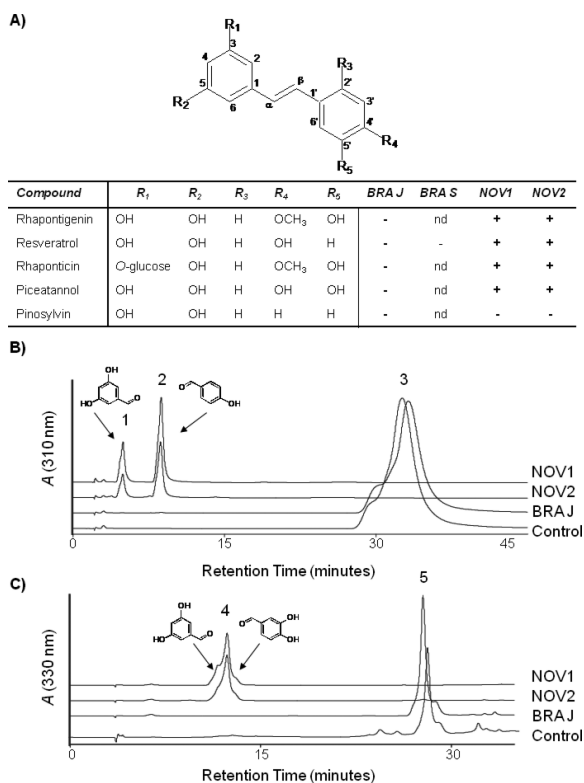


Figure 3.

In vitro cleavage of stilbene compounds. A) Stilbene compounds with different functional groups were chosen as substrates for in vitro assays with NOV1, NOV2, BRA-J, and BRA-S protein lysates. Cleavage of the substrates is indicated by a + in the table based on product identification by HPLC and LC-MS. None of the enzymes cleaved pinosylvin. B) HPLC analysis of in vitro assays with resveratrol as a substrate. Synthesis of 3,5-dihydroxybenzaldehyde (1) and 4-hydroxybenzaldehyde cleavage products (2) from 1 mM resveratrol (3) with NOV1 and NOV2 protein extracts. No residual resveratrol was detected by HPLC or LCMS. C) HPLC trace of in vitro assay with piceatannol as a substrate. Synthesis of 3,5-dihydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde cleavage products (4) from 1 mM piceatannol (5) with NOV1 and NOV2 enzymes protein extracts. Small amounts of residual piceatannol could be detected for NOV2. BRA-J protein extracts did not produce cleavage products with either resveratrol or piceatannol. Protein extracts from *E. coli* served as a control.

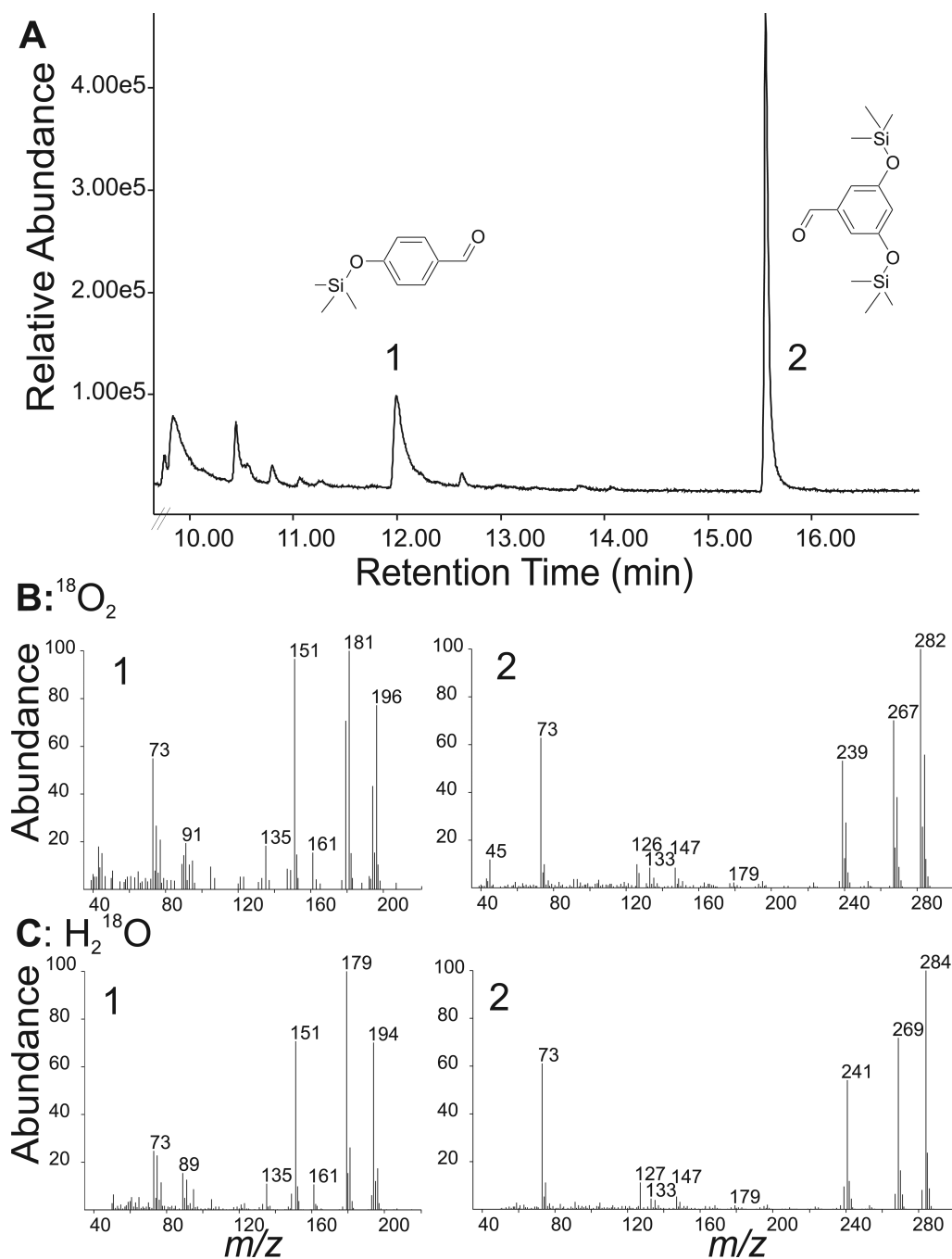
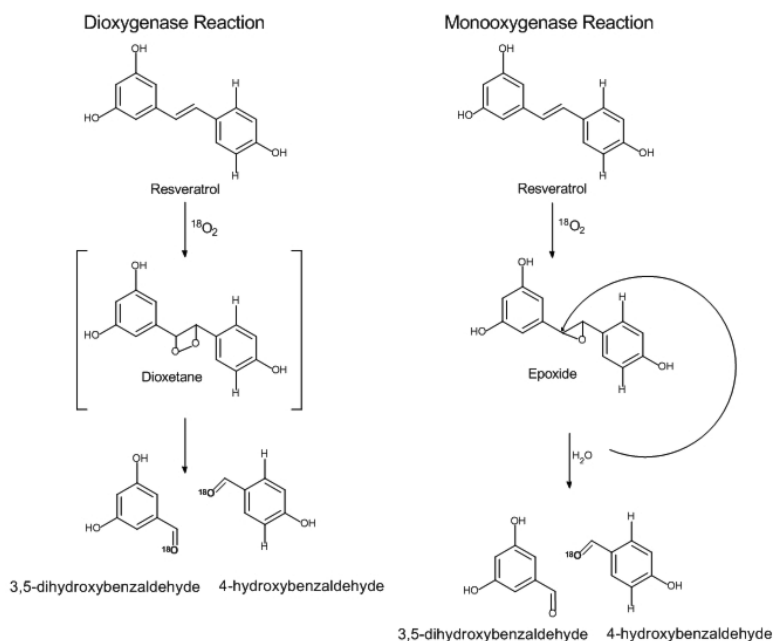


Figure 4. GC-MS analysis of oxygen labeled cleavage products of resveratrol synthesized by NOV2. A) Silylated esters of cleavage products 4-hydroxybenzaldehyde (1) and 3,5-dihydroxybenzaldehyde (2). B) Incorporation of molecular oxygen into 4-hydroxybenzaldehyde. Mass spectra of 4-hydroxybenzaldehyde (left spectrum) and 3,5-dihydroxybenzaldehyde (right spectrum) in $^{18}\text{O}_2$ -atmosphere, showing labeled 4-hydroxybenzaldehyde (m/z 196) and unlabeled 3,5-dihydroxybenzaldehyde (m/z 282). C) Mass spectral data from incorporation of oxygen from water H_2^{18}O . Incorporation of label

into 3,5-dihydroxybenzaldehyde (m/z 284), but 4-hydroxybenzaldehyde remains unlabeled (m/z 194). Note that the main fragments in panels 1B and 1C (m/z 181 and m/z 179) result from the loss of a $[-CH_3]$ group from 4-hydroxybenzaldehyde.

**Scheme 1.**

Possible mechanisms for the oxidative cleavage of resveratrol. Shown are two proposed mechanisms for oxidative cleavage with CCO enzymes. A dioxygenase mechanism results in both aldehyde cleavage products labeled with isotopic ^{18}O when the reactions are performed in an ^{18}O atmosphere. The monooxygenase mechanism results in a single isotopic ^{18}O labeled cleavage product when reactions are performed in an ^{18}O atmosphere.

Table 1

Summary of labeling pattern from isotope experiments

	Product Mass	$^{18}\text{O}_2$	Product Mass	H_2^{18}O
3,5-dihydroxybenzaldehyde	<i>m/z</i> 282	Unlabeled ~65%	<i>m/z</i> 284	Labeled ~92%
4-hydroxybenzaldehyde	<i>m/z</i> 196	Labeled ~69%	<i>m/z</i> 194	Unlabeled ~90%