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MECHANISTIC ASPECTS OF CAROTENOID BIOSYNTHESIS

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1. INTRODUCTION

Carotenoids represent a large class of terpenoids characterized by an extensively conjugated polyene chain. The conjugation system imparts carotenoids with excellent light absorbing properties in the blue-green (450–550 nm) range of the visible spectrum. The light-absorbing properties of carotenoids have been exploited by photosynthetic organisms to extend the range of light absorption by the photosynthetic apparatus beyond that of chlorophyll. [1] Following light absorption the carotenoid excited state undergoes excitation energy transfer to chlorophyll. [2] In addition to their role as accessory pigments, carotenoids protect against excess light by quenching both singlet and triplet state chlorophylls. In plants oxygenated carotenoids, known as xanthophylls, provide additional photoprotective functions by non-photochemical quenching (NPQ) of chlorophyll fluorescence. [2a,3]

Carotenoids carry out light independent functions in scavenging peroxyl radicals and preventing oxidative damage particularly against singlet oxygen ($^1O_2$).[4] Since many organisms and specific animal tissues, such as the macula lutea and the corpus luteum, accumulate large amounts of carotenoids, it was proposed that carotenoids may protect against the damaging effects of oxidative stress in such tissues. [5a,b] These properties have prompted much research in the chemopreventative potential of carotenoids. At high oxygen pressures, however, some carotenoids display prooxidative activity[4b] and some β-carotene formulations have even shown adverse effects in supplementation trials aimed at preventing lung cancer in smokers. [6]

The length of the carotenoid polyene chain corresponds to the width of the phospholipid bilayer, which led to the proposal that carotenoids act as “molecular rivets” to stabilize and add rigidity to the phospholipid membrane. [2b,7] The membrane spanning topology could also allow carotenoids to counteract oxidative damage on either side of the membrane. [4b] It has also been proposed that membrane-spanning carotenoids can mediate proton transfer across the membrane or serve as transmembrane radical channels. [8]

Due to their striking and rich color carotenoids are important floral pigments serving to attract pollinators and seed dispersers. [9] In birds and fish, carotenoids are an important

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The Cover Art and Table of Contents graphic depicts key precursors in the carotenoid synthesis pathway and representative structures of enzymes involved in isoprenoid synthesis.

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signal of good nutritional condition and are used in ornamental displays as a sign of fitness and to increase sexual attractiveness. [10a,b,c,10d–f]

Following oxidative cleavage, carotenoids generate apocarotenoid metabolites which serve important signaling and photoreceptive functions. The 11-*cis* isomers, 11-*cis*-retinaldehyde, 3,4-didehydro-11-*cis*-retinaldehyde or 3-hydroxy-11-*cis*-retinal are used by most animals as a photosensitive moiety coupled to the opsin protein, rhodopsin, cone opsin or melanopsin. [11] These photoreceptor molecules mediate vision and circadian photoentrainment. [12] Bacteria use the light-sensitive carotenoid cleavage product, retinaldehyde, coupled to bacteriorhodopsin and related proteins to transport protons and other ions across the membrane. This ion transport function allows the cell to generate energy, regulate ion balance or sense light. [13a,b,c]

The acidic forms of several apocarotenoids act as signaling molecules in fungi, plants and vertebrates. The apocarotenoid, trisporic acid, signals mating type in fungi. [14] Plants cleave carotenoids such as 9-*cis*-neoxanthin to generate the hormone abscisic acid, which plays important roles in inducing seed dormancy, and allowing the plant to adapt to abiotic stress. [15a,b,c] Other plant apocarotenoid metabolites, such as strigolactones trigger seed germination of parasitic weeds and inhibit shoot branching. [16] Finally, vertebrates use retinoic acid, a ligand for nuclear receptors to regulate gene transcription in physiological processes that include embryonic development, cell differentiation, and immunity. [17a–c,17d,e]

Carotenogenesis occurs in all photosynthetic organisms and in some non-photosynthetic bacteria, archaea, protozoa and fungi. Reflecting their ubiquitous presence and pleiotropic roles there are well over 700 different types of carotenoids generated through variations of their pathways of synthesis. [18] There is even recent evidence for the acquisition of carotenogenic enzymes by metazoans through lateral gene transfer from endosymbiotic fungi. [19] Many excellent reviews have focused on the later steps of the carotenoid synthetic pathways and their regulation in bacteria, and plants. [20,21] In this review we concentrate on the mechanisms of carotenoid synthesis by examining the structure and enzymology of enzymes involved in the production of carotenoids starting from the production of isoprenoid precursors.

2. GENERATION OF C5 ISOPRENOID PRECURSORS

Carotenoids are tetraterpenes derived through the condensation of the five-carbon (C_5) universal isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl pdiphosphate (DMAPP). Two pathways responsible for the synthesis of isoprenoid precursors IPP and DMAPP exist in nature. The acetate-mevalonate (MVA) pathway, originally described by Block, Lynen, Popjak and others, [22] is associated with the production of isoprenoids in animals and yeast and till the late 1990's was considered to be only source of isoprenoid precursors for terpenoid synthesis including carotenoids. [23]

Several early attempts to trace the fate of labeled acetate in forming lycopene, hopanoids, taxol and sterols revealed differences in distribution of the label within the basic isoprene unit inconsistent with a single source of isoprenoid precursors for all terpenoids. [24] These results led to the discovery by several independent research groups of a mevalonate-independent pathway in eubacteria, [25] green algae [26] and higher plants. [24c,27] The studies of the labeling pattern of ubiquinone following incorporation of ^{13}C-labeled glycerol or pyruvate allowed Rohmer *et al*. to identify the conversion of pyruvate to 1-deoxy-D-xylulose-5-phosphate (DXP) as the first step of the mevalonate-independent pathway. [28] In independent studies, by tracing the fate of [1-^{13}C]- and [2,3,4,5-$^{13}C_4$]-DXP in the labeling pattern of β-carotene, lutein, phytol and sitosterol in cell cultures of *Catharanthus roseus*,

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Arigoni *et al*. demonstrated unequivocally the involvement of DXP in the non-mevalonate pathway, also known as the 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway and provided further insight in the compartmentalization of isoprenoid synthesis. [29] Furthermore, the studies by Arigoni *et al* described the occurrence of an intramolecular skeletal rearrangement in the formation of isoprenoid precursors through the non-mevalonate pathway as first proposed by Eisenreich *et al*.. [24c]

Isoprenoid precursors are generated by all organisms with the possible exception of obligate intracellular organisms such as rickettsiae and mycoplasmas. The MEP pathway is utilized by the great majority of eubacteria including photosynthetic cyanobacteria, algae and by all Apicomplexan protists including parasitic pathogens such as *Plasmodium falciparum* and *Toxoplasma gondii.* [30] Archaebacteria, fungi and animals derive isoprenoids strictly via the MVA pathway. [31,32] While most carotenogenic organisms use either the MEP or the MVA pathway to produce IPP and DMAPP, plants and a few bacterial *Streptomyces* species generate isoprenoid precursors using both the MVA and the MEP pathway. [33] The cytosolic (MVA-derived) and plastidial (MEP-derived) plant isoprenoids, however, have different biological fates; plant carotenoids are derived predominantly from isoprenoid precursors generated via the MEP pathway. [27b] There is evidence of limited exchange of isoprenoid precursors as well as higher intermediates between the two plant cell compartments. [34] For example, in the case of carotenoids synthesized by the unicellular alga *Euglena*, carotenoids were derived from both the MVA and MEP pathway. [35] However, neither the MVA nor the MEP pathway can successfully compensate for the absence of the other. Figure 1 illustrates the fate of the radiolabel from [1-^{13}C]–glucose in the formation of isoprenoid units via either the MVA or the more recently discovered MEP pathway.

2.1 The 2*C*-methyl-D-erythritol-4-phosphate-MEP pathway

It is believed that the MEP pathway initially developed in a specific clade of eubacteria and spread through horizontal transmission to other (eu)bacteria. The origin of the MEP pathway in eukaryotes, i.e. plants, is attributed to an ancestral cyanobacterial endosymbiont, which later evolved into plastids. [36] During the course of evolution the genes coding for enzymes involved in the MEP pathway have become integrated by the host nucleus and the enzymes evolved to be post-translationally targeted to plastids via a plastidial targeting sequence. [37] Ancestors of apicomplexan protists also acquired a plastid known as an apicoplast from a red algal endosymbiont. [38] As a result, the MEP pathway is the sole source of isoprenoids in important pathogenic protists such as *Toxoplasma gondii* [18b] and *Plasmodium falciparum* and thus the protozoan MEP pathway represents a promising pharmacological target. The MEP pathway from plants and eubacteria is illustrated in Fig. 2 and shown in comparison with the MVA pathway.

2.1.1 Synthesis of 1-deoxy-D-xylulose-5-phosphate—The enzyme 1-deoxy-D-xylulose-5-phosphate (DXP) synthase, or DXS, catalyzes the condensation of glyceraldehyde-3-phosphate (G3P) and (hydroxyethyl)thiamine diphosphate derived from decarboxylation of pyruvate to produce DXP. G3P and pyruvate are derived from the metabolism of glucose via glycolysis, glyoxylate or the Entner-Doudoroff pathways. [39] The reaction catalyzed by DXS is the rate-limiting reaction in the MEP pathway. [40] Thus changes in the expression of DXS in plants [40b] and bacteria [40a,41] translate directly to changes in the levels of isoprenoids produced. DXS from *E. coli* [42] and higher plants [43] have been cloned and functionally characterized.

DXS was identified through its sequence similarity to pyruvate decarboxylase and transketolases which act on similar substrates and catalyze a similar reaction. [42–43] Like transketolases and pyruvate decarboxylase, DXS is a thiamine diphosphate (TPP)-dependent

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enzyme. [42–43] The reaction mechanism of DXS was proposed to proceed through an ordered ping-pong mechanism [44] via formation of a reactive TPP ylide to attack pyruvate. Following decarboxylation, the DXS-α-carbanion intermediate binds G3P resulting in the elimination of DXP and recycling of the enzyme. [44] Steady-state kinetic studies, inhibition studies, and CO_2-trapping experiments of heterologously expressed and purified DXS from *Rhodobacter capsulatus* demonstrated that G3P is required for the decarboxylation of pyruvate and for the formation of a ternary complex. [44] More recent studies have led others to suggest that DXS uses a random sequential mechanism through reversible rapid binding of substrates. [45]

Analysis of the structures of DXS enzymes from *E. coli* and *Deinococcus radiodurans* revealed interesting differences between the structure of DXS, transketolases and pyruvate dehydrogenases. [46] While the active site of transketolases and pyruvate dehydrogenase is found at the interface between two monomers, the active site of DXS is located within the same monomer along with the binding site for TPP and Mg^{2+}. The structure of DXS revealed that Glu370 and Arg398 are essential for catalysis and that several other polar residues are involved in binding substrate and diphosphate. [46] The mechanism of reaction, structure and key features of DXS in comparison with transketolase are illustrated in Fig. 3

DXS carries out a rate-limiting step in the production of isoprenoid precursors leading to a multitude of important plastidial-derived terpenoid metabolites. [40b,47] Taking into consideration the different roles of such terpenoids, it is perhaps not surprising, that plants code for multiple DXS isoforms which generate precursors destined for various biological fates. It is also important to note that the various DXS isoforms are expressed in different tissues and are differentially regulated. [48] In addition to isoprenoids, DXP can be converted to other essential metabolites such as pyridoxol phosphate and TPP. Therefore, DXS represents a nodal point in the synthesis of vitamin B1, B6 in addition to isoprenoids.

Of the three DXS isoforms found in maize, [48a,49] DXS1 plays a role in the constitutive production of isoprenoid precursors as demonstrated by the *albino* or "*cloroplastos alterados*" (*cla*) phenotype of the *A. thaliana* plants deficient in this enzyme. [50] On the other hand, DXS2 and DXS3 are important in iso-prenoid production during specific temporal and developmental windows. In the case of DXS3, its expression is strongly correlated with the increase in the levels of carotenoids in endosperm. [49] Conversely, the expression of DXS2 correlates with carotenoid accumulation in plant roots colonized by arbuscular mycorrhizal fungi. [48a]

The regulatory mechanisms that govern the expression of the various DXS isoforms have not been elucidated. There is evidence that induction of the expression of phytoene synthase (PSY) enhances the expression [51] and activity of DXS. [52] Therefore, the activity of PSY, an enzyme committed to carotenoid synthesis, can influence the expression of DXS and thus the availability of the universal carotenoid precursors IPP and DMAPP. The expression of DXS can also be upregulated through a post-transcriptional mechanism in response to a block in the MEP pathway. [53]

In addition to the DXS1 (encoded by the *CLA1* gene) there are several DXS-related proteins encoded by the *A. thaliana* genome which evolved through gene duplication. [54] Though it is possible that some of the novel DXS-like proteins from *A. thaliana* contribute to isoprenoid production, it is also possible that they have evolved to aquire additional enzymatic roles beyond DXP synthesis.

Evidence derived from multiple studies suggests that there is limited import of cytosolic isoprenoid precursors or intermediates by plastids. As demonstrated by the lack of

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carotenoids in plants deficient in DXS, [50] cytosolic isoprenoids derived via the MVA pathway cannot fully compensate for the lack of plastidial isoprenoids.

2.1.2 Conversion of 1-deoxy-D-xylulose-5-phosphate to 2*C*-methyl-D-erythritol-4-phosphate—Analysis of the fate of ^{13}C-labeled glucose in isoprenoid metabolites revealed that the labeling pattern of menaquinones by *Corynebacterium ammoniagenes* is identical to that observed in the case of MEP. This observation implicated MEP as the second metabolite of what became know as the MEP pathway. [55] By screening for *E. coli* mutants that display 2*C*-methyl-D-erythritol auxotrophy, Kuzuyama *et al.* identified 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) as the enzyme responsible for the synthesis of MEP. [56]

DXR catalyzes the first committed step of the MEP pathway towards isoprenoid synthesis. DXR carries out the ordered intramolecular rearrangement of DXP and subsequent reduction to produce MEP in the presence of NADPH and a divalent metal ion. [56b,57] Initially proposed to undergo an alpha-ketol rearrangement, the intramolecular rearrangement of DXP catalyzed by DXR is more recently thought to occur through a retroaldol/aldol mechanism based on the measurement of the kinetic isotope effect using [3-^{2}H]- and [4-^{2}H]-DXP. [58] Thus deprotonation of the C4 hydroxyl group is followed by a rate-limiting step of cleavage of the 3–4 bond of DXP to hydroxyacetone and glycoaldehyde phosphate. This is followed by aldol condensation to produce 2*C*-methyl-D-erythritose-4-phosphate. Next, DXR catalyzes the reduction of 2*C*-methyl-D-erythritose-4-phosphate using NADPH to form MEP. Studies of the stereo-specificity of the reaction using recombinant DXR protein of *E. coli* and of the cyanobacteria *Synechocystis sp.* strain PCC6803, revealed that DXR transfers the pro-*S* hydrogen from the *si* face of NADPH to the *re* face of the aldehyde. [59] The importance of DXR in the formation of carotenoids is underscored by the powerful inhibitory effect of the antimalarial drug 3-[formyl(hydroxy)amino]propylphosphonic acid (fosmidomycin), [30b] a specific inhibitor of DXR, [56a] on carotenoid accumulation in ripening tomatoes. [60]

Numerous crystal and one NMR structure of DXR from bacterial and protozoan pathogens have been reported. [61] DXR functions as a homodimer, each monomer containing separate active sites. Highly conserved residues participate in hydrogen bonding to the phosphate group and in coordinating the required divalent metal ion. Structural studies of apo and inhibitor-bound DXR reveal an induced fit model of substrate binding which rely on a flexible “lid” loop that closes upon binding substrate. [61a,b] The mechanism of reaction, structure and key features of DXR are illustrated in Fig. 4.

In addition to DXR, there are other enzymes shown to catalyze the conversion of DXP to MEP which bear no sequence similarity to DXR. This DXR-independent activity is carried out by enzymes found only in bacteria and referred to as DXR-like (DRL). [62] Some species of bacteria like *Brucella abortus* and *Mesorhizobium loti* only code for DRL enzymes while others code for both DXR and DRL. Importantly, *Roseobacter litoralis*, which is known to synthesize carotenoids, codes for a DRL enzyme which was shown to be functional in complementation assays. [62] *R. litoralis* also codes for a DXR-type enzyme, however, this enzyme is not active in complementation assays. [62] Presumably in the case of *R. litoralis* carotenoid synthesis relies solely on the activity of DRL for the formation of isoprenoid precursors. The, distribution, evolutionary history and role of DRL enzymes is relevant for evaluating the potential of targeting enzymes of the MEP pathway for drug discovery. Though DRL enzymes are sensitive to fosmidomycin, several reported structures show only distant structural similarity to DXR. [62] Intriguingly, DRL enzymes which complement DXR-deficient *E. coli* strains can also be found in bacteria which only utilize the MVA

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pathway for isoprenoid synthesis and lack all other MEP pathway genes; this observation suggests that some DRL enzymes might have aquired additional enzymatic roles. [63,64]

It was reported by Ershov *et al.* that isoprenoid synthesis in the cyanobacterium *Synechocystis* strain PCC 6803 grown under photosynthetic conditions is not susceptible to inhibition by fosmidomycin and that isoprenoid production in *Synechocystis* can be stimulated by substrates derived from the pentose phosphate pathway but not by MEP substrates such as pyruvate or DXP. [65] In addition, it was shown that isoprenoid production stimulated by pentose phosphate cycle substrates in *Synechocystis* requires the activity of the product of the *sll1556* gene, which codes for an isopentenyl diphosphate isomerase type 2 (IDI-2). [66] These findings argue that there might be alternate routes for isoprenoid production that bypass MEP, perhaps derived from the pentose phosphate cycle, and which feed into the late steps of the MEP pathway. However, more studies are needed to demonstrate the existence of a DXR-independent MEP pathway as neither the intermediates nor the enzymes involved in this proposed shunt have been characterized.

2.1.3 Conversion of 2*C*-methyl-D-erythritol-4-phosphate to 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate—Radiolabeled MEP incubated with *E. coli* extracts becomes converted to the nucleoside derivative 4-4-(cytidine 5′-diphospho)-2*C*-methyl-D-erythritol (CDP-ME). In turn, radiolabeled [2–14C]CDP-ME incubated with chromoplasts becomes incorporated in the carotenoid fraction demonstrating the involvement of CDP-ME in the MEP pathway for isoprenoid synthesis. [67] Based on these observations, Rohdich *et al.* used a bioinformatics approach to look for unannotated proteins with similarity to ribulose 5-phosphate reductase/CDP-ribitol pyrophosphorylase which carries out a very similar reaction to the MEP → CDP-ME transformation. These studies led them to identify the gene IspD of *E. coli* which codes for 2*C*-methyl-D-erythritol-4-phosphate cytidyltransferase (MCT), the fourth enzyme of the MEP pathway. [67]

Structures of both bacterial [68] and plant MCT [69] have been reported. MCT assumes a dimeric conformation and each subunit of the functional MCT dimer features a globular domain and an extended β-sheet arm. [68a,69a] The two subunits of the MCT dimer are held together by the interlocked β-sheet arms. The two active sites of the dimer involve residues derived from both monomers. A divalent metal ion which is essential for the cytidyltransferase activity of purified *E. coli* MCT, interacts with the α-, β- and γ-phosphate oxygens of the substrate CTP and the α-phosphate oxygen of the CDP-ME product. [68a] The active sites reside at the interface of the subunits. It is still unclear which residues of MCT participate in the coordination of the metal ion. Comparison of the *A. thaliana* and *E. coli* MCT reveals differences in the quaternary structure but the two structures are relatively similar. [69a] The mechanism of reaction, structure and key features of MCT are illustrated in Fig. 4.

Similar approaches to the one used by Rohdich to identify IspD, [67] led to the discovery of the next two enzymes of the MEP pathway. [70] Accordingly, CDP-ME is converted by CDP-ME kinase (CMK) to 4-(cytidine 5′-diphospho)-2*C*-methyl-D-erythritol-2-phosphate (CDP-ME-2P) which in turn is converted through an intramolecular transphosphorylation to 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MECDP) by MECDP synthase (MDS). Radiolabeled CDP-ME-2P and MECDP are efficiently incorporated into carotenoids by isolated chromoplasts from *Capsicum annuum* demonstrating their involvement in the MEP pathway. [70–71] Deleterious mutations in genes coding for enzymes involved in the MEP pathway are not viable in *E. coli* which made genetic attempts at generating MEP pathway mutants in this organism futile. By engineering *E. coli* to also produce IPP using the MVA pathway, however, one can maintain strains deficient in various MEP pathway genes on media supplemented with MVA. This elegant approach was employed by several groups to

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provide definite genetic proof of the involvement of candidate genes in the main trunk of the MEP pathway. [72]

Structure analysis of CMK from several bacterial species indicates the enzyme forms a homodimer of subunits that interact weakly and which surround a large central channel. [73] Each subunit of CMK displays the alpha/beta fold characteristic of the Galacto-, Homoserine-, Mevalonate-, Phosphomevalonate-kinase (GHMP) superfamily [73a,74] and each subunit contains a complete active site including an ATP binding domain. None of the reported structures of CMK indicate an associated metal ion, though a divalent metal ion was shown to be required for the activity of purified tomato CMK. [75]

Reported structures of MDS from bacterial [76] protozoan and plant species [77] reveal a well conserved fold consisting of a homotrimeric protein surrounding a central cavity. The proposed mechanism of reaction of MDS involves an in-line attack on the β-phosphate. A metal ion is required to stabilize a presumed pentacoordinate transition state. [76a] While both Zn^{2+} and Mg^{2+} were found bound to bacterial MDS and are required for catalysis, [76a] only one divalent ion was reported in the structure of plant MDS. [77] The three active sites of MDS are located at the interfaces of the subunits. The mechanism of reaction, structure and key features of CMK and MDS are illustrated in Fig. 5.

2.1.5 Synthesis of isopentenyl diphosphate and dimethylallyl diphosphate— Bioinformatics and genetic approaches led to the identification of two more bacterial genes *IspG* and *IspH* coding for 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate (HMBPP) synthase (HDS) and HMBPP reductase (HDR), respectively, the last enzymes of the MEP pathway. HDS and HDR were identified as essential for isoprenoid production via the MEP pathway in *E. coli* engineered to produce isoprenoids from MVA, [72b–d] in the cyanobacterium *Synechocystis* [78] and in plants. [53] Overexpression of HDS in addition to DXR, MCT, CMK and MDS conferred cells the ability to produce HMBPP, the final intermediate of the MEP pathway. [79] Conversely, deletion of HDR led to the accumulation of HMBPP in *E. coli.* [80] Overexpression of HDR in addition to DXR, MCT, CMK, MDS and HDS in the presence of D-xylulokinase and 1-deoxy-D-xylulose led to production of the universal C5 isoprenoid precursors IPP and DMAPP in a ratio of 5:1. [81] Thus successful reconstitution of the MEP pathway downstream of DXS provided conclusive evidence that the complete set of enzymes involved in the main trunk of the MEP pathway have been identified.

Analysis of the purified HDS and HDR proteins by UV/visible, iron content determination and electron paramagnetic resonance (EPR) spectroscopy indicate that these enzymes possesses a dioxygen-sensitive [4Fe-4S] cluster. [82] Purified HDS and HDR are easily inactivated by oxygen but regain activity upon reconstitution of the iron-sulfur cluster [82b,c,e,83] and in the presence of a reducing system such as the flavodoxin/ flavodoxin reductase in bacteria [82b,c] or ferredoxin in plants and cyanobacteria. [82d,84] Both flavodoxin and an A-type Fe-S protein which transfers the $[4Fe–4S]^{2+}$ cluster to HDS apoprotein are required for the functioning of the MEP pathway in bacteria. [85] The mechanism of HDS and HDR is proposed to involve organometallic species: “metallacycles” in the form of (η(2)-alkenes) and η(1)/η(3)-allyls. [86] The mechanism of reaction, structure and key features of HDS and HDR are illustrated in Fig. 6.

Analysis of the structure of several apo and holo-HDS from bacterial species [87] indicates HDS assumes a dimer conformation. Two structural domains compose each subunit. The N-domain is an eight-stranded β-barrel globular domain with similarity to the a common $(\beta\alpha)_8$-fold adopted by members of the triose phosphate isomerase (TIM) barrel superfamily. The C-domain harbors three cysteines and one glutamate residue which coordinate the [4Fe-4S]

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cluster. [87a] Binding of MECDP to the active site formed by the N-domain of one subunit and the C-domain of the other subunit leads to a substrate-induced rearrangement of the C-terminal domain as seen in the structure of the HDS holoprotein [87c] and also shown by EPR measurements. [86b] Plant HDS contain a third structural domain between the domains homologous to the N- and C-domains of bacterial HDS. The plant specific HDS domain is proposed to form a TIM-barrel structure that plays a primarily structural role. [87b]

The structures of several bacterial HDR proteins have been reported and HDR. [87c,88] HDR is a dimer of two cloverleaf like subunits [88a,b] The subunits are distinguished by three structurally similar domains surrounding a central cavity which contains the [4F-4S] cluster and the active site [88c] The structure of HMBPP-bound HDR indicates that the substrate is presented in a cyclic conformation and forms a complex with the apical iron of the [4F-4S] cluster. [88c,d] Mössbauer spectroscopic studies also indicate that the apical iron of the HDR [4F-4S] cluster exhibits an unusual coordination to several additional O and/or N atoms in addition to three sulfur atoms of the [4F-4S] cluster. [82e,89] Analogues of HMBPP which replace the OH group with poorer leaving groups such as thiol or amino, coordinate to the apical iron group and are potent inhibitors of HDR. [89–90]

The mechanism of reduction of HMBPP by HDR proceeds through a sequential reaction of two electron transfer from HDR to substrate. The first electron transfer generates an allyl radical. Transfer of a second electron allows the formation of an allyl carbanion intermediate [88c,d] Protonation of either C3 or C1 affords IPP or DMAPP, respectively. [88d] Binding of substrate induces the closure of the active site and elimination of solvent to prevent hydrolysis of the diphosphate ester. [88c,d] In addition to its role in the reductive dehydroxylation of HMBPP, HDR can also catalyze the addition of water to acetylenes to form ketones and aldehydes via anti-Markovnikov / Markovnikov addition. [91]

There is evidence that the enzymes of the MEP pathway and carotenoid synthetic enzymes in general associate in multienzyme complexes which are membrane bound in some cases. [68c,92] The role of a multienzyme complex could be to facilitate substrate channeling and prevent the loss of intermediates through side reactions.

2.2 The Mevalonate Pathway

The MVA pathway has evolved in archaea to provide precursors for the synthesis of isoprenoid constituents of the 2,3-dialkyl-glycerol lipids. The pathway contributes to carotenoids production primarily in archaea and fungi and rarely in eubacteria. [93] Based on the species distribution and the compartmentalized nature of isoprenoid metabolism in plants, the bulk of precursors for carotenoid synthesis in nature derive from the MEP pathway. Yet, there is limited exchange of metabolites between cytosol and plastids, hence, the MVA pathway can also contribute to a percentage of carotenoids produced in species that utilize the MEP pathway. Knowledge of the genes involved and regulation of the MVA pathway is also an important consideration for the engineering of carotenogenic pathways in heterologous systems or hosts. [94] The MVA pathway from metazoa and fungi and a variation of the pathway found in archaea are illustrated in Fig. 2.

The specifics of the MVA pathway have been elucidated by an elegant series of labeling experiments by Bloch and Lynen among others investigating the synthesis of isoprenoids in animals and fungi (reviewed in Goodwin and in Beytia and Porter [95]). Though the MVA pathway has been described many years ago, more details are still emerging particularly in the structural understanding of the biochemical details of the MVA pathway and the development of pharmacological inhibitors in the treatment of hypercholesterolemia. In the first step of the MVA pathway two acetyl CoA molecules undergo a Claisen condensation to produce acetoacetylCoA in a reaction catalyzed by acetoacetyl CoA thiolase (AACT).

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Addition of a third acetyl CoA molecule is catalyzed by 3-hydroxyl-3-methylglutaryl CoA synthase (HMGS) yielding 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) which reduced to MVA by HMG-CoA reductase (HMGR). MVA is sequentially phosphorylated by mevalonate kinase (MK) to produce mevalonate-5-phosphate (MVA-P) and then by phosphomevalonate kinase (PMK) to produce mevalonate-5-diphosphate (MVA-PP). Subsequent phosphorylation/dephosphorylation allows the decarboxylation and elimination of phosphate to produce isopentenyl diphosphate (IPP) by mevalonate diphosphate decarboxylase (MVD).

As most archaea lack PMK and MVD, [96] it was proposed that they utilize an alternate route to produce IPP from MVA-P by undergoing first decarboxylation to produce isopentenyl phosphate (IP) followed by phosphorylation of IP to IPP by an IP kinase. [97] The structure of the archaeal IP kinase indicates it belongs to the amino acid kinase family. [98] Mevalonate decarboxylation reaction is proposed to proceed through phosphorylation and formation of a carbocation transition state. [99] Several archaeal gene sequences with similarity to mevalonate kinase were proposed to code for the MVD enzyme, [100] however, the activities of the putative archaeal MVD have not been verified experimentally.

2.3 Interconversion of isopentenyl diphosphate and dimethylallyl diphosphate

Since HDR can synthesize a mixture of both IPP and DMAPP, it is not essential for organisms which can utilize the MEP pathway to be able to interconvert these two isoprenoid precursors. However, IPP-DMAPP isomerase (IDI) activity is indispensable in the formation of DMAPP in animals, archaea and fungi which are restricted to using the MVA pathway. Isomerization of IPP is catalyzed by two very different classes of IDI enzymes which have evolved separately and have different phylogenetic distribution (reviewed in [101]).

Members of the IDI type 1 (IDI-1) enzyme family are found in most species except for some archaea and eubacteria. [102] IDI-1 enzymes originally discovered by Lynen *et al*. [103] and cloned by Hahn *et al*. require Zn^{2+} and Mg^{2+} for catalysis. [104] The mechanism of IDI-1 catalyzed isomerization of IPP to DMAPP is thought to occur through the protonation of substrate and deprotonation of the tertiary carbocation intermediate in a reaction facilitated by Zn^{2+} or Mn^{2+}. [105] A second divalent metal ion, most likely Mg^{2+}, plays a role in binding substrate. [105d] In eukaryotes different isoforms of IDI-1 are targeted to various cellular locations such as plastids, mitochondria or peroxisomes where they contribute to the synthesis of isoprenoids in the respective compartment. In the case of tobacco plants and *A. thaliana*, only one of the two IDI-1 isoforms present becomes targeted to the plastids. [106] Maize IDI-1 contributes to light-stimulated carotenoid synthesis. [107] However, ablation of either or both the plastidial or mitochondrial IDI-1 isoforms in *A. thaliana* activity results in no or very modest reduction in carotenoid synthesis, which is most likely as a result of a secondary metabolic effect. [106b,108] These results would argue that IDI-1 mediated interconversion of IPP and DMAPP plays a limited role in organisms utilizing the MEP pathway.

The structure of several bacterial and human IDI-1 have been reported. [105e,109] IDI-1 acts as a monomer and assumes a compact globular α/β fold. Human IDI-1 undergoes a conformational change upon substrate binding. [105e] The active site of human IDI-1 contains a glutamate (Glu148) and a tyrosine (Tyr136) residue on one side and a cysteine (Cys86) residue positioned on opposite sides of the substrate. The stereoselective antarafacial reaction proceeds through an acid (glutamate) catalyzed protonation; formation of a tertiary carbocation intermediate; deprotonation by a nucleophile (cysteine) and formation of a double bond. [105d,e,109a] A tryptophan residue stabilizes the carbocation intermediate through π-interactions [109a]

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IDI type 2 (IDI-2) enzymes are found in archaea, proteobacteria [102] and cyanobacteria *Synechocystis sp*. strain 6803. [66,110] IDI-2 is often associated with carotenogenic gene clusters in *Enterobacteriaceae* and *Pantoea* species (formerly *Erwinia*). [111] IDI-2 is a flavoprotein [112] which requires reduced FMN and NADPH to catalyze an isomerization reaction which involves no net redox change. [113] Structural studies of IDI-2 indicate it assumes a homooctameric configuration in solution [110a] and in several crystal forms. [114] The IDI-2 octamer dissociates into tetramers upon substrate binding. [114c]

Overexpression of IDI-2 plays an important role in bioengineering *E. coli* to produce carotenoids. *E. coli* utilize the MEP pathway for isoprenoid production and code for an IDI-1 but do not produce carotenoids as they lack the enzymes associates with the later steps. Heterologous expression of carotenogenic genes in *E. coli* can result in production of carotenoids. It was observed that despite the presence of the MEP pathway and despite the presence of a native IDI-1, inclusion of IDI-2 in the carotenogenic gene cluster transformed in *E. coli* results in a dramatic increase in the levels of carotenoids produced [111] The structures and key features of IDI-1 and IDI-2 are illustrated in Fig. 6.

The mechanism of reaction of IDI-2 involves protonation and deprotonation reaction mediated by reduced FMN which in this case functions as an acid/base catalyst. [115] Based on the crystal structure of IDI-2 bound to inhibitors it has been proposed that the proton transfer to substrate involves the N5 proton of FMN. [116] Accordingly, EPR analysis and use of radical clock probes did not detect the presence of a substrate radical-flavin radical pair; [115a,c,d] meanwhile, purified *Sulfolobus shibatae* IDI-2 is not active if reconstituted with 5-deaza-analog of FMN. [113] The mechanism proposed for IDI-2 is the first example of the utilization of reduced FMN in in acid-base catalysis by an enzyme. [115d,e,g,116b] Importantly, several other enzymes involved in carotene synthesis, such as lycopene cyclase, CrtY, [117] and carotene isomerase, CRTISO, [118] depend on reduced flavin moieties to catalyze reactions without concomitant change in the net redox state of the reaction.

3. LYCOPENE SYNTHESIS

3.1 Synthesis of phytoene

Phytoene synthesis represents the first committed step in the production of carotenoids and it is considered as the rate-controlling reaction in the pathway. [51b,92e,119] Phytoene is a C40 isoprenoid generated through the head-to-head 1–1 condensation of two molecules of geranylgeranyl diphosphate (GGPP) to produce first prephytoene and then phytoene. GGPP in turn is generated by the condensation of IPP and DMAPP. Phytoene synthesis is illustrated in Fig. 7.

Three IPP's and one DMAPP molecule undergo 1′-4 head-to-tail condensation to form the C20 iso-prenoid product geranylgeranyl diphosphate in a reaction catalyzed by GGPP synthase (GGPPS). GGPPS was first identified in carotenogenic bacteria *Pantoea agglomerans* (formerly *Erwinia herbicola* [120] and plants [121] and later in yeast [122] and animals [123] (reviewed in Bouvier *et al.* [124]). There are multiple GGPPS enzyme isoforms in plants most of which are plastid located. [125] GGPP is a key intermediate in isoprenoid metabolism of plants, as a precursor of carotenoids, tocopherols, side chain of chlorophylls, plastoquinones and the phytohormones gibberlins. The diverse functions and regulations of these iso-prenoids make the presence of multiple GGPPS isoforms in plants necessary. Different GGPPS isoforms and GGPPS from different organisms vary with regards to the preference for starting allylic substrate. Some plant GGPPS use primarily DMAPP while animals and fungi GGPPS prefer farnesyl diphophosphate (FPP). [124]

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The sequence of GGPPS enzymes indicates the presence of aspartate-rich, DDxx(xx)D, motifs characteristic of *trans*-prenyltransferases that are involved in the coordination of a Mg^{2+} cluster used in substrate binding and catalysis. [126] The 1′-4 coupling reaction catalyzed by GGPPS occurs via nucleophilic attack of the olefinic double bond of IPP onto the carbocation formed through release of the allylic di-phosphate group (DMAPP, GPP or FPP). [127] GGPP is generated through three cycles of ionization-condensation-elimination reaction in which one additional IPP (C5 isoprenoid) unit is added at a time. [128] While FPP synthase (FPPS) limits chain elongation through the presence of a bulky residue before the first DDxx(xx)D motif at the end of the reaction cavity, [129] yeast GGPPS use alternate determinants of chain length. [130]

Several structures of ligand-bound and free GGPPS from yeast, [130,131] human [132] and one plant species have been reported. [133] The plant GGPPS from *Sinapis alba* was crystallized bound to GGPP obtained fortuitously from the bacterial host. Each subunit of the GGPPS dimer adopts a predominantly α-helical fold also seen in FPPS and other *trans*-prenyltransferases. The structure of GGPPS indicates that a Mg2+ cluster mediates binding of the allylic substrate while IPP binds to a second (homoallylic) binding site composed of positively charged residues. [127,130b] In addition to the diphosphate binding sites, both substrates can interact with two distinct hydrophobic binding sites. [132,134] Identification of GGPP bound to the allylic site of GGPPS offers an explanation for the regulation of GGPPS by GGPP through a negative feedback mechanism. [132] The mechanism of cyclic condensations, structure and key features of GGPPS are illustrated in Fig. 8.

Phytoene synthase (PSY) catalyzes the head-to-head condensation of two molecules of GGPP. PSY was initially purified and its gene cloned from plants [135] and bacteria. [136] Later studies showed that PSY is found exclusively in carotenogenic organisms. PSY catalyzes an important rate-controlling step in the synthesis of carotenoids, [119] as a result, its levels are highly regulated at both transcriptional and post-*trans*criptional level. [137] Some plant species code for several paralogs of PSY that are differentially regulated and have different tissue and intracellular expression patterns. [92e,137b,c,138] Importantly, the expression of PSY is upregulated in response to accumulation of downstream intermediates of the poly-*cis* pathway. [139] This is reminiscent of the upregulation of DXS in response to a block in the MEP pathway. [53]

Consistent with the role of carotenoids in photoprotection, the expression of PSY in *A. thaliana* is also upregulated in response to an increase of red and farred illumination. [137b,138c,d,140] The phytochrome interacting factor 1 (PIF1) that represses the expression of PSY is rapidly degraded by photoactivated phytochromes in response to light. [141] This effect results in the derepression of PSY and an increase in carotenoid production. Other carotenogenic and plastidial genes are regulated by PIF-like transcription factors in response to developmental and environmental stimuli. [142]

Despite lack of structural models of PSY, much can be deduced about its activity based on its similarity to dehydrosqualene (4–4′-diapophytoene) synthase (CrtM) and squalene synthase (SQase) which have been structurally characterized. CrtM, is a bacterial enzyme related to PSY which catalyzes the head to head condensation of FPP to generate 15-*cis*-dehydrosqualene, a C30 isoprenoid. Dehy-drosqualene is a precursor for the synthesis of the C30 class of carotenoids, staphyloxanthins, produced by the human pathogen *S. aureus*. The reaction mechanism employed by CrtM is very similar to the one employed by PSY, as the two enzyme share very similar substrates and intermediates. In fact, a heterologous expression system which combines GGPPS, CrtM and the bacterial phytoene desaturase CRTI in *E. coli* can lead to the production of trace amounts of lycopene in such host. [143] Random mutagenesis led to the identification of several mutants of CrtM which, like PSY,

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display increased preference for the synthesis of C40 carotenoid products [143] The enzymatic reaction, structure and key features of CrtM are illustrated in Fig. 9 along with a prediction of the structure of PSY based on based on multiple threading alignments and iterative threading assembly refinement (I-TASSER). [144]

Detailed mechanistic studies and several structures of free and ligand-bound CrtM and SQase have illustrated the key features of the head-to-head terpene cyclase reactions. The structure of CrtM bound to FFPP substrate indicates a typical isoprenoid fold with two distinct substrate binding sites for the allylic donor FFPP and the prenyl acceptor FFPP, respectively. [145] The diphosphate group of the prenyl donor interacts with a divalent metal ion cluster, which is coordinated to DXXXD motifs. [145–146]

The head-to-head terpene condensation proceeds through an ordered reaction which begins with the ionization of the donor GGPP through abstraction of diphosphate to form a geranylgeranyl carbocation. This is followed by nucleophilic attack by the acceptor GGPP to produce prephytoene, a stable cyclo-propylcarbinyl disphosphate intermediate, with a 1′-2–3 structure. [147] Similarly to SQase, PSY and CrtM catalyze a complex rearrangement of the 1′-2–3 cyclopropylcarbinyl diphosphate intermediate via a c1′-2–3^{+} → c1′-1–2^{+} cyclopropylcarbinyl – cyclopropylcarbinyl rearrangement. In the case of PSY and CrtM, the rearrangement is followed by loss of proton and the formation of the 1–1′ product 15-*cis*-phytoene or 15-*cis*-dehydrosqualene, respectively. [148] In contrast, SQase requires NADPH in controlling the stereochemistry of the reaction and to catalyze a further reduction with inversion of configuration of 15-*cis*-dehydrosqualene which affords squalene. [148–149]

3.2 Lycopene synthesis in fungi and bacteria

Two distinct pathways allow for the formation of all-*trans*-lycopene from phytoene. One pathway present in all carotenogenic bacteria with the exception of cyanobacteria relies on one single enzyme, the bacterial-type phytoene desaturase (CRTI), to carry out the desaturation of four bonds of phytoene at position 7, 11, 11′ and 7′. A second pathway present in cyanobacteria, green algae and plants relies on four enzymes, to carry out the same process. The CRTI-based pathway lycopene synthesis pathway found in most carotenogenic bacteria except cyanobacteria is illustrated in Fig. 7.

Screening for mutations leading to carotenoid deficiency in the nonsulfur purple photosynthetic bacteria *Rhodobacter capsulatus* led to the indentification of bacterial-type phytoene desaturase, CRTI. [150] The CRTI enzyme was later purified and shown to require FAD as a cofactor and oxygen (aerobic) or quinones (anaerobic) as final electron acceptors. [151135b,151–152] Analysis of its gene sequence and more recently its structure revealed CRTI bears similarity to protoporphyrinogen oxidoreductases and mono-amine oxidase. [152c,153] The sequence and structural homology between phytoene desaturases like CRTI and monoamine and protoporphyrinogen oxygenases extends beyond the canonical FAD binding domain to include additional conserved residues and structural motifs. [152c,154]

The available structure of CRTI is not ligand or cofactor-bound, but it offered hints with regards to the reaction mechanism of CRTI. In addition to its extended FAD binding domain, the structure of CRTI also indicates a putative substrate binding domain and a possible membrane-binding or regulatory domain. [152c] There is also evidence of a structural rearrangement upon cofactor binding based on evidence that FAD binding to reconstituted CRTI is necessary for its association with membranes. [152c] It has been reported that CRTI catalyzes the desaturation of both ends of the carotenoid molecule simultaneously to produce predominantly symmetric products such as the ζ-carotene intermediate produced by desaturation at 11 and 11′ and the final product all-*trans*-lycopene. [152b] However, docking studies suggest that the ligand binding pocket of CRTI can accommodate only half of the

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carotenoid molecule, [152c] which implies that CRTI might function as a dimer to recognize both ends of the carotenoid molecule simultaneously. More studies are needed to establish the quaternary structure of the active CRTI enzyme and to identify residues and domains involved in catalysis and membrane association. The enzymatic reaction, structure and key features of CRTI are illustrated in Fig. 10.

Based on the analogy of CRTI to monoamine oxidases, the desaturation of phytoene by CRTI could proceed by one of several different mechanisms involving either 1) direct hydride transfer from substrate to FAD, 2) single electron transfer, 3) hydrogen transfer or 4) a nucleophilic mechanism. [155] It was proposed that CRTI employs a hydride transfer mechanism based on the similar α-β dehydrogenation reaction carried out by FAD-dependent acyl-CoA dehydrogenases. [156] The mechanism of CRTI was probed using a 5-deaza-FAD analog which lacks the lone electron pair on N5 thus it is restricted to two-electron transfer chemistry. [157] econstitution of CRTI with 5-deaza-FAD should allow for the proposed hydride transfer mechanism, [158] yet, CRTI is inactive when reconstituted with the 5-deaza-FAD. [152c] It is possible that in the case of CRTI 5-deaza-FAD does not support hydride transfer for steric reasons. Other mechanisms of reaction that could be considered include single-electron transfer through the formation of a radical pair between substrate and semiquinone as proposed for monoamine oxidase. [159] Another mechanism based solely on FAD acting as an acid/base catalyst could also be envisioned. This mechanism was first proposed for IDI-2, [115d,g,116b] which is also inactive when reconstituted with 5-deaza-FAD. [115d] More studies are needed to discern between hydride transfer, single-electron transfer, acid/base, or other potential mechanisms of reaction of CRTI.

It is predicted that in addition to desaturation, CRTI also carries out the isomerization of the 15-*cis* double bond of phytoene. This is based on the fact that all products of CRTI, including partially desaturated intermediates such as phytofluene and ζ-carotene, are found predominantly in the all-*trans* configuration. [152b] Purified CRTI reconstituted with reduced FAD (FAD_{red}) does not isomerize 15-*cis*-phytoene to all-*trans*-phytoene when examined *in vitro* under anaerobic conditions. [152c] However, FA-D_{red}-CRTI does catalyze the isomerization of the non-natural substrate tetra-*cis* lycopene to tri-*cis* lycopene. [152c] Mechanistic studies of the isomerization of tetra-*cis* lycopene by FAD_{red}-CRTI using D_2O indicate that FAD_{red}-CRTI uses an acid-base catalysis mechanism in the isomerization of tetra-*cis* lycopene. [117b] An alternative proposed mechanism for the CRTI-catalyzed isomerization reaction suggests that the isomerization of the 15-*cis* bond occurs concurrently with the desaturation of adjacent double bonds. [160] In this model formation of the allylic carbocation intermediate allows the delocalization of the Π-electrons and free rotation of the 15–15′ bond resulting in *trans* isomerization. [160] More mechanistic studies and especially high resolution structures of CRTI reconstituted with FAD and substrate are needed to establish the geometry of the active site and whether any amino acid residues play roles in the deprotonation of the substrate or in stabilizing the presumed allylic carbocation intermediate.

CRTI from different species display variability with regards to the number of cycles of desaturation catalyzed. CRTI from *Pantoea ananatis* (former *Erwinia uredovora*) carries out four successive desaturation cycles of phytoene to produce lycopene. On the other hand, CRTI from *Rhodobacter sphaeroides* carries out three desaturation cycles which afford neurosporene. [161] Several key residues that control the number of cycles of desaturation were identified. [162] Site-directed mutagenesis of these residues increased the number of desaturation cycles of the neurosporene-producing CRTI from *R. sphaeroides* to four and allowed for the production of lycopene. Further mutations of *Erwinia* CRTI were identified which can increase the number of desaturation cycles to six and afford the production of a 3,4,3′,4′-tetradehydrolycopene, a fully conjugated species. [163] Fungal CRTI is structurally

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similar to bacterial CRTI [152a] and catalyzes 4 or 5 desaturation steps. Fungal CRTI works together with the fungal bifunctional PSY/ lycopene cyclase to yield β-carotene or the monocyclic carotenoid torulene through cyclization of 3,4-didehydrolycopene. [164]

Examination of other CRTI-like enzymes also revealed that substrate recognition by CRTI is not dependent on the length of the polyene chain. CrtN, a homologue of CRTI from *S. aureus*, desaturates C30 dehydrosqualene to produce 4,4′-diaponeurosporene. [165] The C30-directed CrtN and the C40-directed CRTI can substitute for one another to desaturate substrates that are longer, or respectively shorter, than their natural substrate. [166] It is not clear how different length polyene chains can be recognized by the same ligand binding pocket within CRTI or CrtN.

3.3 Lycopene synthesis in cyanobacteria and plants – the poly-*CIS* pathway

In contrast to the all-*trans* pathway carried by CRTI, lycopene is generated in plants and cyanobacteria through a poly-*cis* pathway. [167] The poly-*cis* pathway involves successive dehydrogenations and isomerizations carried out by: two desaturases, the plant-type phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), and by two isomerases, 15-*cis*-ζ-carotene-isomerase (Z-ISO) and CRTISO. PDS, ZDS and CRTISO recognize only one half of the carotenoid molecule thus the sequence of desaturation and isomerization reactions proceeds independently for each half of the carotenoid. The sequence of the reactions is well established. Reactions carried out by Z-ISO and PDS precede ZDS which requires substrates with desaturated 11 and 11′ bonds [168] and 15–15′ in *trans.* [169] The isomerization reaction carried out by CRTISO is the last reaction since it requires substrates to have adjacent 7- and 9-*cis* bonds. [170] Thus for each half of the carotenoid molecule the sequence of desaturation and isomerization reactions generally proceeds in the order PDS→Z-ISO→ZDS→CRTISO. [160,169,171] The poly-*cis* lycopene synthesis pathway from plants and cyanobacteria is illustrated in Fig. 7.

3.3.1 Desaturases/ *TRANS-CIS* isomerases—PDS was initially identified in cyanobacteria as a carotenogenic enzyme target of the bleaching herbicide norflurazon. [172] This was soon followed by cloning a corresponding cDNA from soybean, pepper, tomato and maize. [173] Evidence derived from the effect of PDS inhibitors and the phenotype of PDS mutants indicate that PDS is essential for carotenoid synthesis. [174] Heterologous expression of PDS in *E. coli* indicates it catalyzes a two step saturation of phytoene into ζ-carotene. [173c,e] PDS requires FAD for activity and is a membrane-bound enzyme based enzymatic assays of purified enzyme reconsitituted into liposomes in the presence or absence of redox cofactors. [92b,173b,175] Each cycle of dehydrogenation by PDS generates two electrons which are transferred via a redox chain [176] to plastoquinone [177] and to chloroplast terminal oxidase [178] using oxygen as the final acceptor. [179] The importance of plastoquinone in electron transfer to oxygen from PDS during carotenoid synthesis is underscored by the deficiency in carotenoid production that characterizes mutants with an impaired quinone biosynthetic pathway. [171b,177c] Several point mutations which confer the mutant PDS enzymes resistance to norflurazon also affect its catalytic efficiency. [172b,180] It was proposed that norflurazon inhibits PDS by interfering with quinone cofactor binding. [181]

The activity of ZDS was identified though heterologous expression of a candidate phytoene desaturase in *E. coli* strains that accumulate ζ-carotene. [182] ZDS was shown to introduce *cis*-double bonds in ζ-carotene at position 7 and 7′. The N-terminal domain of ZDS bears striking sequence similarity to the FAD-binding domain of L-amino acid oxidases, however, purified ZDS was shown to be active in the absence of added dinucleotide cofactors. [168] Though ZDS does share significant sequence homology with PDS, it is much more resistant

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(1000-fold) to inhibition by the herbicide norflurazon. [183] Like PDS, the activity of ZDS is required for carotenoid synthesis *in vivo.* [184]

It is not clear why plants and cyanobacteria employ such an elaborate pathway to achieve the same goal as other bacteria and fungi can accomplish with a single enzyme. The most likely explanations for the need of isomerases in the desaturation pathway have to do with the mechanism of reaction of PDS, ZDS, Z-ISO or CRTISO and possible roles of the *cis*-intermediates in regulatory signaling pathways. [139] Unfortunately, still too little is known about the mechanism of the reaction employed by PDS, ZDS, Z-ISO or CRTISO or the possible origin of the 7, 9, 9′ and 7′ *cis*-bonds or the role of *cis*-intermediates beyond lycopene synthesis.

It was originally proposed that the 9 and 9′ *cis*-bonds are not a product of the PDS desaturation reaction and that they originate from a *cis*-GGPP precursor [171a] or, alternatively, that they result from a prenyltransferase reaction. [170] However, the main C40 precursor that can be detected in plants is 15-*cis* phytoene and the presumed 9,15,9′-tri-*cis*-phytoene has not been reported in wild type or PDS-deficient plants. Therefore, the *cis* bonds are most likely formed during the course of the desaturation reactions catalyzed by PDS and ZDS, as first suggested by Goodwin and later by Clough and Pattenden. [167c,185] Biochemical analysis of the activity of heterologously expressed PDS and ZDS provided conclusive evidence of the role of PDS and ZDS in the generation of the 9, 9′ and 7, 7′ cis bonds. [169]

In the current view, the poly-*cis* pathway proceeds as follows. Desaturation of the 11 and 11′ bonds of phytoene by PDS causes the isomerization of the adjacent 9 and 9′ double bonds from *trans* to *cis.* [186] Though, it is still not clear why this is the case, it is possible that desaturation by PDS occurs through a similar mechanism as invoked for CRTI. [160] Hydrogen abstraction from C-10 leads to the formation of an allylic radical and delocalization of Π-electrons allowing free rotation and isomerization of the 9–10 bond from *trans* to *cis*. Desaturation by PDS is followed by the isomerization of the 15-*cis* bond of ζ-carotene to *trans,* which can be mediated either by light or by the Z-ISO isomerase. Then, ZDS carries out the stereospecific abstraction of protons from 7 and 7′ which results in the formation of the 7 and 7′ *cis* bonds in 7,9,9′,7′-tetra-*cis*-lycopene. However, 7,9,9′,7′-tetra-*cis*-lycopene is not a substrate for β or ε-cyclases. [118,187] Therefore, the formation of the poly-*cis* intermediates requires the recruitment of an additional isomerase to generate the all-*trans*-lycopene substrate for cyclization. [169] The last enzyme, CRTISO, fulfills a critical role in converting the *cis*-double bonds introduced by PDS and ZDS to all-*trans* in plants and cyanobacteria.

3.3.2 *CIS-TRANS* isomerases—The source of the 15-*cis*-double bond in phytoene as found in most plants, cyanobacteria and alga can be traced to the PSY activity from such organisms. [188] While PDS is active and in fact prefers 15-*cis* substrates, [169] ZDS is only active against substrates with a 15-*trans* conformation. [169] In sun exposed tissues, the 15-*cis* bond can be photoisomerized to *trans* to provide substrates for ZDS, [169] how-ever, in dark tissues, such as etiolated plants, a light-independent mechanism is required. A mutant in the Z-ISO gene of maize, referred to as *pale yellow 9* (*y9*), was shown to accumulate 9,15,9′-tri-*cis*-ζ-carotene in dark tissues indicating a possible impairment in the light-independent mechanism of isomerization of the 15-*cis* bond. [171b,189] A mutant screen for impaired carotenoid formation in dark germinated seeds led to the identification of the Z-ISO enzyme which converted 9,15,9′-tri-*cis*-ζ-carotene to 9, 9′-tri-*cis*-ζ-carotene in functional complementation assays in *E. coli* also expressing GGPPS, PSY and PDS. [171c]

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Z-ISO is a chloroplast localized transmembrane protein related to the NnrU family of enzymes found in denitrifying bacteria. [171c,190] Expression of *Z-ISO* transcript is highly correlated with the expression of other carotenogenic enzymes. Z-ISO homologs can be found in plants and cyanobacteria. [171c] Based on the central location of the double bond it is predicted that Z-ISO recognizes the carotenoid molecule differently than what is proposed for CRTI, PDS, ZDS or CRTISO which act on one half of the molecule. In addition, the lack of sequence homology of Z-ISO to any known carotenogenic enzyme suggests possibly a different mechanism of reaction.

There has been considerable evidence that tetra-*cis*-lycopene intermediates are formed during carotenogenesis in plants. In fact, fruits of a well known tomato strain called *tangerine* have a bright orange color and had been shown to accumulate *cis*-carotenes by Zechmeister and Pauling. [191] The *tangerine* phenotype is conferred by mutations in the CRTISO gene. Like Z-ISO, the activity of CRTISO is partially redundant in plant tissues that are exposed to high levels of sunlight presumably as a result of the photoisomerization of the tetra-*cis*-lycopene to all-*trans.* [192] Therefore, CRTISO-deficient plants can still synthesize carotenoids, albeit, at slower rate and only in specific tissues. The expression of CRTISO is subject to regulation by a protein belonging to Set Domain Group 8 (SDG8) of histone lysine methyl-transferases suggesting CRTISO plays a key regulatory role in the pathway. [193]

The CRTISO gene was identified independently in cyanobacteria, tomato and *A. thaliana* using different approaches based on biochemical complementation assays or a combination of screens for carotene-deficient strains and map-based cloning. [192,194] Analysis of the sequence of CRTISO indicates significant sequence homology to CRTI-type bacterial phytoene desaturases but less so to the plant desaturases, PDS and ZDS. [192] Like CRTI, CRTISO has an extended FAD binding domain which is also shared by protoporphyrinogen oxidoreductases and monoamine oxidases. [154] However, unlike CRTI, CRTISO carries out a reaction which has no net redox change.

In vitro assays of purified CRTISO reconstituted with cofactors indicate that CRTISO is a FAD_{red}-dependent protein which requires reducing cofactors and a redox system for activity. [118,170] Similarly to CRTI and IDI-2, CRTISO is not active when reconstituted with 5-deaza-FAD [115b,118,152c] indicating the involvement of the lone electron pair of N5 of FAD in a two electron transfer process. [157] In contrast to IDI-2, CRTISO does not incorporate deuterium in the isomerized product which argues against an acid-base mechanism of reaction. [118,195] It was observed that CRTISO recognizes carotenoid precursors with adjacent 7,9-di-*cis* bonds as found in 7,9,9′-tri-*cis*-neurosporene, 7,9,9′-tetra-*cis*-lycopene and 7′,9′-di-*cis*-lycopene. Importantly, CRTISO isomerizes both 7 and 9-*cis* bonds of one end of the molecule simultaneously. Accordingly neither mono-*cis*, tri-*cis* nor symmetric 7,7′-di-*cis*- and 9,9′-di-*cis*-lycopene isomers can be detected as products of CRTISO. [118,196b] Cumulatively these findings suggest that CRTISO catalyzes a two-step reaction recognizing sequentially one end then the other of the carotenoid molecule. [118,196a,b] The process of isomerization depends on the participation of the lone electron pair of N5 of FAD_{red} and there is no evidence of incorporation of deuterium from D_2O into product in the course of isomerization. [118]

The extended FAD binding domain first described in CRTI [154] is also shared by CRTISO and by the mammalian enzyme retinol saturase (RetSat), which catalyzes the double bond saturation of the apo-carotenoid retinol (vitamin A) to produce (13*R*)-all-*trans*-13,14-dihydroretinol [196b,c,d] and requires FAD_{red} and NADPH for hydrolysis (unpublished observations). The sequence similarity of a *cis-trans* isomerase (CRTISO), a desaturase (CRTI) and a saturase (RetSat) which act on similar substrates is consistent with a

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mechanism of *cis-trans* isomerization of isoprenoids with conjugated double bonds that involves saturation of the *cis* bond followed by rotation and desaturation of the double bond in *trans.* [197] A prediction of the structure of CRTISO in comparison with CRTI based on multiple threading alignments and iterative threading assembly refinement (I-TASSER) [144] is shown in Fig. 10.

4. CYCLIZATION OF LYCOPENE

Cyclization of lycopene represents a bifurcation in the carotenogenic pathway leading to carotenoids with either β or ε-ionone rings as initially proposed by Porter and Lincoln [167b] Each ψ-acyclic end of lycopene can be cyclized to produce either β-, ε-, or γ-ionone rings. The type of end groups and type of ring substitutions found in a carotenoid are the most important determinant of the function and fate of the carotenoid compound; meanwhile, the range and type of modifications that can be found in the polyene chain are more limited. Carotenoids that contain at least one unsubstituted β-ionone end group can become a source of vitamin A, retinol, which can be used to generate signaling molecules such as retinoic acid or visual chromophore, 11-*cis*-retinal in vertebrates. [12a,17c,198] Cyclization of lycopene is carried out by enzymes belonging to several distinct families. [124,199] For this reason, increases in the activity of β-versus ε-lycopene cyclases can channel lycopene toward the production of provitamin A carotenoids with increased nutritional value. [200] The mechanism and types of ionone-ring cyclization reactions are indicated in Fig. 11.

CRTY represents a large family of related lycopene β-cyclases found in most proteobacteria but not in cyanobacteria. A related family of lycopene cyclases (LCY) is found in most plants and cyanobacteria. LCY-type enzymes can be further divided into three subtypes based on the type of ionone ring produced. There are LCY-enzymes which generate β-(LCYB) or ε-ionone (LCYE) rings, and bifunctional enzymes that generate carotenoids with either β or ε-ionone rings (LCYB/E). [201]

CRTY was identified though the sequencing of carotenogenic clusters from the photosynthetic bacteria *Rhodobacter sphaeroides* [202] and the non-photosynthetic bacteria *Pantoea agglomerans* (formerly *Erwinia herbicola*). [203] LCY-type activity from the chromoplasts of daffodil and pepper plants has been investigated and shown to require FAD and NADPH cofactors. [196a,204] A LCYB-type enzyme from *Syn-echococcus* sp. was identified as the target of the herbicide 2-(4-methylphenoxy)triethylamine hydrochloride (MPTA) known to inhibit the formation of cyclic carotenoids. [201a,205] Use of introgression lines and map-based cloning led to the identification of a plant β-cyclase, or LCYB, gene responsible for the phenotype of the *Beta* and *old-gold* mutants [201c] and of the ε-cyclase, or LCYE, gene responsible for the *Delta* mutation. [201b] Variations in the expression levels of LCYE are correlated with alterations of the carbon flux towards carotenoids containing either β- or ε-ionone rings. [200] Thus LCYE is a major target in efforts to increase the nutritional value of staple crops through provitamin A biofortification. [200]

The LCYB-type β-cyclases, catalyze the cyclization of one end of lycopene to γ-carotene and subsequently the other to produce β-carotene. The LCYE-type ε-cyclases catalyze the cyclization of one ring of lycopene to produce δ-carotene. A subsequent cyclization of the ψ-acyclic end of δ-carotene by β-cyclases generates α-carotene. The LCYE enzyme of lettuce (*Lactuca sativa*) can cyclize both ψ-ends of lycopene to generate an ε,ε-carotene-precursor of lactucaxanthin. [206] Several specific domains and residues have been identified which determine whether LCYE introduces one or two ε-ionone rings. [206] A fascinating bifunctional LCYB/E-type enzyme employed by the cyanobacterium *Prochlorococcus marinus* cyclizes lycopene to either β- or ε-ionone end groups to generate β,β-, β,ε- or ε,ε-

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carotene. [201d] The amino acid sequences of all LCY-type enzymes includes a conserved motif FLEET motif which is important for activity. [207]

CRTY and LCY enzymes also show sequence homology to enzymes involved in ring substitutions such as neoxanthin synthases (NSY) and capsanthin-capsorubin synthases (CCS). CCS enzymes convert 5,6-epoxycarotenoids, such as antheraxanthin and violaxanthin, into the corresponding κ-cyclic carotenoids, capsanthin and capsorubin found in pepper plants. [208] CCS also exhibit β-cyclase activity. [209] A gene highly similar to CCS was discovered in potato and tomato plants [210] and the encoded enzyme converted the 5,6-epoxycarotenoid violaxanthin into neoxanthin, hence referred to as NSY. [210] In fact the gene coding for tomato NSY [210b] is the same as the gene that codes for tomato LCYB. [201c,211]

Though the overall homology between the monomeric lycopene cyclases CRTY and LCY-type enzymes is relatively low (20–25% identical and 40–50 % conservative substitutions) there are several well conserved domains shared by both types of lycopene cyclase enzymes. [199a,201c,212] Amongst these conserved domains, the pentapeptidic sequence, LIEDT, includes an essential Glu residue which is required for the activity of CRTY. [117b,207b] The LIEDT motif is similar to the essential FLEET motif of LCY enzymes. [207b] Studies of purified LCY- and CRTY-type enzymes reconstituted with cofactors indicate these cyclase enzymes share the use of FAD_{red} as a cofactor. [117]

LCY, CRTY and CCS have been shown to require FAD_{red} and NADPH for activity. [117,213] Cyclization of lycopene by flavobacterial CRTY in the presence of D_2O leads to the stereospecific incorporation of deuterium in the zeaxanthin product. [214] Lycopene cyclization by LCYB or CCS is inhibited by ammonium analogs. [207b,208,214] It has been proposed that the mechanism of cyclization for CRTY and LCY-type enzymes is initiated by proton attack [213a,214] involving the formation of tertiary carbocation which is stabilized by the enzyme. [117] This is then followed by proton abstraction from C-6 or C-4 to afford β- or ε-ionone end group, respectively. [207b,213a,214] In contrast to IDI-2, CRTI or CRTISO, CRTY reconstituted with 1-deaza-FAD or 5-deaza-FAD is, in fact, active. This observation does not support a role for FAD as an acid-base catalyst or in the formation of a radical pair though single-electron transfer. [117b] In line with this observation, it was proposed that the role of the FAD_{red} is most likely to stabilize the carbocation intermediate. [117b,207a] The essential glutamate residue of the LIEDT motif was proposed to act as an acid base catalyst. [117b] Based on the highly similar sequence and response to inhibitors it is likely that CCS and NSY also employ a similar mechanism. [117a,208,210]

Gram-positive bacteria, archaea and halophilic bacteria employ a heterodimeric β-cyclase encoded by two different genes CrtYc and CrtYd. [215] Members of this family are generally small proteins (approx. 12 kDa) and bear a signature hPhEEhhhhhh motif where “h” is a hydrophobic residue. The CrtYc and CrtYd-like domains of the equivalent β-lycopene cyclases of archaea are fused into a single polypeptide. [215c,d]

Fungi encode an intriguing family of enzymes that combine phytoene synthesis activity with lycopene β-cyclization. [216] Two discrete enzyme domains are responsible for the phytoene synthase and lycopene cyclase activities. [124,217] Studies of the CarRA enzyme of *Phycomyces* suggest that the bifunctional enzyme evolved from the fusion of a gene coding for a PSY and one coding for a lycopene cyclase. [216c,217] It is not clear if the fused polypeptide CarRA is the mature catalytically active form of the protein or whether the translation product is a protein precursor that requires cleavage to produce individual PSY and lycopene cyclase enzymes. A putative protease cleavage site that separates the two domains has been identified but not demonstrated. [216c] The cyclase domain of the

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bifunctional fungal enzyme bears homology to the CrtYc subunit of bacterial heterodimeric cyclases.

The genome of the green sulfur phototrophic bacterium *Chlorobium tepidum* does not code for any homologs of the CRTY, LCY or of bifunctional phytoene synthase/lycopene cyclase enzymes found in fungi. [218] Instead *C. tepidum* utilizes a novel β-cyclase enzyme, CruA, which converts lycopene to γ-carotene and very low amounts of β-carotene. [199b] Interestingly, *C. tepidum* is one of the few bacteria that employs a poly-*cis* pathway dependent on PDS-, ZDS- and CRTISO-type enzymes to convert phytoene to lycopene. [199b] Green sulfur bacteria also code for a paralog of CruA referred to as CruB which catalyzes β-cyclization of the ψ-acyclic end of γ-carotene and neurosporene (7′,8′-dihydrolycopene) but not lycopene. [219] Thus, CruB requires that the opposite end of the molecule than the one cyclized to be either cyclic or unsaturated. Presumably CruA and CruB act sequentially to cyclize lycopene to γ-carotene and then β-carotene.

CruA and CruB are part of the FixC dehydrogenase/oxidoreductase superfamily, [220] which includes mostly electron transfer flavoprotein (ETF) oxidoreductases. ETF oxidoreductases are a large family of flavoproteins which carry out one or two electron transfer to oxidize or reduce various substrates. [221] The mechanism of cyclization by CruA, CruB is not currently established, therefore, we do not know if the FAD cofactor is involved in electron, proton or hydride transfer or whether it serves to stabilize a presumed carbocation intermediate. It is important to note the frequent use of flavoproteins that carry out isomerization reactions with no net redox change to perform biochemical transformations in the carotenoid synthesis pathway.

In addition to green-sulfur bacteria, a paralog of CruA, referred to as CruP is also present in cyanobacteria and in plants. [199b] *C. tepidum* CruA has been shown to convert lycopene to γ-carotene in complementation studies [199b,222] and is required for lycopene cyclization *in vivo*. [199b] However, the lycopene cyclization activity and physiological role of the CruP paralog from cyanobacteria and plants is not quite clear. Neither complementation studies with CruP [222] nor the carotenoid profile of CruP-deficient *Synechococcus* sp. PCC 7002 [199b] support a role for CruP in cyclization of lycopene [222] despite earlier evidence of biochemical activity. [199b] There is, however, evidence that plant CruP plays a role in the reduction of oxidative stress associated with cold or anoxic conditions. [222]

5. RING SUBSTITUTIONS

Introduction of keto and hydroxyl groups are the most common ring substitutions seen in carotenoids. The hydroxylation of β- or ε-ionone rings at the C-3 position leads to the production of xanthophylls lutein and zeaxanthin. Xanthophylls play essential roles in protection against photooxidative damage. [223] Two distinct classes of enzymes are involved in the hydroxylation of carotenoids namely ferrodoxin-dependent non-heme diiron enzymes and enzymes belonging to the cytochrome P450 family. Substitutions of the β-ionone ring of provitamin A carotenoids, such as β-carotene, lead to formation of nonprovitamin A xanthophylls. Accordingly, specific members of the β-ionone hydroxylase family have been recognized as a critical target in breeding plants with a high provitamin A carotenoid content. [224] The mechanism and types of ionone-ring substitutions reactions are indicated in Fig. 11.

5.1 Carotene hydroxylation by nonheme diiron hydroxylases

The first carotene hydroxylases identified were those encoded by the carotenogenic clusters of the *Erwinia urdovora/herbicola* (*Pantoea*) species [225] and later those of plants. [226] The bacterial and plant enzymes share sequence homology (40% identity and 60% conserved

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substitutions) to each other. Their sequence also indicates similarity to nonheme diiron oxidases (HYD) which are involved in the hydroxylation or desaturation of fatty acids, sterols and other substrates. [226a,227] Specifically carotenoid β-hydroxylases from the HYD family contain conserved histidine cluster motifs HX (2–3)(XH)H, which is thought to coordinate a diferric oxo-bridged cluster. [228] The mechanism of reaction of HYD enzymes was first examined in the case of methane monooxygenase [229] and shown to proceed through a radical rebound (hydrogen atom abstraction–oxygen rebound) mechanism reminiscent of the one also employed by cytochrome P450 enzymes.

Based on studies of the alkane monooxygenase AlkB [230] and the L-p-aminophenylalanine hydroxylase, [231] a possible mechanism of carotenoid hydroxylation by HYD enzymes would proceed through binding of dioxygen to the iron cluster to form a peroxodiferric intermediate. This is followed by hydrogen abstraction from C-3 and formation of an alkyl radical which rebounds to form the hydroxylated ionone ring. Both plant and bacterial HYD enzymes hydroxylate β-ionone rings at the C-3 position and are not efficient at hydroxylating ε-ionone rings. [226a] Analysis of purified and reconstituted HYD enzymes showed that HYD hydroxylases are ferredoxin-dependent. [226b] A related family of HYD enzymes coded by *Adonis aestivalis* carry out the desaturation of the 3–4 bond or the introduction of hydroxyl group at the C-4 position in β-ionone. [232] The fact that HYD enzymes can also carry out the desaturation of the β-ionone ring offers support for the mechanism proposed for the HYD β-cyclases.

Cyanobacteria also code for enzymes, represented by CrtR, which are involved in the hydroxylation of C-3 of β-ionone rings. The sequence of CrtR indicates the presence of the histidine-rich motifs found in the HYD family. [228b,233] However, the overall sequence of the CrtR is homologous to β-carotene ketolases of the CrtW-type rather than to the β-hydroxylases HYD enzymes from other bacteria and plants. [228b,233a]

Members of, yet, another family of carotenoid hydroxylases found in marine proteobacteria were shown to catalyze the hydroxylation of the β-ionone at the C-2 position. [234] The sequence of these enzymes also indicates the presence of the histidine rich motifs associated with enzymes belonging to HYD family but the overall homology to other HYD-type β-hydroxylases is low. The C-2 hydroxylase recognizes β-ionone groups with a 4-keto or a 3-hydroxy substitution.

These examples illustrate that HYD family enzymes with apparently different evolutionary origin have been adapted to carry out carotene hydroxylation in various organisms. Perhaps use of HYD enzymes in ionone ring hydroxylation is a reflection of convergent evolution. Other HYD enzymes that have been recruited in carotenoid synthetic pathways include the plastid terminal oxidase which oxidizes plastoquinol to plastoquinone thus transferring the electrons generated by phytoene desaturases to oxygen, the terminal acceptor. [178,235]

5.2 Carotene hydroxylation by cytochrome P450 enzymes

The dihydroxy α-xanthophyll lutein is the most abundant xanthophyll found in plants, [236] yet, for a long time the identity of enzymes responsible for the hydroxylation of ε-ionone rings remained elusive. Genetic evidence suggested that the enzyme responsible for hydroxylation of ε-ionone rings in *A. thaliana* was associated with the *lut1* locus. [237] This observations led to the identification of the gene coding for the ε-hydroxylase through map-based cloning. [238] In contrast to the previously known carotenoid hydroxylases, the novel ε-hydroxylase was identified as a cytochrome P450 enzyme member of the CYP97C family. [238] CYP97C preferentially hydroxylates ε-ionone rings but it can also carry out β-ring hydroxylation *in vivo*. [239] A second P450 enzyme from the CYP97A family was found to hydroxylate β-ionone rings. [240]

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It is not clear what dictates the substrate specificity of the CYP97C family for ε-ionone rings while the related CYP97A prefers β-ionone rings. It has been suggested that CYP97C works in conjunction with CYP97A β-hydroxylases form lutein by preferentially hydroxylating the ε-ionone rings of substrates whose β-ring is already hydroxylated. [240–241] There is also evidence that the various carotenoid hydroxylases work cooperatively in the production of xanthophylls. [242] Ablation of the four carotene hydroxylases of *A. thaliana* namely the two HYD and the two P450 family members results in a complete lack of xanthophylls. Thus, the four genes represent the full complement of carotenoid hydroxylases in this plant. [236] Nevertheless, there is evidence that a related plant P450 enzyme belonging to the CYP97B family can catalyze the hydroxylation of the β-ionone ring of α-carotene when overexpressed in transgenic plants but the physiological role of the CYP97B-mediated hydroxylation is still unclear. [243]

Interestingly, other P450 superfamily members found in vertebrates and cyanobacteria carry out the hydroxylation of the β-ionone ring of the C20 apocarotenoid retinoic acid. [244] The sequence homology and similar mechanism of enzymes involved in the similar biotransformation of carotenoids and apocarotenoids reflects an important functional and evolutionary relationship. [197b]

5.3 Carotene ketolases

The C-4 keto substitution is a very common modification of carotenoids synthesized by cyanobacteria. The ketocarotenoids include many species which have high commercial value and potential clinical use. Carotenoid ketolases that belong to two distinct families have been described. One family of carotene ketolases represented by the *Synechocystis* CrtO acts asymmetrically to introduce a 4-keto group in the β-ionone ring of one end of β-carotene to produce at first echinenone and subsequently ketolates the other end to produce canthaxanthin. [245] The enzymes involved in the introduction of keto-groups are indicated in Fig. 12.

CrtO shows similarity to CRTI and is proposed to employ a similar mechanism based on the formation of a carbocation intermediate which reacts with OH- to form a 4-hydroxy and subsequently a 4-dihydroxy derivative. [246] Though, the 4-dihydroxy derivative was not detected in the course of the reactions catalyzed by purified CrtO, [246] it was shown to be substrate for CrtO leading to the formation of 4-keto group by elimination of water. [246]

The second group of carotenoid ketolases are represented CrtW-type enzymes found in marine bacteria and green alga. Analysis of extracts of *E. coli* which overexpress CrtW indicates CrtW catalyze the introduction of keto groups at C-4 to produce canthaxanthin. [247] Complementation assays in *E. coli* that express carotenogenic genes indicate that, in contrast to CrtO, CrtW can also introduce 4-keto groups into the substituted β-rings of zexanthin leading to the formation of ketocarotenoids such as astaxanthin. [233b,248] Also differently than CrtO, the CrtW-catalyzed reaction requires oxygen but not dinucleotide cofactors based on the enzyme activities of lysates of *E. coli* expressing CrtW. [247d] The sequence of CrtW enzymes shows homology to the HYD-type β-hydroxylase CrtR from cyanobacteria. [233a] Similar to the CrtR several histidine-rich motifs commonly found in HYD family of oxidases are required for the activity of CrtW [249] possibly suggesting CrtW employs a similar mechanism to one proposed for HYD-type β-hydroxylase enzymes. [247d,249]

5.4 Epoxidases and de-epoxidases

Zeaxanthin can be further modified by the epoxidation of its β-ionone rings at the 5–6 position to successively produce antheraxanthin and later violaxanthin in reactions catalyzed by zeaxanthin epoxi-dase (ZEP). [250] The reverse reactions are catalyzed by the enzyme

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violaxanthin de-epoxidase (VDE). [251] The interconversion of zeaxanthin and violaxanthin by ZEP and VDE creates a xanthophyll cycle which plays a central role in the regulation of photosynthetic energy conversion. [3a,252] Plants with mutations in the ZEP and VDE genes are characterized by alterations in NPQ of chlorophyll fluorescence. [3]

Based on the analysis of crude preparations of thylakoid membranes, ZEP is a flavin-dependent monooxygenase which requires NADPH and oxygen. [253] Like other flavin-dependent monooxygenases, it is proposed that ZEP carries out electron transfer from reduced flavin to oxygen to form a hydroper-oxyflavin species which then carries out electrophilic attack on the ionone ring to incorporate epoxide. [254] There is little data regarding the residues involved in the epoxidation reaction due to difficulties in both heterologous expression and purification of ZEP. [250]

Based on the analysis of the sequence of ZEP and VDE it was predicted that that both enzymes contain a lipocalin fold found in proteins that bind hydrophobic ligands. [255] Analysis of the crystal structure of the central domain of VDE confirmed this prediction as the central domain of VDE assumes the characteristic eight stranded antiparallel β-barrel found in lipocalins. [256] The lipocalin domain of VDE was crystallized at both pH 5 and 7, [256] respectively. The two structures show that the lipocalin fold undergoes a pH-dependent transition from closed (neutral conditions) to open (acidic conditions). [256] This transition provides a mechanistic explanation for the effects of pH on the activity of VDE which is active and associated with the thylakoid membrane at low pH and inactive and not membrane bound at neutral pH. [257] Several key residues involved in the pH-dependent transition have been identified. [257b] In addition to the lipocalin fold, the sequence of VDE indicates the presence of a conserved cysteine-rich N-terminal domain whose role is not understood and a glutamate-rich C-terminal domain which is important for the association with the thylakoid membrane. [258] The enzymatic mechanism involved in the de-epoxidation reaction carried out by VDE is not entirely clear.

5.5 Other modifications

In addition to the classic carotenoid ring substitutions described above, there are many more possible substitutions found in less common carotenoids. Purple sulfur bacteria code for a β-ionone desaturase/methyltransferase, CrtU, which converts β-carotene to isorenieratene, a carotenoid with aryl end groups. [259] CrtU is a flavoprotein which bears some similarity to CRTI. [259]

The acyclic ψ-end groups of neurosporene and lycopene can also be further modified to produce acyclic carotenoids such as spheroidene and spirilloxanthin, respectively, by the purple photosynthetic bacteria. [260] These transformations require a 1,2-hydratase, a 3,4 desaturase and a methyltransferase, respectively, represented by the CRTI/CrtC, CrtD and CrtF enzymes. [261]

There are over 700 carotenoid species in nature representing one of the most diverse classes of biological compounds and many continue to be discovered. This diversity is a result of the large number of possible substitutions and the combinatorial use of a finite enzymatic repertoire by living systems to produce alternate outcomes. As we will continue to discover more enzymes responsible for carotenoid modifications and we will begin to explore their structure and mechanisms, we will have an unprecedented molecular understanding of these pathways and we will be in a position to predict the enzymes necessary to synthesize any carotenoid species desired.

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6. CONCLUDING REMARKS

One of the most important questions that has puzzled the carotenoid field for decades is in regards to the origin and advantage of the poly-*cis* pathway for lycopene synthesis in plants and cyanobacteria. [160] Why would cyanobacteria and plants use four separate enzymes and a convoluted mechanism to form lycopene when bacteria can accomplish the same goal with one single enzyme, CRTI. An explanation proposed is that the early intermediates of carotenoid synthesis are more energetically stable as *cis* rather than as *trans*-isomers. [262] Alternatively, it was proposed that one of the advantages of the poly-*cis* pathway is that the use of redox cofactors by the desaturases (PDS and ZDS) and isomerases (CRTISO) of the poly-*cis* pathway ties lycopene formation to the availability of redox cofactors, hence, the metabolic state of the photosynthetic cell. [160] This is a persuasive argument which is reminiscent of other regulatory models proposed for the regulation of secondary metabolism. However, in light of the requirement of redox cofactors by CRTI, it is apparent that a similar mechanism of redox regulation of carotenogenesis could operate even in the case of the CRTI pathway. Therefore, it is still not clear why cyanobacteria would need to develop a more complex pathway.

A recently developed concept that could explain the role of the poly-*cis* pathway in carotenoid synthesis is that the intermediates of this pathway have additional physiological roles that extend beyond serving as precursors of lycopene. [139] This concept is based on the analysis of the phenotype of several mutant strains of tomato lacking carotenoid synthetic genes. It has been known since 1950's that the *tangerine* mutation of tomato plants is epistatic to the *yellow-flesh* mutation in tomato plants. [263] The *yellow-flesh* mutation was identified as a mutation in the PSY gene [264] and, more recently, the *tangerine* mutation was identified as a mutation in CRTISO. [192a] Bearing in mind the sequence of reactions in the lycopene synthetic pathway in plants proceeds in the order PSY→PDS→Z-ISO→ZDS→CRTISO, the epistatic effect of *tangerine* on *yellow-flesh* is very unusual. This is because plants deficient in both PSY and the downstream gene CRTISO resemble the phenotype of plants deficient in CRTISO when logic dictates it should be the other way around. Though the exact nature of the recessive *yellow-flesh* mutation in PSY is not known, it appears that it is not a result of deleterious mutation in the PSY coding region. Instead it is proposed that the *yellow-flesh* mutation causes a decrease in PSY expression by altering a *cis*-acting transcriptional element or epigenetic factor. Moreover, plants lacking both PSY and Z-ISO resemble PSY in accordance with the sequence of synthetic reactions. The best explanation, thus far, for the epistatic mechanism of *tangerine* on *yellow-flesh* is that lack of CRTISO causes accumulation of intermediates of the poly-*cis* pathway which leads to the upregulation of the expression of PSY. [139] The feedback regulation of early carotenoid synthetic genes in response to a block in upstream metabolism represents a paradigm shift in our understanding of the mechanism and regulation of carotenoid synthesis, and of metabolic regulation in general. A similar phenomenon is encountered in the case of the MEP pathway where DXS protein levels are upregulated in response to a block in the MEP pathway or in response to an overexpression of carotenogenic genes such as PSY. [51b,53]

The molecular details of a signaling pathway that regulates carotenogenesis in response to the levels of carotenoid precursors are still unclear. It is not known if poly-*cis* pathway intermediates signal through specific receptors or whether they are converted to other bioactive metabolites. In addition to known intermediates of the poly-*cis* pathway, such as, tetra-*cis*-lycopene and tri-*cis*-neurosporene, CRTISO-deficient, *tangerine*, tomato fruits also accumulate apocarotenoids derived from their degradation. Some of the norisoprenoids which accumulate in *tangerine* fruits include pseudoionone, dihydro-*apo*-farnesal 6-methyl-5-hepten-2-one and significant levels of geranylacetone. [265]. It is, therefore, possible that such apocarotenoid metabolites act as a readout of the carbon flux through the

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carotenoid pathway allowing the organism to further tune carotenoid production. In support of this hypothesis, genes of poly-*cis* pathway enzymes (PSY, ZDS and PDS) were also shown to be induced in another mutant of CRTISO, namely the *yellow–orange fruit flesh* (*yofl*) mutant which also accumulates significant levels of apocarotenoids. [266]

On a fundamental level, the basic question that needs to be addressed first is with regards to the mechanism of catalysis by the desaturase/isomerase enzymes PDS and ZDS. Could the poly-*cis* pathway be a simple consequence of the choice by cyanobacteria and plants to use of mixed function desaturase/isomerase enzymes such as PDS and ZDS for lycopene synthesis – A mechanistic and structural understanding of the enzymes involved in the later steps of carotenoid metabolism would answer many of these questions.

Despite much progress in the understanding of the structure and mechanism of enzymes involved in the early steps of carotenoid synthesis involving isoprenoid and phytoene precursor synthesis, we still have a limited understanding of the later steps of carotenoid synthesis. Crystal or NMR structures have been reported for all of the enzymes involved in IPP and DMAPP synthesis. This knowledge has provided a detailed molecular understanding of the chemistry of isoprenoid precursor synthesis. Though the structure of PSY, the first enzyme of the pathway committed to carotenoid production, has not been reported, the mechanistic and structural studies of squalene synthase and CRTM have shed light on its mechanism and structure.

With the exception of the recently reported structure of CRTI and of a domain of VDE, there are no reports of structures for the enzymes involved in the synthesis of lycopene, lycopene cyclization or ionone-ring substitutions. Much of the paucity of structures and the limited enzymological understanding of enzymes involved in the later steps of carotenoid synthesis can be attributed to the notorious difficulty in handling such enzymes. Many of the enzymes involved in transformations of C40 isoprenoids are membrane-bound and use ligands that are some of most light sensitive and hydrophobic compounds known in biology. In addition, many carotenogenic enzymes require specific redox cofactors for activity. The fact that we know or strongly suspect the role of a great majority of the carotenoid enzymes and that for the most part we can predict the pathways that can generate a novel carotenoid is truly remarkable. It is also a testament to the tenacity and superb skills of the many biochemists and geneticists which have dedicated their studies to carotenoid biology over the past century.

Metabolic engineering of carotenoid synthetic pathways using a combinatorial approach has led to the efficient production of interesting carotenoids of high commercial value and pharmaceutical potential. [267] The occurrence of many possible xanthophyll enantiomers have made synthetic chemical approaches too laborious, costly or simply not feasible for industrial scale production. In addition, many of the natural sources of highly sought after carotenoids are difficult to obtain or to culture on an industrial scale. Heterologous expression in bacteria, yeast or plants can overcome many of the problems associated with cultivation or use of natural sources and allows for strict pathway control. [49,268] Combinatorial use of enzymes involved in the carotenogenic pathway has also led to the production of unique carotenoids that have, so far, not been documented in nature. Such examples include the novel violet phillipsiaxanthin and carotenoids with C45 and C50 backbones. [269] Many of these unusual carotenoids have very interesting physical and chemical properties which could be exploited for research, food colorant or clinical use.

Provitamin A carotenoids such as β-carotene and β-cryptoxanthin are the most important sources of vitamin A in the world population. [270] Dietary provitamin A carotenoids are oxidatively cleaved by carotenoid monooxygenase CMO1 to retinaldehyde. [271]

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Retinaldehyde is reduced to vitamin A, retinol which can be further esterified for storage as retinyl esters or can be converted to several active retinoids, such as 11-*cis*-retinal, the visual chromophore, [11e,f,12a,272] or all-*trans*-retinoic acid (ATRA), a ligand of nuclear hormone receptors which carries out essential roles in a multitude of biological processes throughout life. [11b17a,b] Despite the well recognized role of vitamin A in carrying out essential functions in embryonic development, vision, immunity and tissue differentiation, vitamin A deficiency is still one of the most important human health problems. Though, vitamin A supplementation prevents 350,000 childhood deaths annually in developing countries, yet, another 500,000 children still die and 250,000 become blind as a result of its deficiency [273]

One very important advance in combating vitamin A deficiency in developing countries relies on the redirecting carotenoid synthesis towards the synthesis of provitamin A carotenoids in staple food crops. Biofortification efforts through genetic engineering of major food crops such as rice, tomato, maize potato and cassava have identified key rate-limiting steps, genes and regulatory factors that can lead to increase production of provitamin A carotenoids. [21d,119b,200,224,274] Today, the content of β-carotene in *Golden Rice* approaches 37 μg/ g milled uncooked rice. Supplementation of school children diet with Golden Rice supplies vitamin A as effectively as a supplementation of an emulsion of pure β-carotene in oil and is even better than spinach. [274i,275] Much of this progress would not have been possible without an intimate knowledge of the carotenoid synthetic pathways tediously worked out by carotenoid biologists for the past century.

Our current knowledge of the carotenoid synthetic pathways is still limited in two important aspects which are essential considerations in devising approaches to meet plant biofortification goals. The steady state levels of carotenoids that accumulate in a cell are determined not just by synthetic but also by factors and pathway that control the degradation of carotenoids. Carotenoids can be oxidatively cleaved by both enzymatic and non-enzymatic means [18a,276] to produce many apocarotenoid metabolites. Some of these apocarotenoids include strigolactones and abscisic acid that play vital roles in plant metabolism. [15b,277] Others apocarotenoids include volatile norisoprenoid compounds such as β-ionone, geranial and citral that impart a pleasant aroma to fruits and vegetables. [265a,278] Elucidation of the enzymatic and non-enzymatic processes that govern carotenoid cleavage could help achieve higher levels of carotenoids in staple crops. Alternatively, creating metabolic storage sinks to sequester carotenoids and protect them from degradation can also lead to significantly higher content of carotenoids in food crops. [279]

Though we know the role of many of the individual enzymes involved in carotenoid synthesis, we do not know what the complete pathway looks like in terms of its three dimensional multi-enzyme organization. There is extensive evidence that many of the plant carotenogenic enzymes interact with one another forming complexes associated with the plastid membrane. [92,235] Association of enzymes that carry out consecutive steps in the pathway can allow for substrate channeling and avoid loss of intermediates to side reactions. It is also clear that many of the carotenogenic enzymes require redox factors for activity which need to couple to a redox chain and final electron acceptors. Therefore, besides focusing on individual enzymes, future carotenoid research will need to further investigate the macro-molecular structure and assembly of the multiprotein carotenogenic complex. Future avenues of research need to involve post-reductionist approaches using systems biology, structural biology, cryoelectron tomography and high-resolution optical imaging. [280] Such knowledge will provide a better understanding of the cellular environment of carotene synthesis and its complex interactions with other pathways within the cell.

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Biographies

Alexander Moise is a graduate of the Department of Zoology at the University of British Columbia, Vancouver, Canada, where he received his PhD (2000) for research on the mechanisms of evasion of the immune response by adenovirus. This was followed by a postdoctoral research project on cancer immunotherapy conducted jointly at The Michael Smith Laboratories and the Biomedical Research Centre at the University of British Columbia. In 2003, he joined the lab of Kris Palczewski at the University of Washington researching the metabolism and physiological roles of carotenoids and retinoids as they pertain to the processes of vision and gene regulation. In 2005, he moved to Case Western Reserve University to continue his training with Kris Palczewski and was promoted to the position of instructor in 2007. His postdoctoral studies led to the identification of retinol saturase (RetSat), a vertebrate homolog of CRTISO and the identification and characterization of the physiological role of the *dihydroretinoid* pathway in retinoid metabolism. His research also contributed to several important studies on the biochemistry and function of other critical enzymes involved in vitamin A metabolism. In 2009, he established his own lab at the University of Kansas where he continues to investigate the structure, biochemistry and physiological role of enzymes involved in carotenoid/retinoid and lipid metabolism in general.

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Salim Al-Babili studied Biology at the University of Freiburg, Germany. In 1996, he received his PhD in plant molecular biology and biochemistry from the same University. Between 1996 and 1998, he was a Rockefeller Post-Doc Fellow in the lab of Dr. Peter Beyer, working on developing "Golden Rice". After that, he held different positions as Post-Doc and lab leader heading an independent research group at the University of Freiburg. In 2007, he received his Habilitation in Cell Biology at the University of Freiburg. Dr. Al-Babili is currently Associate Professor for Bioscience at King Abdullah University of Science and Technology (KAUST), Saudi Arabia. Dr. Al-Babili made essential contributions to engineering of crops with enhanced provitamin A content, and his work on apocaotenoids formation unraveled the presence of retinoid metabolism in cyanbacteria and led to the elucidation of different related metabolic processes in fungi. His breakthrough discovery of carlactone deciphered the biosynthesis of the novel plant hormones strigolactones and revealed a new branch in the plant carotenoid pathway, which is initiated by enzymatic isomerization of all-*trans*-β-carotene.

Eleanore T. Wurtzel received a Ph.D. from SUNY Stony Brook in biochemistry and molecular biology (1982) where she conducted pioneering work on the first two-component signal transduction system in bacteria. Dr. Wurtzel switched to plant biology with postdoctoral stays at Brookhaven National Laboratory and Cold Spring Harbor Laboratory and then established her lab (1987) on plant carotenogenesis at Lehman College, The City University of New York, where she is currently Professor of Biological Sciences, Chair of the CUNY Plant Sciences Ph.D. subprogram and serves on the Biology and Biochemistry Doctoral faculty. Dr. Wurtzel's research on plant provitamin A carotenoids supports development of sustainable solutions to global vitamin A deficiency and has been funded by the NIH, Rocke-feller Foundation International Rice Biotechnology program, American Cancer Society, McKnight Foundation, and NSF. For long-standing contributions to carotenoid research and to the scientific community, Dr. Wurtzel was elected a AAAS fellow (2006) and was awarded "Fellow of ASPB" by the American Society of Plant Biologists (2012). Dr. Wurtzel founded and chaired the first Gordon Research Conference on Plant Metabolic Engineering and its first Gordon-Kenan Seminar (GRS) for early career scientists. She co-organized the 2010 Gordon Research Conference on Carotenoids, chaired the 2013 meeting and founded its new GRS. Wurtzel is a current member of the Gordon

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Research Conference Board of Trustees and serves as a Monitoring Editor of the journal, *Plant Physiology*.

ABBREVIATIONS

AACT	acetoacetyl CoA thiolase
ATP	adenosine 5′-triphosphate
CCS	capsanthin-capsorubin synthase
CDP-ME	4-(cytidine 5′-diphospho)-2*C*-methyl-D-erythritol
CDP-ME-2P	4-(cytidine 5′-diphospho)-2*C*-methyl-D-erythritol-2-phosphate
CMK	4-(cytidine 5′-diphospho)-2*C*-methyl-D-erythritol kinase
CMP	cytidine 5′-monophosphate
CRTI	bacterial-type phytoene desaturase
CRTISO	carotene isomerase
CrtM	dehydrosqualene synthase
CrtO	β-carotene ketolase, only unsubstituted β-rings
CrtW	β-carotene ketolase, including carotenoids with substituted β-rings
CRTY	bacterial-type lycopene β-cyclase
CTP	cytidine 5′-triphosphate
DMAPP	dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
FAD	flavin adenine dinucleotide
FAD_{red}	reduced FAD (FADH• or FADH2)
FMN	flavin mononucleotide
FPP	farnesyl diphosphate
FPPS	farnesyl diphosphate synthase
G3P	glyceraldehyde 3-phosphate
GGPP	geranylgeranyl diphosphate
GGPPS	geranylgeranyl diphosphate synthase
HDR	1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase
HDS	1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase
HMBPP	1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGS	3-hydroxy-3-methylglutaryl-CoA synthase
HPP	p-hydroxyphenylpyruvate

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HPPD	p-hydroxyphenylpyruvate dioxygenase
HYD	nonheme diiron oxidase
IDI-1	IPP-DMAPP isomerase type 1
IDI-2	IPP-DMAPP isomerase type 2
IPP	isopentenyl diphosphate
KET	β-carotene ketolase
LCY	plant-type lycopene cyclase
LCYB	lycopene β-cyclase
LCYE	lycopene ε-cyclase
MCT	2*C*-methyl-D-erythritol-4-phosphate cytidyltransferase
MDS	2*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase
MECDP	2*C*-methyl-D-erythritol-2,4-cyclodiphosphate
MEP	2*C*-methyl-D-erythritol-4-phosphate
MK	mevalonate kinase
MVA	mevalonate
MVD	mevalonate diphosphate decarboxylase
NADP+	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NPQ	non-photochemical quenching
NSY	neoxanthin synthase
P450	heme-binding cytochrome P450
PDS	plant-type phytoene desaturase
PMK	phosphomevalonate kinase
PSY	phytoene synthase
SQase	squalene synthase
VDE	violaxanthin de-epoxidase
ZDS	ζ-carotene desaturase
Z-ISO	15-*cis*-ζ-carotene-isomerase
ZEP	zeaxanthin epoxidase

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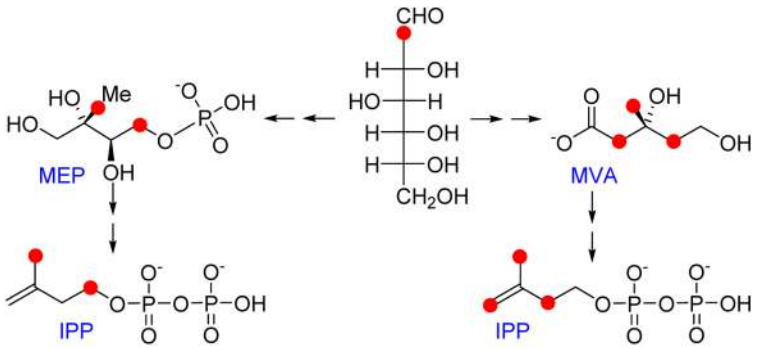

Figure 1.

Fate of the radiolabel from [1-13C]–glucose (red circle) in the formation of IPP via either the MEP pathway (*left* pathway) or via MVA pathway (*right* pathway). Adapted and reprinted with permission from Reference 35. Copyright 2004 American Chemical Society.

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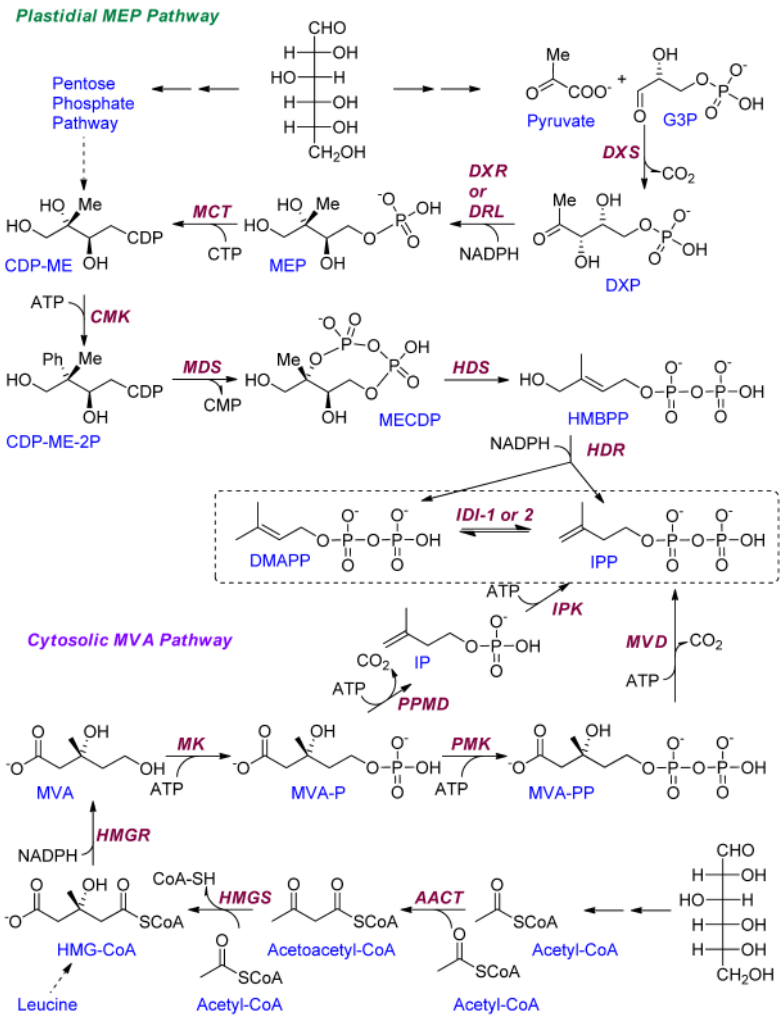

Figure 2.

Synthesis of the C5 isoprenoid precursors IPP and DMAPP. The plastidial MEP pathway from plants and eubacteria (shown in top diagram) leads to the production of both IPP and DMAPP (indicated in inset dashed-line box). A possible source of CDP-ME from the pentose phosphate pathway is also suggested. The MVA pathway from metazoa and fungi and a variation of the pathway found in archaea leading to the formation of IPP are illustrated in bottom diagram adapted from [281]. HMG-CoA can also be obtained from branched-chain amino acid leucine via 3-methylglutaconyl CoA. IPP and DMAPP can be interconverted by IDI-1 or IDI-2. Adapted and reprinted with permission from Reference 281. Copyright 2008 Springer.

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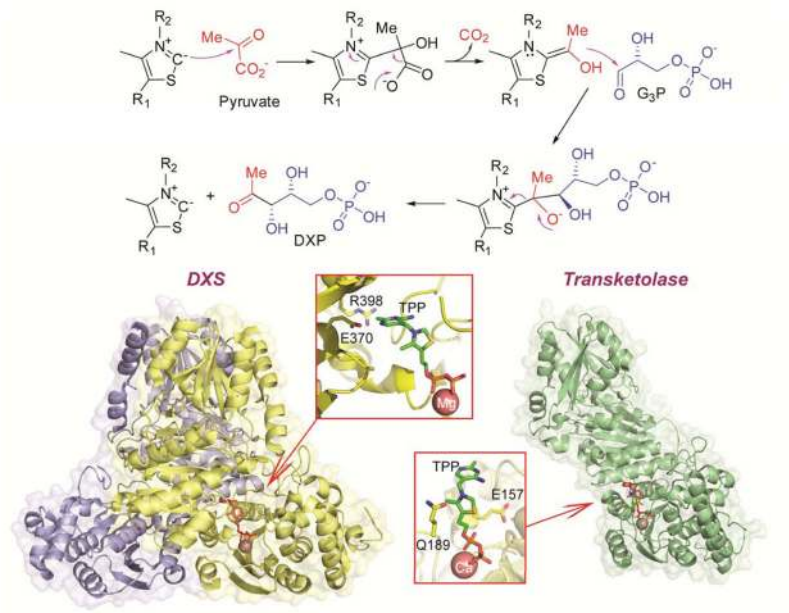

Figure 3.

The mechanism of reaction, structure and key features of DXS in comparison with transketolase. At left, the structure of the DXS dimer in complex with TPP (*red*) based on its reported structure (PDB 2O1S) with the subunits colored either *blue* or *yellow*. [46] Inset shows the orientation of TPP and the critical E370 and R398 residues of DXS colored by heteroatoms. Right structure shows a monomer of the related transketolase structure (PDB 3MOS) (*green*) in combination with TPP (*red*). [282] Inset shows the important transketolase E157 and Q189 residues involved in binding TPP colored by heteroatoms. The mechanisim of reaction of DXS is adapted and reprinted with permission from Reference 44. Copyright 2003 American Chemical Society.

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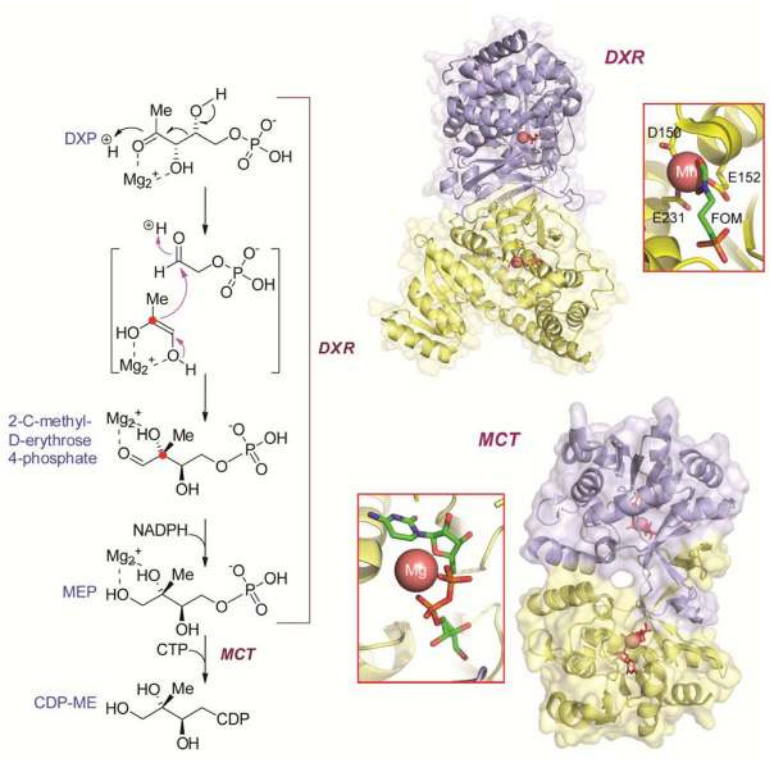

Figure 4.

The mechanism of reaction, structure and key features of DXR and MCT. The top structure shows the DXR dimer with the subunits colored either *blue* or *yellow* in association with the inhibitor fosmidomycin (FOM in *red*) based on its reported structure (PDB 1ONP). [61c] Inset indicates FOM and the critical residues D150, E152 and E231 (colored by heteroatoms) that coordinate the divalent ion (Mn^{2+}) within the active site of DXR. The MCT dimer structure with the subunits colored either *blue* or *yellow* in complex with its product CDP-ME (*red*) is based on its reported structure (PDB 3Q80). Inset shows the orientation of CDP-ME in relation to the Mg^{2+} ion within the active site of MCT. The mechanism of reaction of DXR is adapted from with permission from Reference 58. Copyright 2008 American Chemical Society.

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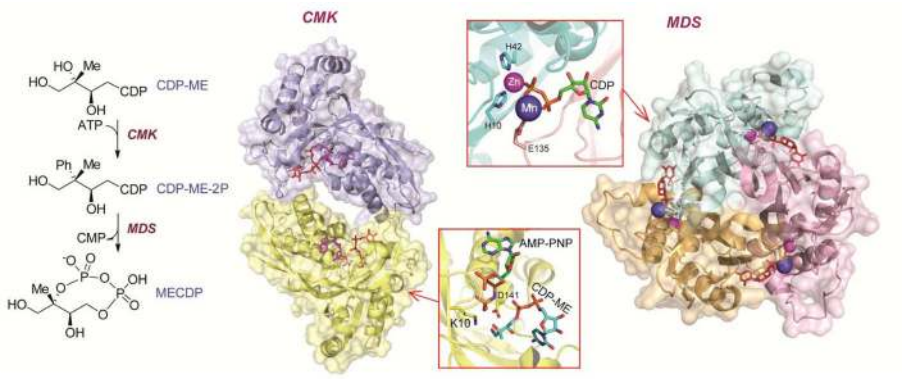

Figure 5.

The mechanism of reaction, structure and key features of CMK and MDS. The structure of the CMK dimer with the subunits colored either *blue* or *yellow* in association with a nonhydrolyzable ATP analogue (AMP-PNP) and substrate (CDP-ME) (both in *red*) based on its reported structure (PDB 1OJ4) [73a] Inset shows the orientation of AMP-PNP and CDP-ME forming hydrogen bonds with residues K10 and D141 (colored by heteroatoms) the active site of CMK. The MDS trimer structure with subunits colored *blue*, *yellow* or *red* in complex with cytidine 5′-diphosphate (CDP) (*red*) based on its reported structure (PDB 1GX1). [76a] Inset indicates the orientation of CDP in relation to the Mn^{2+} and Zn^{2+} ions and the MDS active site residues E135, H10 and H42 (colored by heteroatoms). Please note the active site of MDS is formed by residues derived from adjacent subunits.

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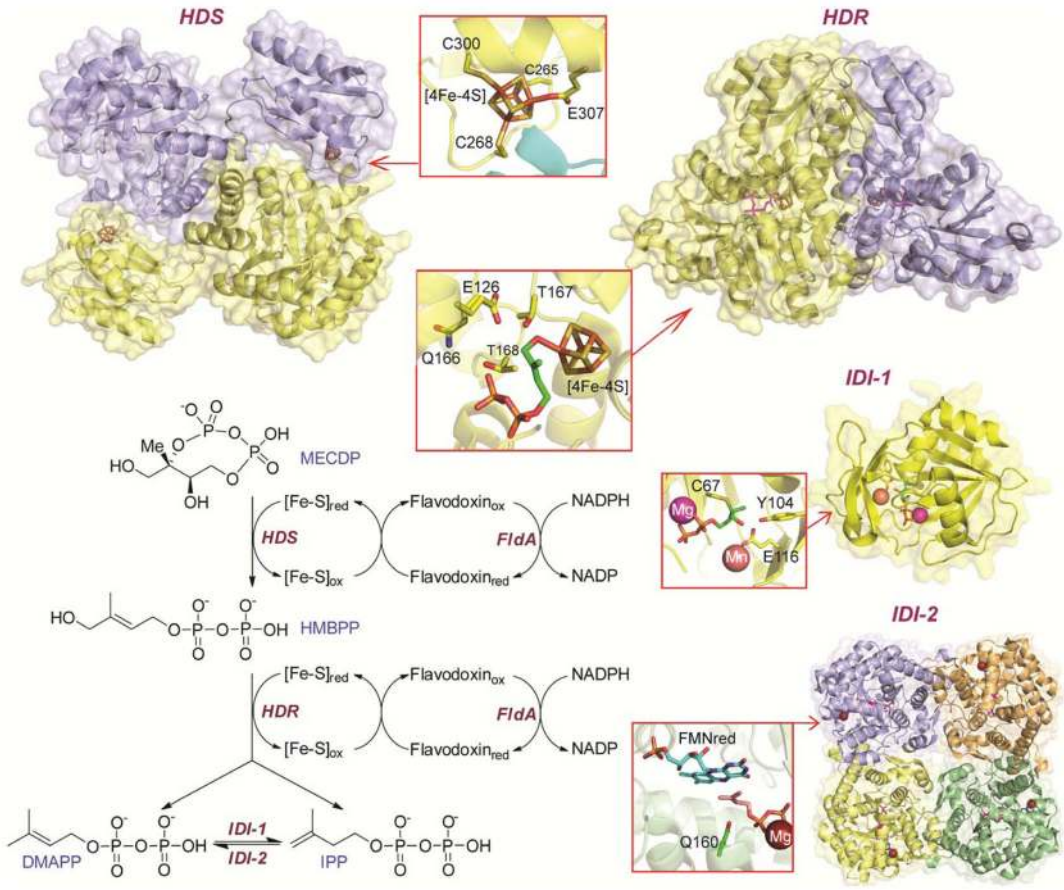

Figure 6.

The mechanism of reaction, structure and key features of HDS, HDR, IDI-1 and IDI-2. The reductive reactions carried out by HDS and HDR indicate the coupling to electrons obtained via flavodoxin and flavodoxin reductase (FldA) from NADPH. *Top left,* the HDS dimer structure with the subunits colored either *blue* or *yellow* based on its reported structure (PDB 3NOY). [87a] Inset indicates the critical C265, C268, C300, and E307 residues of HDS that coordinate the iron sulfur cluster ([4Fe-4S]) (colored by heteroatoms). *Top right,* the HDR dimer structure with the subunits colored either *blue* or *yellow* based on its reported structure (PDB 3KE8) in complex with HMBPP substrate (*red*). [88c] Inset indicates the iron sulfur cluster ([4Fe-4S]) and the critical Q166, T167, T168 and E126 residues (colored by heteroatoms) of HDR. *Middle right*, the structure of *E. coli* IDI-1 monomer (PDB 1NFZ) following treatment with the irreversible inhibitor 3,4-epoxy-3-methyl-1-butyl diphosphate. [109a] Inset indicates the orientation of 4-hydroxy-3-methyl butyl diphosphate linked to the sulfur group of C67 and in relation to the Mg^{2+} and Mn^{2+} divalent ions and the critical residues E116, Y104 (colored by heteroatoms) of IDI-1. *Bottom right*, the structure of IDI-2 tetramer with each subunit colored in either *blue*, *orange*, *teal* or *yellow* based on its reported structure (PDB 3B05). [116a] Inset indicates the critical residue Q160 and the reduced FMN (FMNred) cofactor of IDI-2. *Bottom left,* diagram indicates the mechanism of the HDS, HDR and IDI-1 and IDI-2 catalyzed reactions adapted from reference with permission from reference 283. Copyright 2007 American Society for Biochemistry and Molecular Biology.

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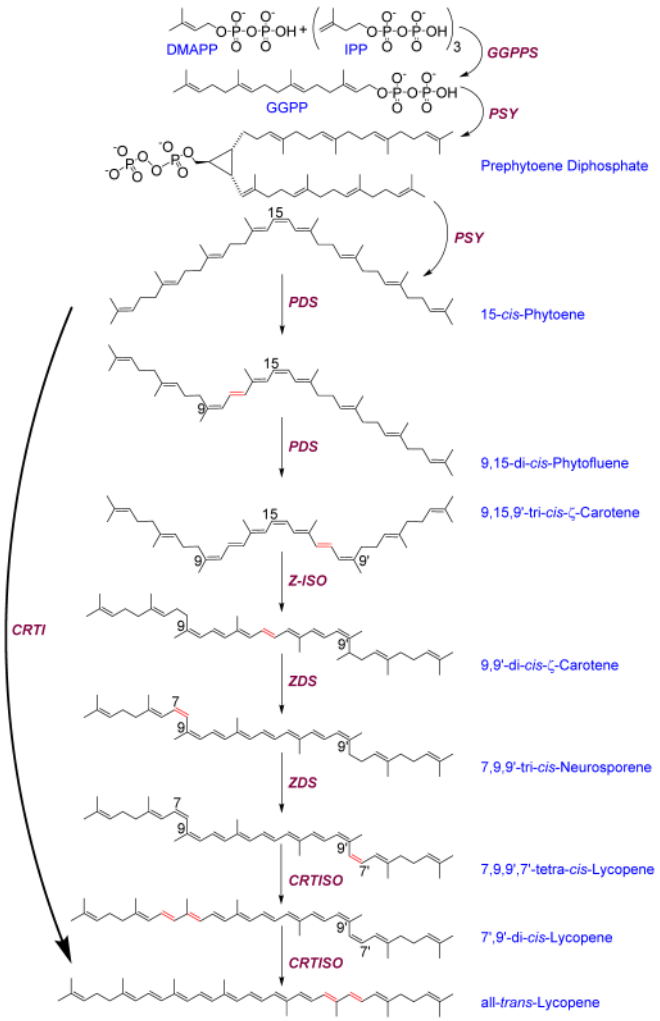

Figure 7.

The poly-*cis* phytoene synthesis pathway from plants, cyanobacteria and the CRTI-based phytoene synthesis pathway from other carotenogenic bacteria. Newly desaturated or isomerized bonds are colored red.

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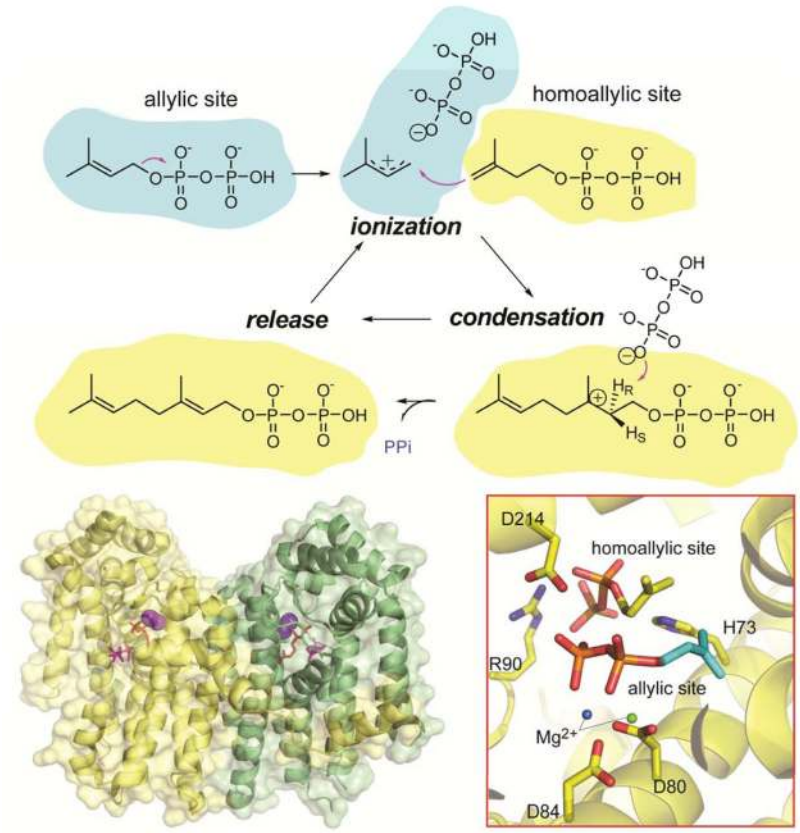

Figure 8.

The mechanism of cyclic condensations, structure and key features of GGPPS. *Top*, mechanism of the 1′-4 head-to-tail condensation condensation reaction catalyzed by GGPPS. The allylic substrate (DMAPP shown) binds the allylic binding site in blue while IPP binds to the homoallylic binding site indicated in yellow. The condensation cycle proceeds through ionization, condensation and release steps. *Bottom*, the structure of GGPPS dimer (*green* and *yellow* subunits) in association with isoprenoid substrates (*red*) based on its published structure (PDB 2E8U). [130b] Inset shows the orientation of IPP bound to either the natural, homoallylic binding site or the non natural allylic binding site of GGPPS. Binding to the allylic site is mediated by the Mg^{2+} cluster which is coordinated by the residues D80, D84 of the DDxxD motif. Active site residues R90, D214 and H73 that are proposed to participate in substrate binding are also shown (residues and substrate IPP are colored by heteroatoms). The mechanism of reaction of GGPPS is adapted and reprinted with permission from Reference 284. Copyright 2008 American Chemical Society.

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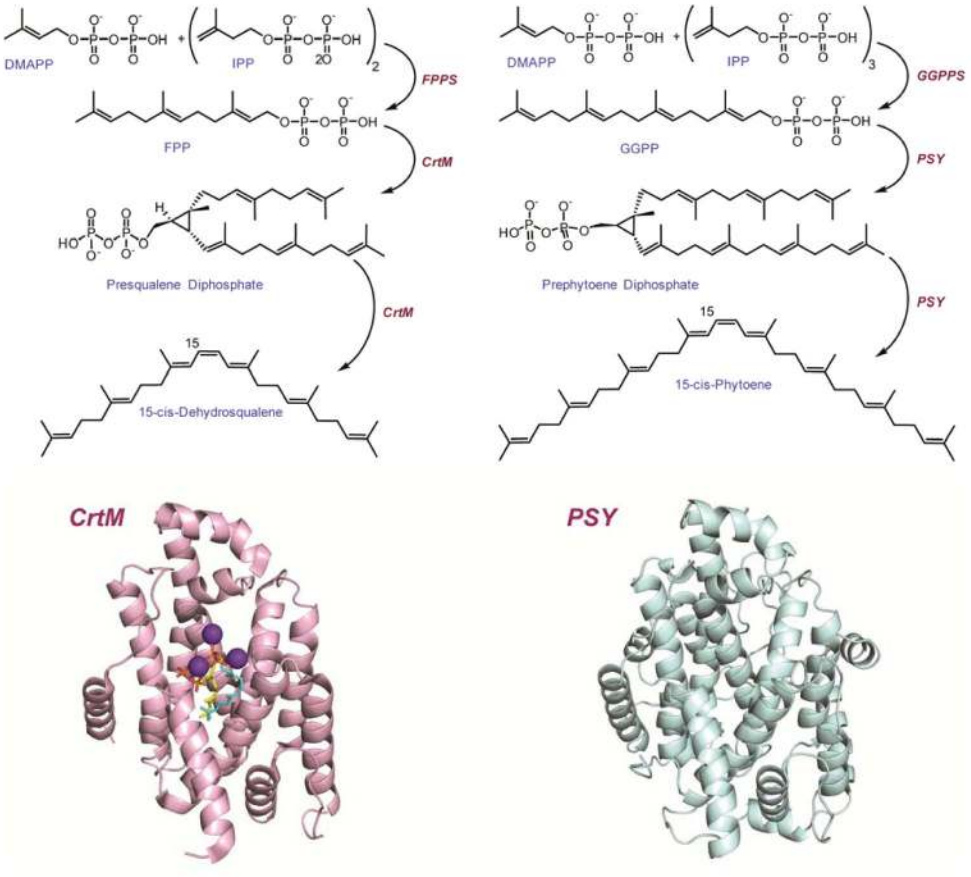

Figure 9.

The enzymatic reaction, structure and key features of CrtM and a model of PSY. *Top*, diagrams illustrate the CrtM and PSY-catalyzed reactions. *Bottom left*, the structure of CrtM bound to farnesyl thiodiphosphate in either the allylic or homoallylic site based on published structure (PDB 2ZCP). [146] The divalent metal ion cluster is also indicated. *Bottom right*, a prediction of the structure of *A. thaliana* PSY based on multiple threading alignments and iterative threading assembly refinement (I-TASSER) [144] is also shown for comparison. The mechanism of reaction of CrtM is adapted and reprinted with permission from Reference 285. Copyright 2009 American Chemical Society.

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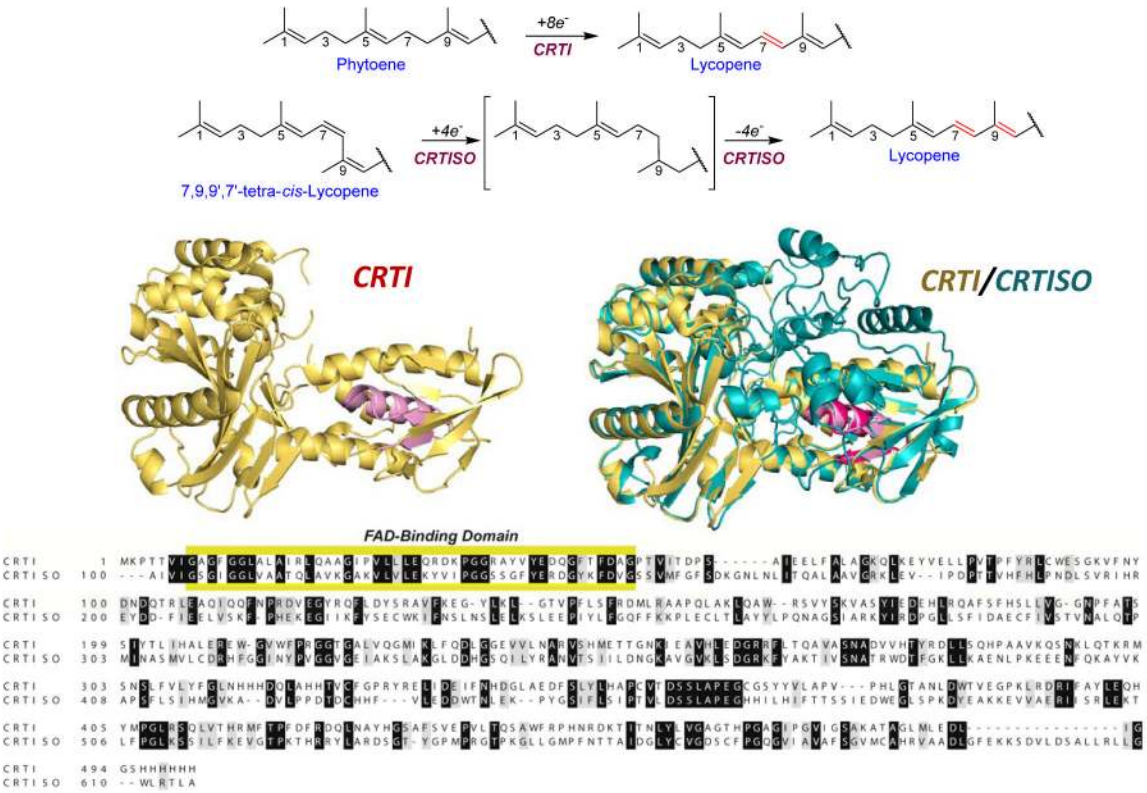

Figure 10.

The enzymatic reaction, structure and key features of CRTI and CRTISO. The desaturation reaction catalyzed by CRTI is shown in comparison with the saturation-isomerization-desaturation reaction catalyzed by CRTISO. *Bottom left*, the structure of CRTI is based on its reported structure (PDB 4DGK) indicating the FAD binding β-α-β fold (*pink*). [152c] *Bottom right*, a prediction of the structure of tomato CRTISO using I-TASSER [144] is colored in teal and is superimposed on the CRTI structure colored in gold. The FAD binding domain of CRTISO (*purple*) and that of CRTI (*pink*) are indicated. *Below*, the alignment of the sequence of hexahistidine tagged-CRTI (as used to generate the PDB 4DGK structure) and tomato CRTISO with identical residues in white letters on black background and conserved substitutions in black on grey background. The extended FAD binding domain shared by CRTI, CRTISO and protoporphyrinogen oxidoreductases and monoamine oxidase is highlighted in yellow. The diagrams depicting reactions of CRTI and CRTISO is adapted and reprinted with permission from reference from reference 197b. Copyright 2005 Elsevier.

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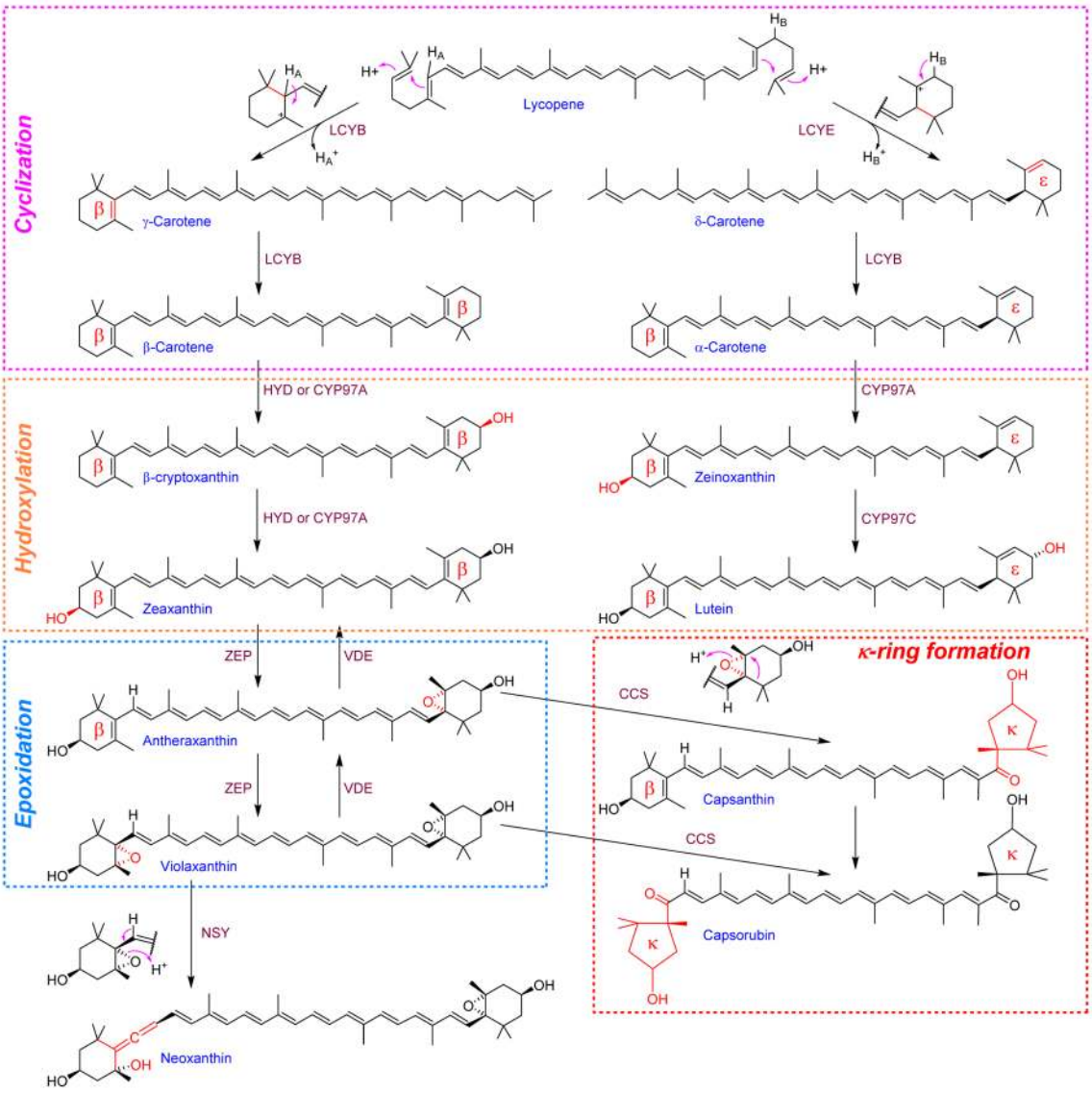

Figure 11.

The mechanism of main types of ionone-ring cyclization and substitution reactions. Cyclization and formation of β- or ε-ionone rings, hydroxylation and epoxidation reactions are boxed in *purple*, *orange* and *blue* respectively. The reactions occurring in pepper plants leading to the conversion of epoxidized ionone rings to κ-rings are shown in *red* box. Newly acquired substitutions or modifications are colored *red*. The scheme of ionone-ring cyclization and substitution reactions is adapted from and reprinted with permission from reference 18a. Reproduced by permission of The Royal Society of Chemistry

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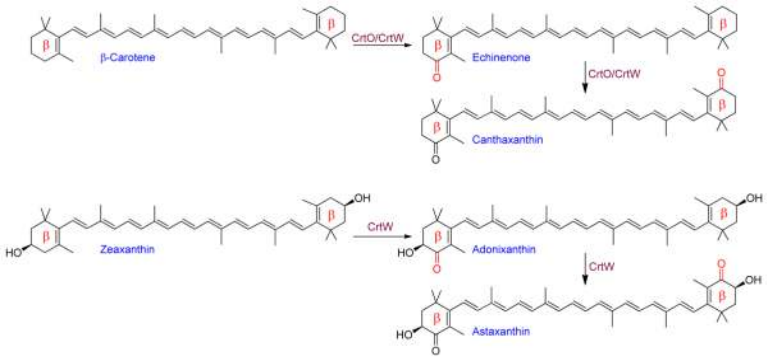

Figure 12.
The main enzymes involved in the introduction of keto-groups in carotenoids in cyanobacteria. Newly acquired keto-groups are colored *red*.