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Catalytic properties and reaction mechanism of the CrtO carotenoid ketolase from the cyanobacterium *Synechocystis* sp. PCC 6803

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ABSTRACT

CrtW and CrtO are two distinct non-homologous β-carotene ketolases catalyzing the formation of echinenone and canthaxanthin. CrtO belongs to the CrtI family which comprises carotene desaturases and carotenoid oxidases. The CrtO protein from *Synechocystis* sp. PCC 6803 has been heterologously expressed, extracted and purified. Substrate specificity has been determined in vitro. The enzyme from *Synechocystis* is basically a mono ketolase. Nevertheless, small amounts of diketo canthaxanthin can be formed. The poor diketolation reaction could be explained by the low relative turnover numbers for the mono keto echinenone. Also other carotenoids with an unsubstituted β-ionone ring were utilized with low conversion rates by CrtO regardless of the substitutions at the other end of the molecule. The CrtO ketolase was independent of oxygen and utilized an oxidized quinone as co-factor. In common to CrtI-type desaturases, the first catalytic step involved hydride transfer to the quinone. The stabilization reaction of the resulting carbo cation was a reaction with OH^- forming a hydroxy group. Finally, the keto group resulted from two subsequent hydroxylations at the same C-atom and water elimination. This reaction mechanism was confirmed by in vitro conversion of the postulated hydroxy intermediates and by their enrichment and identification as trace intermediates during ketolation.

Introduction

Ketocarotenoids derived from β-carotene are major carotenoids in cyanobacteria [1,2]. Canthaxanthin (4,4′-diketo-β-carotene) found predominantly in filamentous groups is the most efficient photo-protective carotenoid preventing pigment degradation and inhibition of photosynthesis under high-light conditions and UV radiation [3]. Echinenone (4-keto-β-carotene) exerts a somehow lower but still substantial protective effect [4]. However, as a 3′-HO derivative it is a functional component of the water soluble orange carotenoid protein which participates in energy dissipation from light-harvesting phycobilisomes [5]. Ketolation of β-carotene at position 4 of the β-ionone ring is catalyzed by several types of ketolases. The most abundant is the CrtW/bkt ketolase present in cyanobacteria, in other prokaryotes and in green algae. It is a non-heme iron protein with sequence homologies to cyanobacterial β-carotene hydroxylase CrtR [6] acting as an oxygen-dependent dioxygenase [7]. Most CrtW ketolases insert the keto group preferentially into unsubstituted β-ionone rings. However, some are able to ketolate 3-HO-β-carotene to astaxanthin [8]. Another β-carotene ketolase dominating in cyanobacteria is CrtO. It was first cloned from *Synechocystis* sp. PCC 6803 where it acts as a monoketolase in the synthesis of echinenone [9]. However, in other cyanobacteria it is a typical diketolase catalyzing the formation of canthaxanthin [10]. CrtW and CrtO show no significant sequence homologies. But the latter is structurally related to the CrtI type phytoene desaturase [9] both sharing six conserved regions of the amino acid sequences [11]. Like CrtI, CrtO possesses a nucleotide binding fold close to the N-terminus. The catalytic mechanism of CrtI is well-understood. Allylic double bonds are formed stepwise by removal of two hydrogens as a *trans* elimination. This process involves first a hydride transfer from an allylic carbon. Then, the generated carbo cation is stabilized by proton abstraction from the adjacent carbon atom leading to a new double bond in the polyene system [12]. In analogy to this mechanism, CrtO may also start its catalysis by hydrid abstraction of allylic C4 of the β-ionone ring. A different stabilization reaction of the resulting carbo cation should then lead to the formation of the 4-keto group.

In the present investigation, the catalytic mechanisms of the CrtO ketolase from *Synechocystis* sp. PCC 6803 was elucidated. This involved heterologous expression in *Escherichia coli* of the ketolase and kinetic studies with the purified enzyme focussing on cofactor requirement, substrate specificity and the enrichment and identification of traces of reaction intermediates in *Synechocystis*.

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Experimental procedures

Growth of organisms

Cultivation of *E. coli* was in darkness in LB medium either routinely at 37 °C for 3 days or for only 1 day for better accumulation and isolation of 4-hydroxy-β-carotene or for protein expression. The strain DH5α was used for cloning and synthesis of carotenoids and JM101 for protein expression.

Plasmids

The expression vector pPEU30 was performed constructed by PCR amplification of the whole promoter region of the carotenogenic gene cluster from *Pantoea ananatis* formerly *Erwinia uredovura* out of plasmid pACCRT-EBI [13] with primers forward TACCGCTCGAGCTGCCAA and revers GAATTCTCGGGCTGTCCTTAT generating a *Xho*I and *Eco*RI restriction site, subcloning into the T-overhang vector pMON38201 [14], cut out with *Xho*I/*Eco*RI and ligated into the *Xho*I/*Eco*RI sites of plasmid pPQE30 (Quiagen, Hilden, Germany). Then, the *crtO* coding region was PCR amplified from plasmids pslr0088 [9] with primers forward CCATGGGATCCATCACCACCG and revers TTCTGCAGTTACCAAAAACGACG generating a *Bam*HI and *Pst*I restriction site, and cloned into the *Bam*HI/*Pst*I sites of pPEU30 generating the expression vector pPEU30crtO. Other plasmids used for co-expression in *E. coli* were pACCAR16ΔcrtX to generate a β-carotene background [13], pQE970 containing a combined lycopene ε- and β-cylase [15], together with pACCRT-EBI [13] for the synthesis of α-carotene, pRKcrtR [16] for the synthesis of β-cryptoxanthin from β-carotene, and pCRBKT for the synthesis of canthaxanthin from β-carotene [17].

Enzyme purification after heterologous expression in E. coli

Escherichia coli cells were re-suspended 1:30 of the culture volume in 100 mM phosphate buffer pH 7.8 containing 0.1% Nonidet P40, broken in a French pressure cell at 95 MPa and the homogenate centrifuged at 40,000 g for 20 min at 4 °C. After incubation of 15 ml of the supernatant with 1.2 ml of the talon resin (BD Clontech, Heidelberg, Germany) for 45 min on ice, the mixture was packed in a column and non-bounded proteins were washed four times with 5 ml buffer containing 100 mM potassium phosphate buffer pH 7.8, 300 mM sodium chloride, 10% glycerol and 10 mM imidazole. For elution of proteins (in 1.5 ml fractions), increasing imidazole concentrations (50, 100, 150 and 200 mM) were used. The 200 mM fraction used for in vitro assays was desalted on Sephadex G25 (PD 10 column, Pharmacia). For the documentation of the different purification steps 270 μl of each fraction was precipitated after addition of 6% of trichloric acid and applied for separation on a 12.5% SDS–PAG [18]. The relative amounts of the expressed proteins were estimated after separation on the SDS-gel by staining with Coomassie Brilliant Blue and scanning using a densitometric software. Protein concentrations were determined with the BioRad protein assay.

In vitro enzyme assay

The ketolase assays contained 230 μl of the desalted 200 mM imidazole fraction (equivalent to 250 μg protein), 4 μg of β-carotene or other carotenoids as indicated, 0.1% Nonidet P40, 1 mM cofactor (in 20 μl buffer or 10 μl methanol for ubiquinone and plastoquinone) in a final volume of 1.5 ml 0.1 M potassium phosphate buffer pH 7.8. After incubation for 16 h at 28 °C, the reaction was terminated by addition of 2.5 ml of methanol. The remaining substrate and the products formed were extracted from the incubation mixture with diethyl ether/petroleum ether (b.p. 35–60 °C) (1:1, v/v) and analyzed in HPLC system II as described below.

For determination of the K_m value for β-carotene and echinenone, substrate concentrations varied in the range from 1 to 20 μM. K_m and V_{max} values were obtained as mean from three double reciprocal Lineweaver–Burk plots of the reaction rates versus the substrate concentration with five data points and a correlation coefficient for linearity of >0.97. I_{50} values for inhibition of the ketolase reaction by diphenylamine (DPA)[2] were determined with concentrations from 50 to 600 μM.

Carotenoid analysis

Carotenoids were extracted from freeze-dried cells by heating in acetone (60 °C, 20 min) and partitioning into 10% ether in petrol. The carotenoids from the upper phase were either used directly for analysis or fractionated by TLC to concentrate the ketolation intermediates 4-hydroxy-β-carotene and 4-hydroxy-echinenone. TLC was carried out on activated silica plates with the solvent toluene/ethylacetate/methanol (90:9:1, v/v/v). The TLC band containing the 4-hydroxy derivatives with an R_f-value of 0.45 above a 0.35 band containing *cis* cantaxanthin was scraped off and the carotenoids eluted with acetone. The fraction from a 1-day old culture was used to identify 4-hydroxy-β-carotene and a 3-day old culture for 4-hydroxy-echinenone. This was achieved on a HPLC system I on a 15 cm Nucleosil C18, 3μ column with a mobile phase of acetonitrile/2-propanol/methanol/water (82:5:10:3, v/v/v/v), flow 1 ml/min, at 10 °C column temperature. The other carotenoid separations were carried out with HPLC system II on a 25 cm Vydac 201TP C18, 10μ column with 2% water in methanol, flow 1 ml/min, at 25 °C. Spectra were recorded on-line with a Kontron DAD 440 diode array detector (Kontron Instruments, Neufahrn, Germany). Peaks were identified by co-chromatography with reference compounds together with their spectra. Authentic carotenoid standards for HPLC were generated by combinatorial biosynthesis in *E. coli* [19] or in case of 4-hydroxy-β-carotene, 4-hydroxy-4′-keto-β-carotene and 4,4′-dihydroxy-β-carotene by reduction of echinenone or canthaxanthin with $NaBH_4$ [20].

Software

Data base searches were carried out with the similarity search tool BLAST P 2.2.10 [21]. Phylogenetic analysis of amino acid sequences were performed with the program Clustal X [22] and the alignments visualized with TreeView.

Results

The phylogenetic tree of Fig. 1 with CrtI-related enzymes from bacteria consists of four branches starting from a common origin. CrtO type ketolases from different prokaryotes group well together. CrtO from *Synechocystis* is closest to the other cyanobacterial monoketolase from *Gloeobacter violaceus*. Protein identity among the cyanobacterial enzymes in this cluster is around 60%. The cyanobacterial ketolases join with CrtOs from other bacteria to a common branch. Another cluster represents bacterial phytoene desaturases (CrtI). It is close to the 4,4′-diapophytoene desaturases (CrtN). In a functional sense, these enzymes differ by their specificity for a C40 or C30 substrate. Connected to them are the CrtNb ketolases which show sequence homology to these desaturases [11]. In contrast to the ketolation activity of CrtO, CrtNb enzymes form an aldehyde group at the terminal C-atom of acyclic

[2] *Abbreviations used:* DPA, diphenylamine.

Fig. 1. Phylogenetic tree of β-carotene 4-ketolase CrtO and other enzymes related to bacterial phytoene desaturases CrtI including CrtN (4,4′-diapophytoene desaturase), CrtNb (4,4′-diapolycopene oxidase) and CrtNc (4,4′-diapolycopene-4,4′-dial oxidase). The following genes with their accession number were used for the construction of the tree: *crtI Pantoea ananasi* YP_003522456, *crtI Paracoccus marcusii* CAB56062.1, *crtI Rhodopseudomonas palustris* NP_946860.1, *crtI Enterobacter helveticus* CAZ90572.1, *crtO Gloeobacter violaceus* NP_923340.1, *crtO Synechocystis* PCC 6803 NP-442491.1, *crtO Rhodococcus erythropolis* YP_002767014.1, *crtO Deinococcus radiodurans* YP_293819.1, *crtO Nostoc* PCC 7120 NP_487784.1, *crtO Nostoc* PCC 73120 YP_001867077.1, *crtNa Methylomonas* sp. 16A AAX46183.1, *crtNa Halobacillus halophilus* YP_006180382, *crtNb Halobacillus halophilus* ACM07427.1, *crtNb Methylomonas* sp. 16A AAX46185.1, and *crtN Staphylococcus aureus* CAA52098.1.

C30 carotenes and not at position 4 of the β-ionone ring of a C40 molecule.

Prior to enzymatic characterisation of CrtO from *Synechocystis*, the enzyme was expressed in *E. coli*. A prominent polypeptide with an apparent molecular mass of 61 kDa was detected in cells of the *crt*O transformant (Fig. 2 lane C+) in comparison to *E. coli* carrying the empty cloning vector pPEU30 (Fig. 2 lane C−). When plasmid pQE30crtO with a *lac* Z promoter was expressed (data not shown), very little of the enzyme was recovered in the soluble fraction S since most of the protein was sequestered in inclusion bodies. Therefore, the *lac* Z promoter was replaced by the weak promoter of the *P. ananatis* carotenogenic gene cluster [23] in pPEU30crtO. With this expression plasmid, CrtO amounts of around 10% of the total protein were obtained of which more than half was recovered from the soluble fraction after cell disruption and centrifugation (Fig. 2 lane S). Purification of CrtO was carried out by cobalt chelate affinity chromatography. After binding to the Talon resin and washing, CrtO was eluted with increasing imidazole concentrations (Fig. 2 lanes 2–5). CrtO was enriched to 93% purity in the 200 mM imidazole elution fraction.

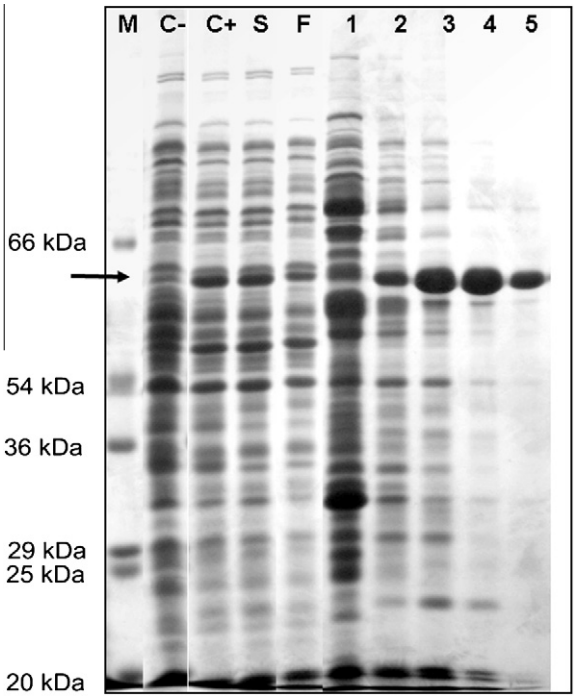

Fig. 2. Heterologous expression in *E. coli* and purification of CrtO ketolase from *Synechocystis* sp. PCC 6803. M marker, C− control cells without CrtO, C+ cell expressing CrtO, S soluble fraction, F flow through the Ni–NTA column, (1) washing (including 10 mM imidaziole), elution with imidazole: (2) 50 mM, (3) 100 mM, (4) 150 mM and (5) 200 mM.

Activity of the purified ketolase was assayed in vitro with β-carotene as substrate. Carotenoids extracts obtained after incubation were separated by HPLC (Fig. 3A). In addition to residual β-carotene (peak 3 all-*trans*, peak 3′ *cis* isomer), echinenone was predominantly formed (peak 2 all-*trans*, peak 2′ *cis* isomer) together with very small amounts of canthaxanthin (peak 1). A specific activity of the purified ketolase for formation of ketolated β-carotene derivatives was calculated as 0.134 nmol mg^{-1} h^{-1} (Table 1A). The purified CrtO enzyme is also capable of ketolating other carotenoids with an unsubstituted β-ionone ring like α-carotene and β-cryptoxanthin with conversion rates of about 5% (Fig. 4B).

Several oxidized or reduced cofactors were analyzed in the ketolase reaction including nicotine nucleotides and water soluble and lipophilic quinone derivatives. Although a high back-ground activity in control, a significant increase of the reaction was obtained with oxidized benzoquinone as cofactor. All other oxidized or reduced compounds in Table 1A had no stimulating effect. Also anaerobic conditions had no influence on the activity of CrtO. K_m

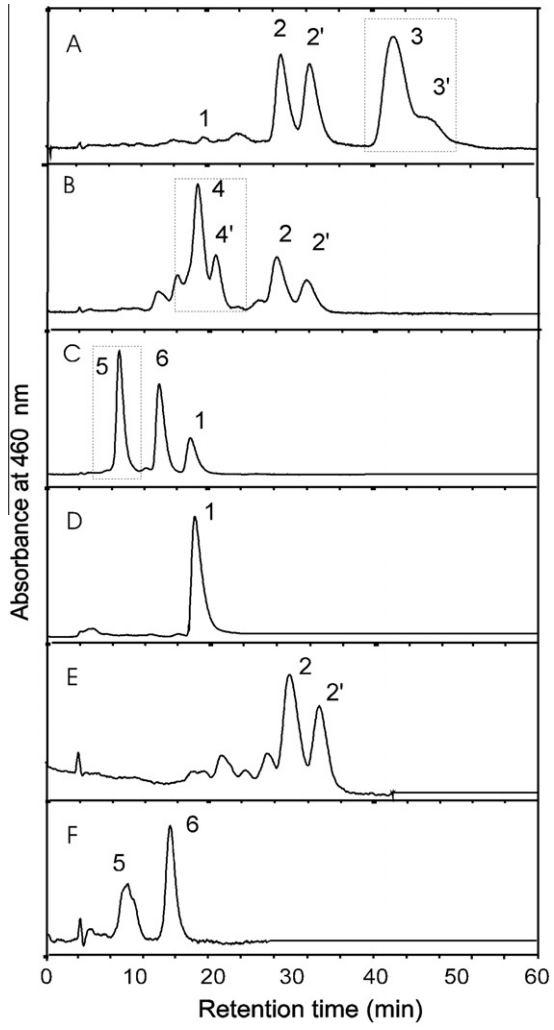

Fig. 3. HPLC separation and identification of products resulting from in vitro conversion of substrates in the pathway to canthaxanthin by CrtO from *Synechocystis* sp. PCC 6803 in HPLC system II. Substrates: (A) β-carotene, (B) 4-HO-β-carotene, (C) 4,4′-diHO-β-carotene as indicated by boxes. (D–F) Standards for identification. Peak assignment: (1) canthaxanthin, (2) echinenone, (3) β-carotene, (4) HO-β-carotene, (5) 4,4′-diOH-β-carotene and (6) 4-HO-4-keto-β-carotene. Peaks with primed numbers resemble *cis* isomers.

and V_{max} values were determined for the substrates echinenone and canthaxanthin in the presence of benzoquinone as cofactor and specificity values V_{max}/K_m [9] calculated (Table 1B). These values represent relative turnover numbers and are useful for the direct comparison of catalytic activity for a given substrates. K_m values were rather similar for both substrates. The main difference was the high V_{max} value for β-carotene compared to echinenone which determined the higher specificity value for β-carotene. Diphenylamine (DPA) is an inhibitor of CrtI type phytoene desaturases [24]. Due to the homology of CrtO to CrtI, the I_{50} inhibitory constant of DPA inhibition of CrtO was determined and a high value of about 405 μM obtained (Table 1B).

Based on the enzymatic properties in Table 1 and the relationship of CrtO–CrtI, a ketolation mechanism without oxygen is proposed and outlined in Fig. 4C. It involves hydride transfer in analogy to CrtI to plastoquinone yielding an allylic carbocation at C4 of the β-ionone ring. This cation is then stabilized by reaction with OH^-. The resulting 4-HO derivative should be an intermediate which finally is hydroxylated at the same carbon in the same way in a second reaction. Finally, the dihydroxy intermediate eliminates water yielding the 4-keto end product of the entire reaction sequence. This reaction

Table 1
Co-factor requirements (A) and kinetic properties (B) of purified CrtO from *Synechocystis* sp. PCC 6803.

Additions		Specific activity* (nmol mg^{-1} h^{-1})	
A			
None		0.134 ± 0.014	
Benzoquinone		0.239 ± 0.023	
Plastoquinone		0.088 ± 0.005	
Ubiquinone		0.127 ± 0.014	
FAD		0.114 ± 0.010	
NADP		0.123 ± 0.004	
NADH		0.140 ± 0.010	
NADPH		0.126 ± 0.005	
Substrate	K_m (μM)	V_{max} (μmol h^{-1} mg^{-1})	V_{max}/K_m
B			
β-Carotene	41.6	1.318	0.032
Echinenone	35.3	0.339	0.010
Inhibition constant for diphenylamine		I_{50} = 405 μM	

* Values represent means of three determination from plots of five data points with a correlation coefficient for linearity of >0.97.

sequence was tested in two ways, by enrichment and identification of 3-HO intermediates by pathway reconstruction in *E. coli* and the utilization of 4-HO-β-carotene and 4,4′-diHO-β-carotene as substrates in in vitro reactions as shown in Fig. 4A.

Separation of a carotenoid extract from a complementation experiment in *E. coli* carrying a combination of plasmid pACAR16ΔcrtX for β-carotene generation and pPEU30crtO is shown in Fig. 5A. β-Carotene is completely ketolated and converted to echinenone (peak 2) and canthaxanthin (peak 1). Other carotenoids and intermediates of the reaction may be present but only at trace amounts. By chromatographic carotenoid concentration and separation, fractions enriched in hydroxy derivatives were obtained. In this fraction from a 1-day old culture, a prominent peak 4 was obtained together with echinenone and canthaxanthin (Fig. 5B). This carotenoid has the same retention time as 4-hydroxy-β-carotene obtained by chemical reduction of echinone (Fig. 5D). Both had the same chromophore and spectrum as β-carotene (absorbance maxima at 429, 452 and 478 nm). The hydroxy fraction from a 3-day old culture was used to identify 4-hydroxyechinenone. The chromatogram shows residual amounts of canthaxanthin (peak 1) and a small peak 6 in front of canthaxanthin at a 0.5 min earlier retention time (Fig. 5C). 4-HO-4′-keto-β-carotene obtained by canthaxanthin reduction runs as a standard at the same retention time (Fig. 5E) and exhibited the same echinenone-like spectrum (absorbance maximum at 465 nm) as compound six. In none of the fractions, 4,4′-diHO-β-carotene peak 5 was detectable which due to its higher polarity runs 2 min ahead of echinenone (Fig. 5F).

The mono- and 4-dihydroxy β-carotene derivatives were applied as substrates in the in vitro ketolation reaction. 4-Hydroxy-β-carotene which was available as an all-*trans* (peak 4) and *cis* isomer (peak 4′) was converted to the corresponding *trans* and *cis* echinenone (peaks 2 and 2′, Fig. 3B). Also 4,4′-diHO-β-carotene was utilized by CrtO yielding 4-HO-4′-keto-β-carotene (peak 6) as an intermediate and canthaxanthin (peak 1) as the final reaction product (Fig. 3C). All products were identified by same retention times and spectra as their standards canthaxanthin (peak 1, Fig. 3D), echinenone *cis* and *trans* isomers (peak 2 and 2′, Fig. 3E) and 4-HO-4′-keto-β-carotene (peak 6, Fig. 3F).

Discussion

The CrtI enzyme family comprises different desaturases and ketolases. The phylogenetic tree (Fig. 1) shows that all of them, CrtI, CrtN and CrtNb are related to CrtO. They share everal homologous

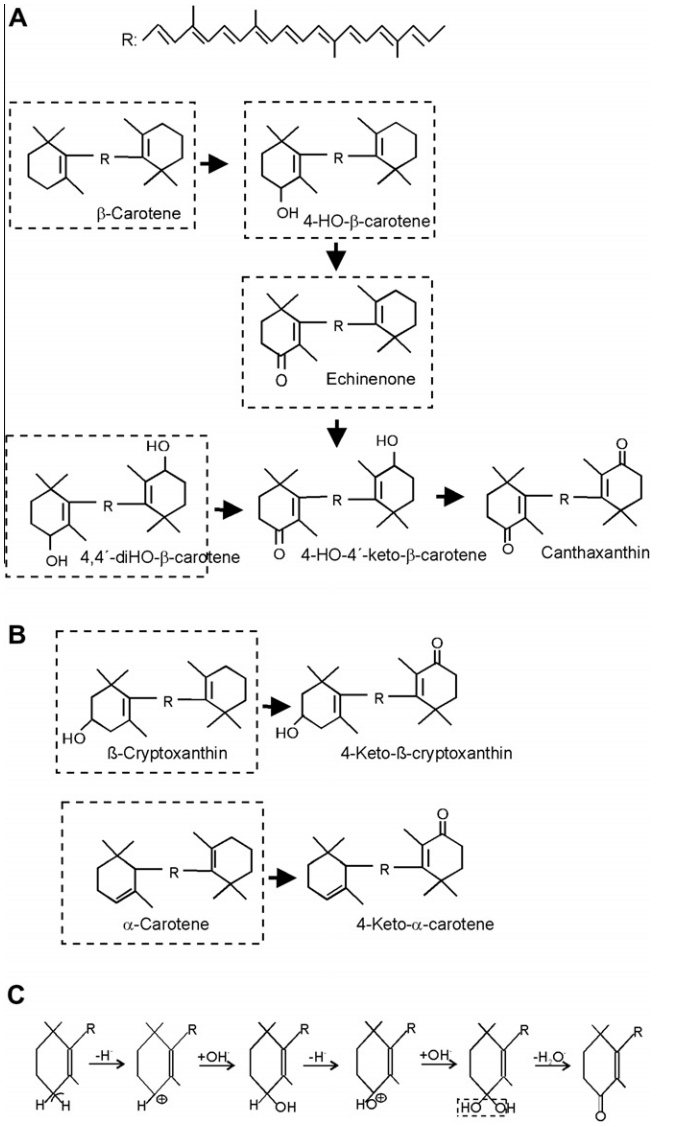

Fig. 4. Carotenoid structures and in vitro reactions by CrtO from *Synechocystis* sp. PCC 6803, (A) from β-carotene to canthaxanthin, (B) of other substrates. All substrates are boxed. (C) Proposed mechanism for the formation of the 4-keto group at the β-ionone ring.

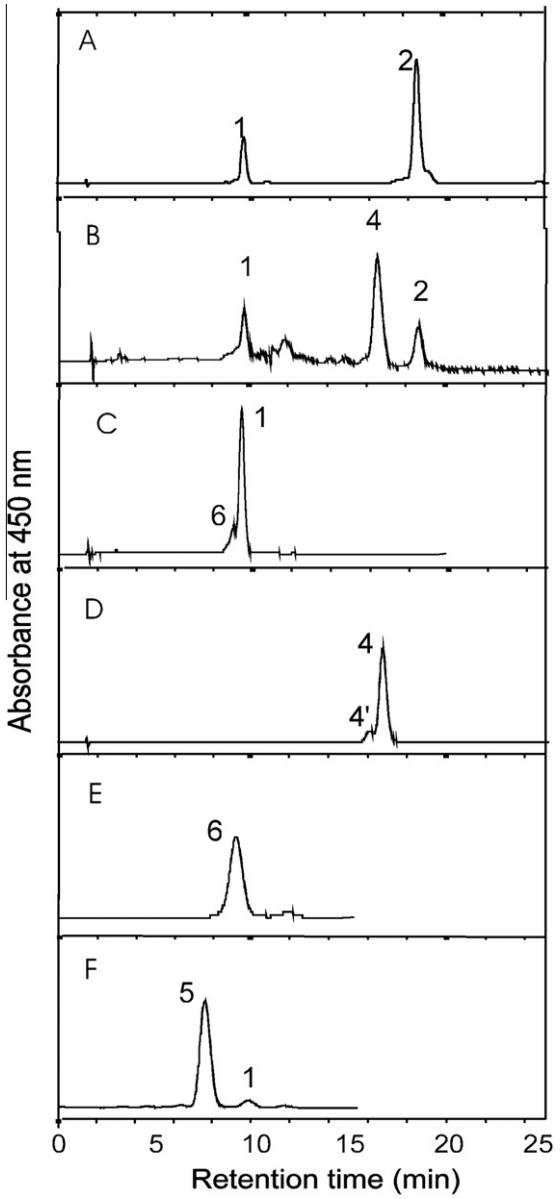

Fig. 5. HPLC separation and identification of carotenoids from *E. coli* co-transformed with plasmids pACCar16ΔX for formation of β-carotene and pPEU30crtO for substrate conversion in HPLC system I. (A) Main carotenoids from extracted cells of a 3-day culture, (B) enrichment in a TLC fraction from a 1-day old culture and (C) enrichment by TLC from a 3-day old culture. Standards: (D) 4-HO-β-carotene, (E) 4-HO-4′-keto-β-carotene and (F) 4,4′-diHO-β-carotene. Numbering of peaks is the same as in Fig. 3.

domains in their amino acid sequences [9,11]. The CrtI desaturase is the best-studied enzyme with a well-established reaction mechanism [12]. Double bonds are introduced to extend the central conjugated polyene chain. This involves an oxidized cofactor like FAD for a bacterial enzyme [25] or NAD for a fungal enzyme [26]. The starting reaction is hydride transfer to the co-factor generating an allylic carbo cation. In the case of CrtO, benzoquinone is another oxidized cofactor for the ketolation reaction (Table 1A). An initial reaction of generating a carbo cation can also start a ketolation reaction (Fig. 4C). A common feature of the desaturases and the CrtO ketolase is their sensitivity against diphenylamine. This inhibition is another indication for an intermediate carbo cation in both types of reactions. The I_{50} value for CrtO inhibition is comparably high (Table 1B) but also among the CrtI enzymes, there is a variability of I_{50} values from 64 to 230 μM [24,27]. The second reaction step includes stabilization of the carbo cation. For CrtI and CrtN, this proceeds by proton abstraction from the adjacent C-atom yielding a new double bond. In the CrtO catalyzed reaction, the positively charged C-4 reacts with OH^- to a C4 hydroxy derivative (Fig. 4C). After a second hydroxylation at the same C4 carbon, water is eliminated finally yielding a C4 keto group. The entire reaction mechanism proposed in Fig. 4C involves different 4-HO intermediates as outlined in Fig. 4A. Their existence could be demonstrated by enrichment and HPLC analysis (Fig. 5). Furthermore, they were all converted in vitro by the purified ketolase to the corresponding keto products (Fig. 3). Both results strongly support the mechanisms of double-serial hydrid transfer, reaction with OH^- and water elimination. This is fundamentally different to the CrtW ketolase reaction which ketolates the same substrate to the same product in an oxygen-dependent dioxygenase reaction [7]. Unlike the C4 mono hydroxy compounds, 4,4′-diOH-β-carotene was not detectable (Fig. 5) although it can be utilized as substrate by CrtO. This suggests that the preferential sequence of canthaxanthin formation is complete processing of one β-ionone ring at one side before the other one is ketolated.

In many cyanobacteria, CrtO acts as a diketolase in the synthesis of canthaxanthin [10]. However, in *Synechcocystis* echinenone is the only ketocarotenoid [9]. The in vitro reaction with the purified CrtO demonstrates that this enzyme is a monoketolase forming echinenone as the major product (Fig. 3A). This is reflected by the relative turnover numbers for the substrates β-carotene and echinenone indicated by the specificity values K_m/V_{max} (Table 1). The direct comparison of these values which are 3-fold higher for β-carotene indicated that this carotene is the preferred substrate for CrtO from *Synechocystis*. Nevertheless, substantial amounts of diketo canthaxanthin are formed by this CrtO upon expression in *E. coli* (Fig. 5A). This is due to the expression of high CrtO amounts in *E. coli*. It has been shown for CrtI in vitro that with increasing enzyme concentration, it can be forced to synthesise an additional desaturation step which does not occur in vivo [28]. CrtO from *Synechocystis* can also utilize carotenoids other than β-carotene and echinenone with an unsubstituted β-ionone ring regardless of the substitutions at the other end of the molecule with low conversion rates (Table 1A).

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