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Evolutionary aspects and enzymology of metazoan carotenoid cleavage oxygenases

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Abstract

The carotenoids are terpenoid fat-soluble pigments produced by plants, algae, and several bacteria and fungi. They are ubiquitous components of animal diets. Carotenoid cleavage oxygenase (CCO) superfamily members are involved in carotenoid metabolism and are present in all kingdoms of life. Throughout the animal kingdom, carotenoid oxygenases are widely distributed and they are completely absent only in two unicellular organisms, *Monosiga* and *Leishmania*. Mammals have three paralogs 15,15'-β-carotene oxygenase (BCO1), 9',10'-β-carotene oxygenase (BCO2) and RPE65. The first two enzymes are classical carotenoid oxygenases: they cleave carbon-carbon double bonds and incorporate two atoms of oxygen in the substrate at the site of cleavage. The third, RPE65, is an unusual family member, it is the retinoid isomerohydrolase in the visual cycle that converts all-*trans*-retinyl ester into 11-*cis*-retinol. Here we discuss evolutionary aspects of the carotenoid cleavage oxygenase superfamily and their enzymology to deduce what insight we can obtain from their evolutionary conservation.

Keywords

carotenoids; carotenoid oxygenases; phylogenetic; BCO2; RPE65; enzymes; palmitoylation; membrane associated proteins

Carotenoid cleavage oxygenase superfamily and their versatility

Members of the carotenoid cleavage oxygenase (CCO) superfamily are present in all kingdoms of life [1], including lignostilbene dioxygenases (LSDs) in eubacteria [2] and apocarotenoid oxygenases (ACOs) in cyanobacteria [3, 4]. In plants, there are diverse carotenoid cleaving enzymes (CCDs) [5–8], and epoxycarotenoid-cleaving enzymes (NCEDs) that are required for plant abscisic acid biosynthesis [9,10]. CCOs, such as

torulene oxygenases (TOs) [11], are also widespread in fungi. CCOs exist widely in other eukaryotes, pointing to important common functions for CCOs among these organisms [12]. Interestingly, we have observed only two instances of complete loss of the carotenoid oxygenase superfamily members in the genomes of eukaryotes (in the choanoflagellate *Monosiga* and the trypanosome *Leishmania*). In metazoans, carotenoid cleaving and double bond isomerizing enzymes (such as NinaB (=neither inactivation nor afterpotential B, from the electrophysiological phenotype of mutant Drosophila) are found in insects [13], and other BCO-related paralogs are widespread in invertebrate and vertebrate animals (BCOs) [14–16]. In vertebrates we have an unusual member of the family-Retinal pigment epithelium-specific 65 kDa protein (RPE65) retinoid isomerohydrolase, which is required for production of visual pigment chromophore [17–19].

All CCOs enzymes use polyenes with conjugated double bonds as their substrates and most of the enzymes cleave carotenoids (C40) or apocarotenoids analogs shortened by oxidative cleavage (<C40) [20]. On the other hand, some bacterial enzymes use lignostilbenes (diarylethenes) or stilbenoids (hydroxyl derivatives of stilbenes)[21, 22]. Only vertebrate RPE65 utilizes a C20-retinoid (retinyl ester) which, strictly speaking, can be considered a form of apocarotenoid. RPE65 is not an oxygenase; historically RPE65 was termed an isomerohydrolase [17, 19], and now is termed an isomerase [23, 24]. Specifically, it is confusing to term it a hydrolase as it performs an O-alkyl cleavage, cleaving between the retinoid and the ester oxygen rather than between this oxygen and the acyl group (an O-acyl cleavage, which is implied for a classical hydrolase) [25–27]. It has been described in the literature that various oxygenases (incorporation of oxygen) work as hydroxylases (incorporation of hydroxyl group), especially in the presence of a second reductant, e.g. NADH [28]. For example, the enzyme methane monooxygenase catalyzes the incorporation of one atom of an oxygen molecule into the substrate, while the other atom is reduced to water, and works as hydrolase for alkanes but as an oxygenase for carbon monoxide [28].

Insect NinaB and vertebrate RPE65 members have acquired an intrinsic isomerization activity and produce visual chromophores (11-*cis* retinoids) in their respective taxa. Insect chromophore (R-11-*cis*-3-hydroxy-retinal) is produced by isomerization and cleavage of zeaxanthin (3,3'-R,R-dihydroxy-β,β-carotene), though in some species the product of the NinaB enzyme will require further enantiomerization or hydroxylation [29]. Similarly, to produce the vertebrate visual chromophore (11-*cis*-retinal), RPE65 cleaves the O-alkyl ester bond of all-*trans*-retinyl ester and then isomerizes the retinyl moiety to produce 11-*cis*-retinol [26, 27] that is subsequently oxidized to 11-*cis*-retinal by retinol dehydrogenase 5. It has been proposed that only one-half of the carotenoid substrate is isomerized by the moth *Galleria* NinaB enzyme (gNinaB) [29], and, recently, this has been reported for several other insect species [30]. Therefore, both enzymes are able to isomerize a conjugated C20 fragment from all-*trans* to the 11-*cis* configuration. Isomerase activity has been described for BCO1 with 9-cis-beta-carotene substrate, though the possibility of thermal isomerization after the enzymatic reaction cannot be excluded [31, 32]. The mechanism of isomerization by RPE65, on the one hand, had been controversial [25,26] but more recent structural studies with inhibitors [33, 34] and using site-directed mutagenesis [27, 65] point to the formation of a delocalized carbocation and the transient destabilization of the double bond order upon O-alkyl cleavage as prerequisite to isomerization. For gNinaB, on the other hand, it is

possible the all-*trans* to 11-*cis* isomerization could be driven by the oxidative cleavage of the carotenoid, though this is much less well established [29, 30].

Recently, we described a new ancestral clade within the animal CCO superfamily, the BCO-like (BCOL) carotenoid oxygenases [1]. These enzymes were first identified in lancelet along with the several other non-chordate animals (8 BCOLs are present in lancelet with 23–28% amino acid identity with lamprey BCO2). All the BCOLs lack the conserved PDPCK motif (palmitoylation site for RPE65 [35]; see below) present in all other animal CCOs. Moreover, the N-terminal sequences of the various BCOLs are predicted by the TargetP [36, 37] program as being signal peptides [1] and overexpression of BCOLg in HEK293F leads to significant secretion of the protein in the medium (unpublished results). Significantly, no other metazoan CCOs are secreted. BCOLs are only known to occur in marine non-vertebrate organisms, and it is possible that they only occur when extracellular carotenoid metabolism is required. Among these, the BCOLg enzyme preferentially cleaves *cis* isomers of beta-carotene. Previously, ferret BCO2 demonstrated higher activity towards 5-*cis* and 13-*cis* isomers of lycopene than to all-*trans*-lycopene [38]. We propose that individual enzymes have evolved to metabolize cis isomers of carotenoids depending on their availability in environment [1].

We and others have cloned various metazoan and chordate CCOs [1, 39]. Lancelet and *Ciona* CCOs are β-carotene cleaving enzymes while *Petromyzon* BCO2 can cleave lycopene but not β-carotene, and *Nematostella* BCOc strongly prefers lycopene over β-carotene. On the other hand, all the cloned CCO members of the tunicate *Oikopleura dioica* demonstrated no activity towards β-carotene [39]. Marti-Solans *et al.* suggested that the BCO1 lineage has been lost in *O. dioica* [39], but we question whether the true BCO1 lineage starts in the ancestral Chordata because the RPE65, BCO1 and BCO2 clades are clearly defined only in vertebrates (Figure 1) [18, 40]. A common method to investigate the function of genes putatively involved in carotenoid biosynthesis and metabolism is the so called color complementation assay in *Escherichia coli* [41]. In this assay, the gene under investigation is expressed in *E. coli* strains genetically engineered to synthesize and accumulate potential carotenoid substrates [42, 43], followed by analysis of color changes in the carotenogenic bacteria visually and by high-performance liquid chromatography (HPLC). The method has an array of limitations including possible insolubility of overexpressed enzymes and a limited variety of substrates, however it is the most widely used activity assay for carotenoid oxygenases because of comparatively simple high-throughput methodology [21, 44–46]. In color complementation activity assays we do not see a significant difference between *Ciona* ciBCOa and ciBCOb catalytic activity towards β-carotene, and we do not detect all-*trans*-retinal for either enzyme while all-*trans*-retinal is clearly detected in the case of mouse BCO1 [18]. We used phylogenetic analysis to reconstruct the evolutionary history of the CCO superfamily, a commonly used approach to define lineage-specific losses and gene duplication events (see the “Concluding Remarks” section for more details). *Ciona* BCOb is close to vertebrate BCO1 members on the phylogenetic tree, however it is hard to claim that they are true orthologs based on bootstrap confidence level, even with the large bootstrap value (89%; Figure 1), because of the established functional differences discussed above. We cannot exclude long branch attraction artifacts [47], taking into account that many internal branches have low bootstrap values and the branch leading to the putative lamprey BCOl is

also relatively long (Figure 1). Thus, an ancestral function (and substrate specificity) for the last common ancestor of the *Ciona*, lamprey and jawed vertebrates BCO1 clade cannot be inferred.

In general, detection of functionally similar genes is a difficult problem for functionally volatile gene families. Statements about losses of genes that perform certain functions could be misleading unless a putative gene loss event is nested deep in the well-defined clade of (mostly) single-copy genes. All claims about the presence or absence of functionally similar genes should be made with much caution, especially for multigene families [48–50] (see the "concluding remarks" for more details).

Evolutionary conservation of catalytic site and mechanism of carotenoid oxygenases

The first crystal structure obtained of a CCO member was that of the apocarotenoid-15,15'-oxygenase (ACO) from *Synechocystis* sp. PCC 6803, solved in early 2005 [3]. The authors described an unusual Fe^{2+}-4-His arrangement at the center of a seven-bladed β-propeller chain fold [3]. Concurrently, working on mouse BCO1 catalytic activity, we demonstrated that the 4 histidines perfectly conserved in the whole CCO family are required for the catalytic activity of mouse BCO1 and proposed that these residues participate in iron coordination [51]. As expected, the mutation of paralogous conserved histidines (H180A, H241A, H313A, H527A) in dog and human RPE65 in a non-RPE cell culture system (minimal visual cycle model) led to abolishment of RPE65 activity [19, 52]. Since then bovine RPE65 [24], maize VP14 [53], stilbenoid-cleaving NOV1 [22] and NOV2 [54] from *Novosphingobium aromaticivorans* and *Neurospora crassa* carotenoid oxygenase 1 (CAO1) [54] crystal structures confirmed that the overall fold and active site arrangement is conserved throughout the superfamily. Most characterized non-heme Fe(II) dioxygenases exhibit sequential binding of substrates and dioxygen molecule and in these cases substrate binding affects the electronic properties of the iron atom and can gate O_2 binding [55]. Dioxygen was observed in two crystal structures (VP14 and NOV1), with or without substrate and binding of substrate did not cause significant conformational changes to the enzymes [22, 53]. The question of the presence of dioxygen in NOV1 structure is still open to discussion in the literature [56, 57]. Substrate binding in the stilbenoid-cleaving NOV2, CAO1 and apocarotenoid ACO oxygenases did not induce a significant electronic change in the iron as observed by Mossbauer spectroscopy, nor did it induce significant protein structural changes and therefore such gating mechanisms are not likely to apply for these enzymes [54]. These authors hinted that the direct interaction of oxygen and substrate could stabilize its binding to the iron center in these cases [54]. The observed formation of an Fe-O_2 moiety in the absence of substrate in NOV1 suggests that the reaction starts with a Fe^{3+}-superoxo intermediate and proceeds through a deprotonation step of 4'-OH by Y101 and K135 in activation of the substrate [22] (Figure 2A). The proposed VP14 mechanism follows the same oxygen activation pattern where Fe^{3+}-O_2^-, a radical superoxide, attacks the double bond resulting in a radical species that can react with the second oxygen to form the dioxetane species that spontaneously produces the two aldehydes [53] (Figure 2A). The Mossbauer spectroscopy implied the differences in apparent charge at the Fe(II) center

between the substrate-binding site in CAO1 and ACO [54]. The substrate binding site in CAO1 is more polar and involves hydrogen bonding, compared to ACO where substrate recognition mainly involves nonpolar and π-stacking interactions [54]. DFT analysis of the ACO mechanism predicted that binding of dioxygen promotes one-electron oxidation of carotenoid to a radical cation ($car^{\cdot+}$) and therefore activation of oxygen occurs by interaction with a suitable substrate (carotenoid) rather than by one-electron oxidation of iron [58]. Further, while it can proceed through a dioxetane or epoxide species, DFT analysis slightly favored a dioxetane pathway for ACO [58] (Figure 2B). BCO1 was first proposed to be a monooxygenase [59] but later was described as a dioxygenase [60]. Several plant CCOs also were shown to be dioxygenases [61, 62], as well as NinaB enzyme [63]. Recently ACO and NOV2 were shown to be dioxygenases and the former monooxygenase hypothesis was explained by the fast exchange of oxygen and water in the resulting aldehydes [60, 64]. No evidence for incorporation of molecular oxygen in the product of RPE65 has been presented so far and, moreover, it was shown that water is the source of the hydroxyl group in the isomerized product [24]. Several years ago, we proposed that a retinoid cation radical intermediate participates in the catalytic mechanism of RPE65 as opposed to a carbocation intermediate [26], and subsequently demonstrated effective inhibition of RPE65 catalytic activity by spin trap compounds [27, 65, 66]. Additionally, inhibition by spin trap compounds has been shown for NinaB enzyme and the authors concluded that NinaB followed a dioxygenase mechanism with a cation radical intermediate [63] (Figure 2B). Such a mechanism would allow oxygen to be the electron acceptor, generating a retinoid cation radical intermediate similarly to the one discussed in the DFT analysis for ACO [58]. We speculate that isomerization is an intrinsic property of carotenoid oxygenases, where a permanent product isomerization by NinaB and a temporary one by ACO (still highly contentious [67]) are enforcing cases for the cation radical intermediate [3, 29, 63]. The most plausible explanation is that the isomerization reaction catalyzed by RPE65 and NinaB involves a radical cation intermediate stabilized around the 11, 12-carbon double bond by specific residues [27, 63, 66]. However, RPE65 activity was shown to be indifferent to oxygen depletion or supplementation, which complicates the implication of oxygen in the mechanism [64].

Substrate specificity and intrinsic flexibility in substrate recognition

The specificity of the carotenoid cleavage oxygenase family is very broad and evolutionarily flexible. Mammalian and chicken BCO1 prefer β-carotene but additionally can cleave other provitamin A and non-provitamin A carotenoids such as lycopene and some β-apocarotenals, as well as substrates with one β-ionone substituted ring such as cryptoxanthin [45, 68–70]. Substrate specificity of vertebrate BCO2s is even more broad, encompassing oxidative cleavage of β-carotene, α-carotene, β-cryptoxanthin, and the xanthophylls lutein and zeaxanthin, but not apocarotenoids [15, 71, 72]. A recent study on β-cryptoxanthin described in detail the Interaction of mammalian BCO1 and BCO2 with ionone ring sites and the stepwise production of Vitamin A from an asymmetric substrate [73], Additionally, purified chicken BCO2 and murine BCO2 were reported to cleave 9-*cis*-β-carotene [31, 72], and ferret BCO2 was shown to cleave 5-*cis*- and 13-*cis*-isomers but not the all-*trans* isomer of lycopene [38]. A distant relative of BCO2 from the new branch of BCOL proteins,

Lancelet BCOLg also preferred *cis* isomers of β-carotene [1]. Even though CCO subfamilies share preferred substrates between orthologs (Table 1), the elucidation of substrates for even evolutionarily related paralogs is much trickier.

The fusarium carotenoid cleavage enzyme CarX and NCS3 *Nostoc* ACO can cleave β-carotene, torulene and β-Apo-8'-carotenal [74, 79, 80]. PpCCO from the marine myxobacterium *Plesiocystis pacifica*, which cleaves hydroxy and keto carotenoids (C40 as well as C50 carotenoids) [81], and the cyanobacterial NCS3 *Nostoc* ACO, with apocarotenoid and torulene cleavage activity [79], are closely related in sequence and evolutionarily close (boot strap support = 80, Figure 1) [1]. Bacterial CCOs have an even more complex pattern of preferred substrates and products. For example, *M. tuberculosis* carotenoid cleavage oxygenase (MtCCO) processes β-carotene, hydroxylated and aromatic carotenoids and apocarotenoids and cleaves C15-C15' and/or the excentric double bond at the C13-C14 position, depending on substrate [82]. Its close relative (boot strap support = 87, Figure 1) *Sphingopyxis alaskensis* RB2256 carotenoid cleavage oxygenase (SaCCO), encoded by *sala_1698*, was shown to cleave acyclic and monocyclic substrates such as lycopene, hydroxylycopene, dihydroxylycopene and the monocyclic substrate apo-8'-carotenal [81]. RPE65, in addition, has been shown to have isomerase activity towards lutein, isomerizing it to *meso*-zeaxanthin for use as an antioxidant in the macula lutea of the primate retina fovea [83]. Thus, the CCO family has intrinsic flexibility in substrate recognition and can fine-tune substrate specificity for local carotenoid needs. We could observe such a fine-tuning with two non-active-site amino acid changes in the polymorphic variant of mouse BCO2 (BCO2/P108S+N190D) cloned from the Sugano cDNA library derived from the C57BL/6 mouse [1]. The resulting enzyme exhibits a significant change in preferred substrate, from β-carotene to lycopene [1].

Evolutionary conservation of PDPCK-motif and palmitoylation

In general, BCOs and RPE65 are soluble proteins and manifest a hydrophobic tunnel to channel their lipophilic substrates, carotenoids, from the hydrophobic lipid bilayer core or from lipid droplets to the catalytic site. The mouth of the tunnel consists largely of hydrophobic patches formed by protruding non-polar residues, which allows for membrane binding. In addition to their conserved overall structural architecture and catalytic center, previous comparative evolutionary analyses revealed the presence of a short conserved motif PDPC(K) in all metazoan BCO/RPE65 proteins [1]. Structural inspection revealed that this motif is surface-exposed and located in the center of a highly mobile loop. In the case of RPE65, this motif is still unresolved in the crystal structures known so far [24, 84]. However, several previous reports and, recently, our study reveal that RPE65 is a palmitoylated protein and that the palmitoyl moiety may help anchor the protein to the endoplasmic reticulum (ER) membrane, ostensibly to extract substrate [35, 85, 86]. In our experiments [35, 85, 86], we and others have clearly demonstrated that palmitoylation at the C112 residue, located within the PDPC(K) motif, is crucial for the membrane association of RPE65. Because of this conserved nature, we speculate that the PDPC(K) motif may be essential for the palmitoylation of the cysteine residue within this motif in BCOs in general. In confirmation of this hypothesis, we have detected the palmitoylation of mitochondrial BCO2 protein [71, 87], which also contains the PDPC(K) motif, using a simple and robust

biochemical palmitoylation detection method: acyl-resin assisted capture (RAC) assay. As shown in Figure 3, recombinant expressed wild type BCO2 protein showed a prominent protein band in the hydroxylamine-treated sample compared to almost no protein in the untreated control sample, indicating S-palmitoylation of BCO2 protein similar to RPE65. We also assessed the palmitoylation level of BCO2 protein upon treatment with its substrate, 0.2 μM β-carotene, and, interestingly, observed a significant decrease with almost no protein signal in the hydroxylamine-treated sample (Figure 3). In our experiments with BCO2 protein, we used a lower concentration (0.25 M) of hydroxylamine to release the palmitoyl moiety from the protein in the untreated and β-carotene-treated samples, instead of the 0.5 M concentration as used earlier for RPE65 [35]. Upon treatment with 0.5 M hydroxylamine, BCO2 protein displayed inconsistent results in the acyl-RAC assay (data not shown). It has been reported that some proteins undergo rapid degradation upon treatment with high concentration of hydroxylamine due to sensitivity of Asn-Gly bond to hydroxylamine hydrolysis [88, 89]. It is possible that BCO2 protein with several Asn-Gly sites is more sensitive to degradation by 500 mM hydroxylamine than RPE65 protein which might correlate to differences in the protein structure and localization in membrane between the two proteins.

To our surprise, both RPE65 and BCO2 proteins displayed similar palmitoylation pattern i.e., the loss of palmitoylation signal in the active system when provided with their respective substrates, thus highlighting the fact that the role of palmitoylation in BCO2 might be analogous to that in RPE65. This needs further investigation in detail. Based on our results, we believe that the PDPC(K) motif in metazoan BCO/RPE65 proteins serves as a signature motif providing specificity for palmitoylation of the cysteine residue within this motif and thus may mediate interaction with the membrane for extraction of their substrates.

Concluding Remarks

The evolutionary history of genomes combines vertical descent (speciation) with numerous lineage-specific losses and gene duplication events [90]. The genes that are related via vertical descent are known as orthologs whereas genes that evolved via duplication in a certain lineage are called paralogs [90]. These definitions are straightforward in theory but the actual evolutionary relationships between genes are often extremely complex and involve not only one-to-one (orthologous genes) but also one-to-many and many-to-many correspondence (“mixtures” of orthologous and paralogous genes) due to the complicated combinations of lineage-specific duplication and gene loss [48]. Robust identification of orthologs is of central importance for functional genomics due to a rarely stated but almost universally implied concept that orthologous genes perform equivalent functions. Accordingly, experimentally determined functions of a gene can be transferred to its experimentally uncharacterized orthologs in other species (taking into account the biological differences between the organisms involved) [48]. The ortholog concept effectively forms the foundation of all functional annotations of sequenced genomes given that experimental characterization of the functions of any sizable fraction of the genes in the huge number of sequenced genomes remains unrealistic.

Orthologous relationships can be established for some CCO clades. One example is the RPE65 clade (although some fish genomes contain diverged copies of the RPE65 gene suggesting some functional variability of these copies [49]). Much caution is required when we discuss other CCO clades. For example, there is a substantial change of substrate specificity associated with two SNPs in mouse BCO2 protein (BCO2P108SN190D) where acyclic lycopene is preferred substrate (Figure 4A and 4B) [1].

RPE65 is described as a monotopic membrane-associated protein and its structure in lipidic matrix has been investigated [84]. Mouse BCO2 is associated with the mitochondrial membrane [91] and the mobility of its loops combined with the palmitoylation site is the most probable face for membrane association. To address this we modeled the orientation of mBCO2 and RPE65 in relation to the lipid membrane by PPM server (Figure 5; the parameters calculated for the proteins are described in Supplemental Table S1) [92]. Additionally, we performed a sequence alignment of mBCO2 and RPE65 with structural representation based on the RPE65 structure (PDB ID: 4F30) [93] and highlighted predicted membrane-interface residues by the MODA server in both proteins [94] (Supplemental Figure S2).

Even within the well-defined clade of BCO2 enzymes there is a substantial variability of the very 5’ end of the protein coding region (Figure 6). The start codon in human and mouse have different locations: the mouse protein coding region is shorter (Figure 6). There are even signs of positive selection in this region: the rate of nonsynonymous changes (aligned human - mouse coding fragments, 0.61, Figure 6) is higher than the rate of nonsynonymous changes (human - mouse, 0.53) (analyses were conducted using the Pamilo-Bianchi-Li model) [95, 96]. Positive selection is a strong indicator of functional changes in this region [97]. High variability of the 5’ end (Figure 6) may be associated with the presence of an N-terminal mitochondrial signal [1]. Various BCO2 proteins and their isoforms differ in the length of their N-terminal sequences (from 58 aa to 1), with a proposed putative proteolytic site at a position of 521 amino acids away from the C-terminal [91].

CCO is a functionally volatile multigene family. There is likely to be a substantial variation of substrate specificity even within well-defined clades especially when several diverged copies are present in some genomes. Thus, claims about the presence or absence of functionally similar CCO genes require much caution [48–50]. On the other hand, a high degree of amino acid identity in protein alignments through the whole family or certain paralogs could add to the plethora of information about the studied protein as is shown for the catalytic site in the CCO family [51, 52] or for the palmitoylation site as presented here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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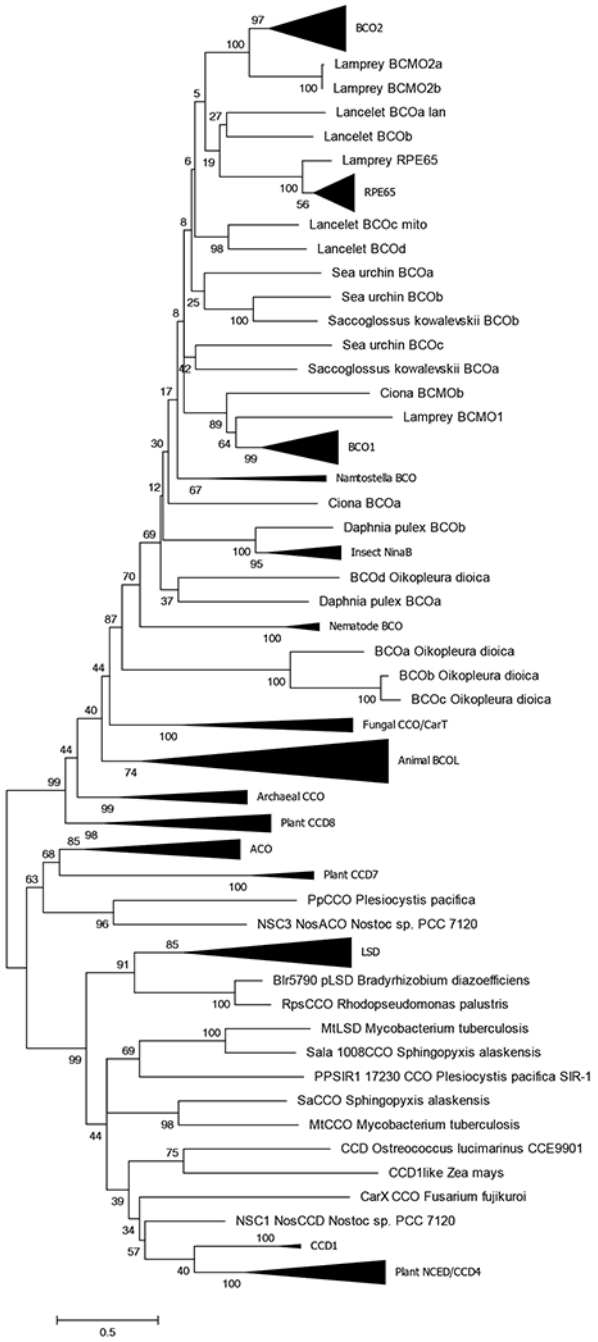

Figure 1. The phylogenetic tree of CCO proteins.

The phylogenetic tree of CCO proteins was inferred by use of the Maximum Likelihood method based on the WAG matrix-based model. The percentage of trees in which the associated taxa clustered together (bootstrap support values) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the WAG model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5

categories). The rate variation model allowed for some sites to be evolutionarily invariable. Black triangles indicate collapsed clades (names of clades are shown near triangles, the complete tree is shown in the Supplementary Figure S1). The scale bar at the lower left of the figure represents the amount of change per one position in the alignment. The MEGA package (www.megasoftware.net) was used for the tree reconstruction.

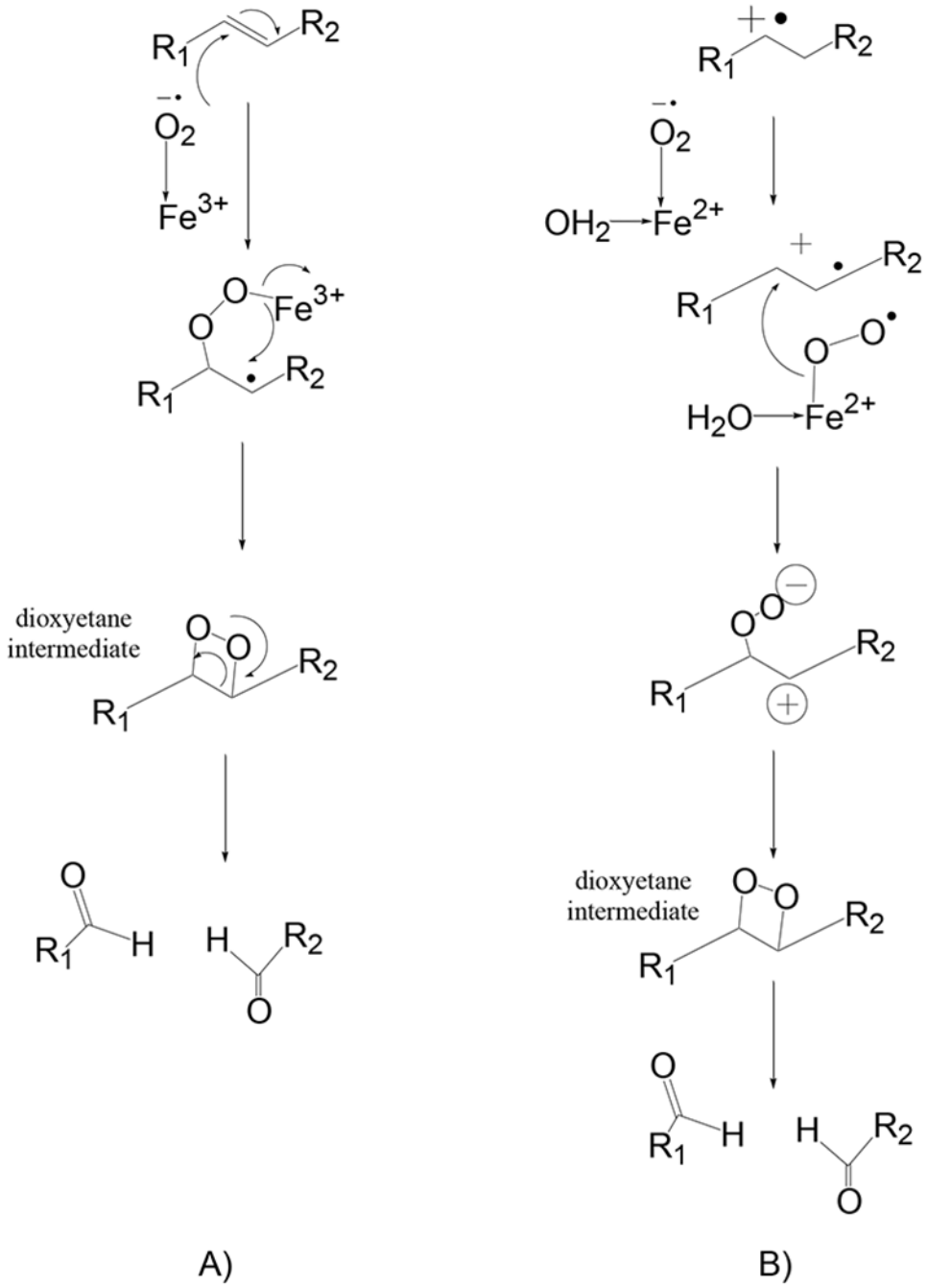

Figure 2. Proposed mechanisms for carotenoid cleavage oxygenases.

A) Representative proposed mechanism for VP14 [53] and NOVI [22] oxygenases. B) Representative proposed mechanism for ACO [58], NinaB oxygenase [63] and mammalian β-carotene oxygenases.

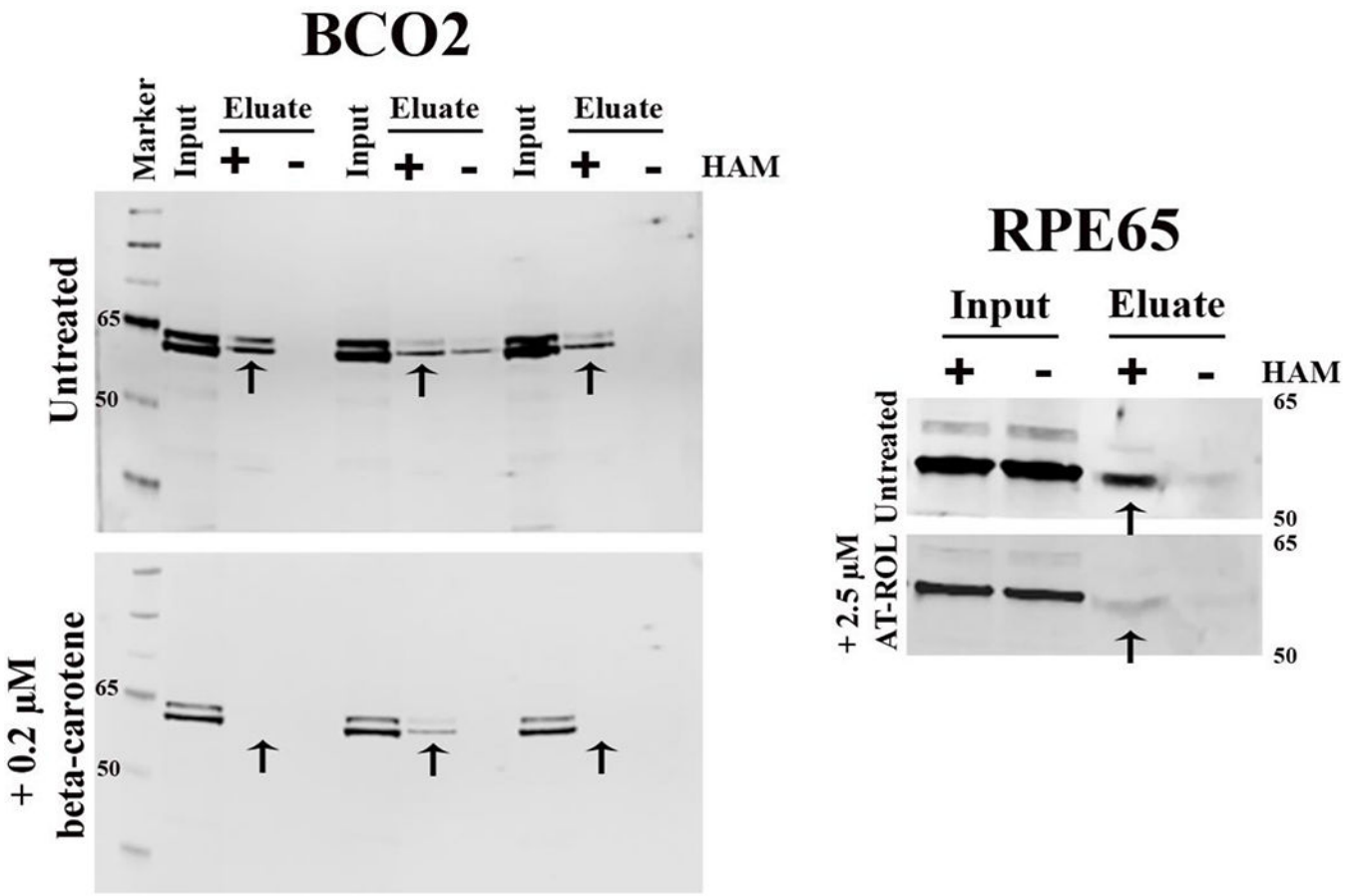

Figure 3. BCO2 is a palmitoylated protein.

HEK293F-cell cultures transfected with pVitro2/BCO2 wild type plasmid were incubated with 0.2 μM β-carotene for 5h. Cell extracts were used for the detection of palmitoylation by the acyl-RAC assay. Samples were treated with 0.25 M hydroxylamine (HAM; indicated as "+") or 0.25 M NaCl (control; indicated as "−"). The palmitoylated proteins were enriched by affinity purification using thiopropyl-sepharose beads and eluted using 2.5% β-mercaptoethanol in SDS-PAGE sample buffer. Equal amounts of total (indicated as "input") and eluate samples were subjected to SDS-PAGE followed by western blotting with custom-made primary antibody (7055, anti-rabbit; 1:1000 dilution) to BCO2 protein. The experiment was carried out in triplicate. The upper protein band represent a nonspecific band and lower protein represent the specific BCO2 protein as confirmed by mass spectrometry (data not shown). Acyl-RAC results for RPE65 is taken from ref. [35]. RPE65 was co-expressed with lecithin:retinol acyltransferase (LRAT) and treated with 2.5 μM all-trans retinol (AT-ROL) for 7h.

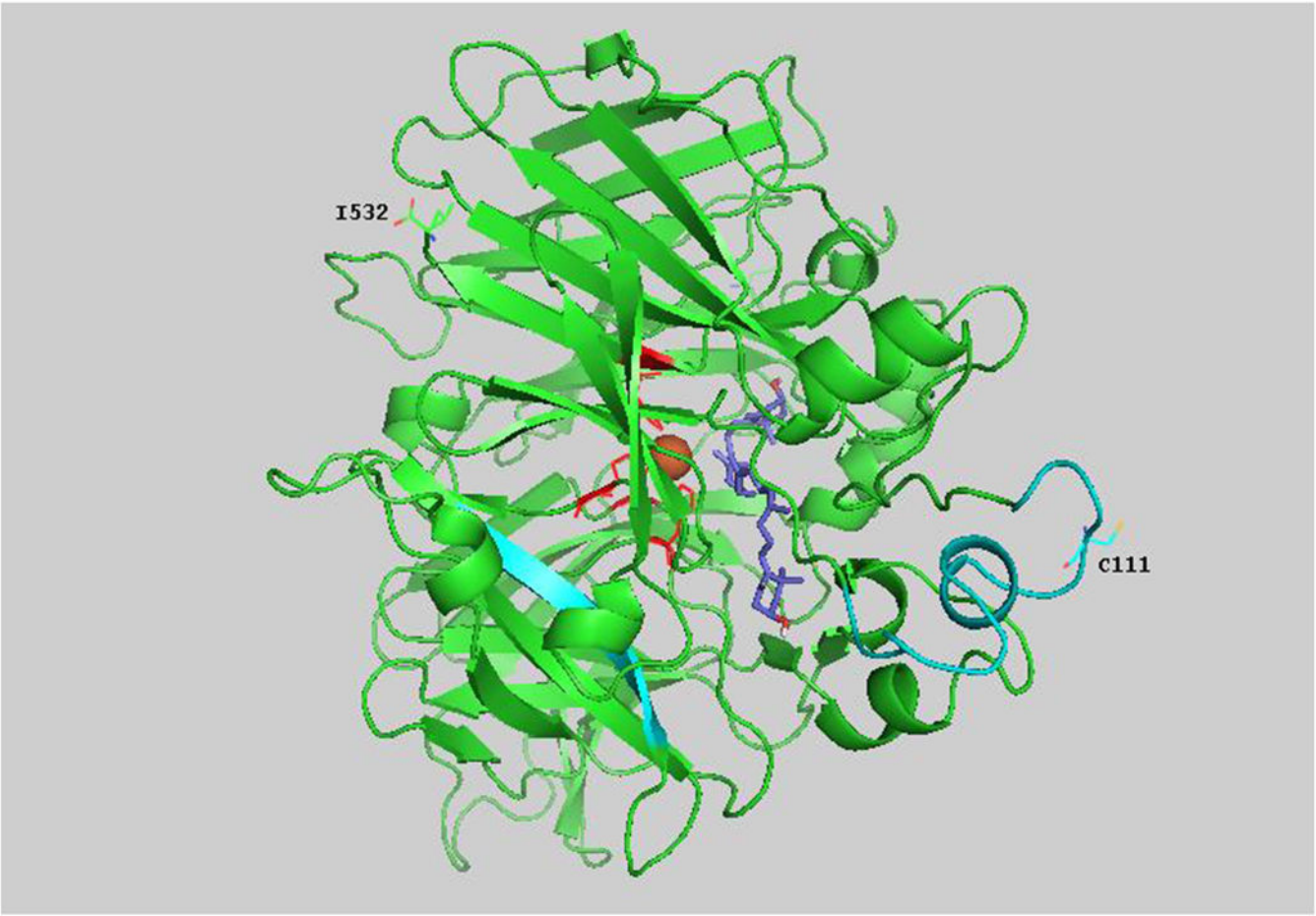

Figure 4A. Visualization of a model of the mBCO2 protein (in green) based on an RPE65 crystal (PDB ID 4F30).

Residues in the region consisting of aa109-125 are generated by the I-TASSER server [35] and changes due to P108S and N190D mutations in the mBCO2SD protein model are indicated in cyan; coordinating histidines are in red; meso-zeaxanthin substrate is in purple, iron atom is a brown sphere, C-terminal I532 and C111 labeled on the figure [1].

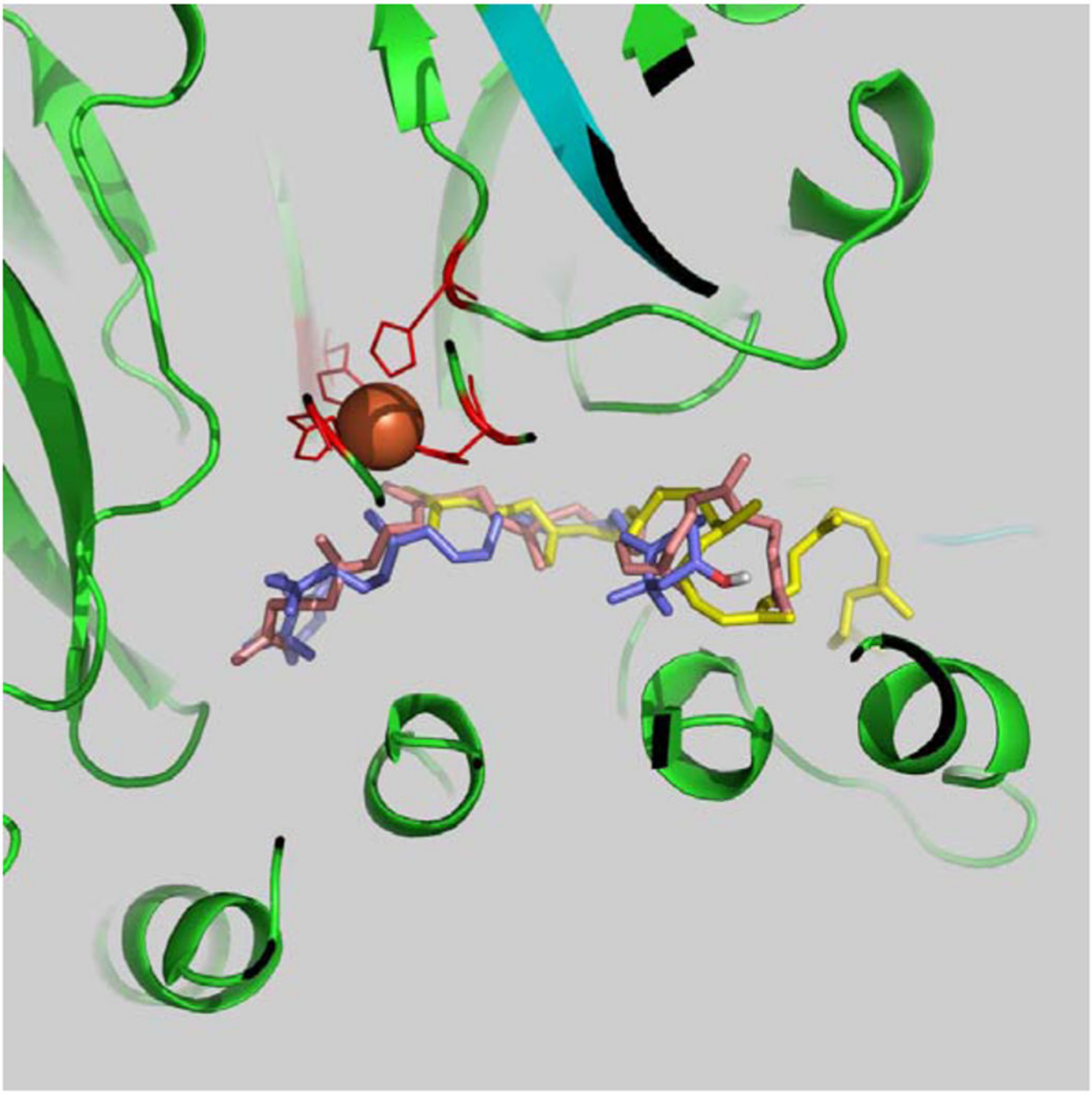

Figure 4B.
Visualization of lycopene docking on the mBCO2 protein model (yellow) and on the mBCO2SD protein model (pink) [1] compared to meso-zeaxanthin docking position (purple) on mBCO2 protein model.

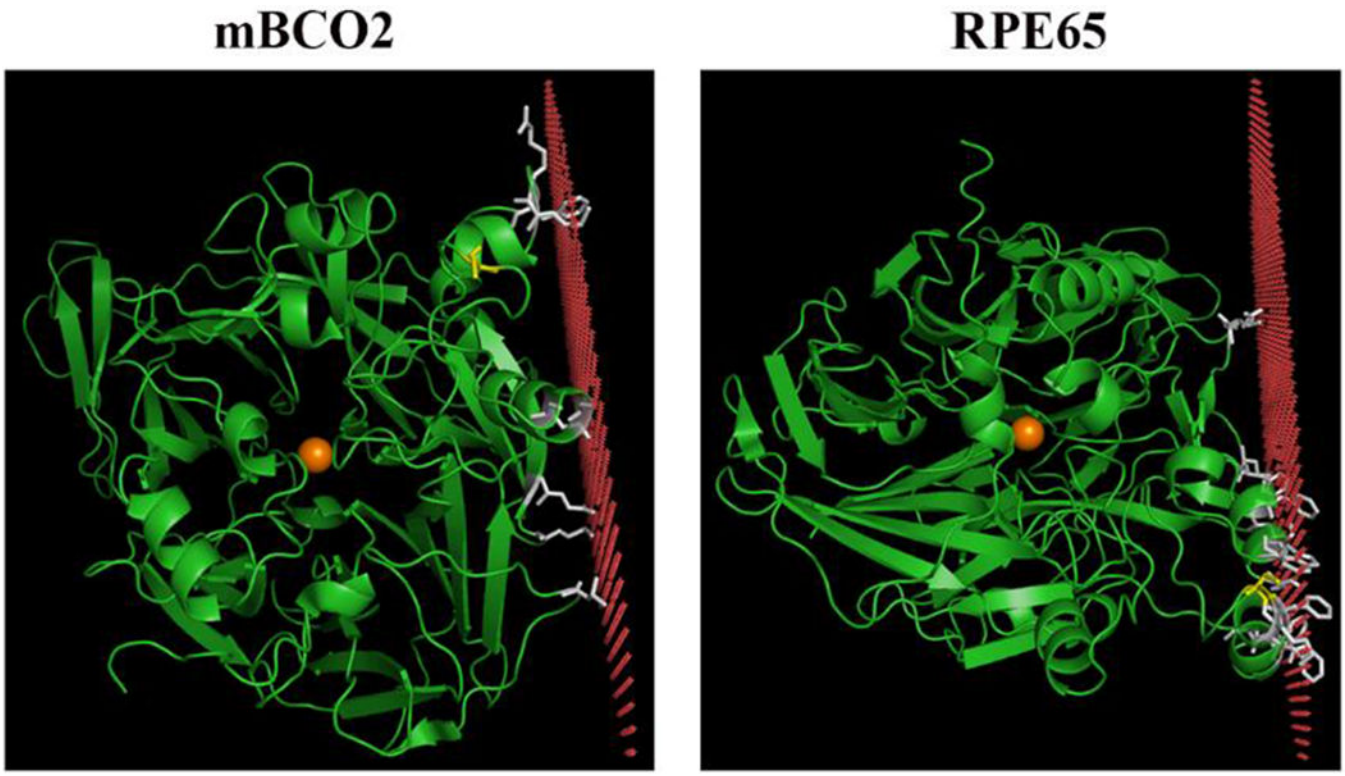

Figure 5. Orientation of mBCO2 (left) and RPE65 (right) in membrane.

The position of the peripheral proteins in membrane is predicted by PPM server (https://opm.phar.umich.edu/ppm_server) using their PDB coordinate file. The iron (Fe) atom in the center of the protein is depicted as an orange ball. The membrane-interface residues are shown as sticks in gray color. RPE65 PDB ID: 4F30

Figure 6.

A genomic alignment of the 5’ region of mammalian BCO2 genes. Human BCO2b and mice BCO2 start codons (according to the Refseq NM_001037290 and NM_133217 respectively) are underlined.

Table 1.

Orthologous enzyme groups and their preferred substrates.

Enzymes	Preferred substrate
B-carotene oxygenase 1 (BCO1)	B-carotene, lycopene [68]
B-carotene oxygenase 2 (BCO2)	Xanthophylls (lutein and zeaxanthin), β-carotene [72]
B-carotene oxygenase-like proteins (BCOL)	9-cis- β -carotene and 13-cis- β -carotene [1]
Cyanobacterial apocarotenoid oxygenases (ACO)	apo-β-carotenals (β-apo-8'-carotenal) [4]
Fungal CarTs	Torulene [74]
LSDs (RCOs)	Lignostilbene, 4-hydroxystilbene, (resveratrol) [75]
Insect NinaB	Zeaxanthin, lutein, β-carotene [29]
CCD4	β-Carotene [76]
Plant NCED	9-cis-violaxanthin, 9-cis-neoxanthin [77]
RPE65	All-trans-Retinyl esters [78]