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ORIGINAL PAPER

Contribution of two ζ-carotene desaturases to the poly-*cis* desaturation pathway in the cyanobacterium *Nostoc* PCC 7120

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Abstract The presence of two completely unrelated ζ-carotene desaturases CrtQa and CrtQb in some *Nostoc* strains is unique. CrtQb is the ζ-carotene desaturase, which was acquired by almost all cyanobacteria. The additional CrtQa can be regarded as an evolutionary relict of the CrtI desaturase present in non-photosynthetic bacteria. By reconstruction of the carotene desaturation pathway, we showed that both enzymes from *Nostoc* PCC 7120 were active. However, they differed in their preferred utilization of ζ-carotene Z isomers. CrtQa converted ζ-carotene isomers that were poorly metabolized by CrtQb. In this respect, CrtQa complemented the reactions of CrtQb, which is an advantage avoiding dead ends in the poly-*cis* desaturation pathway. In addition to ζ-carotene desaturation, CrtQa still possesses the Z to E isomerase function of the ancestral desaturase CrtI. Biochemical characterization showed that CrtQb is an enzyme with one molecule of tightly bound FAD and acts as a dehydrogenase transferring hydrogen to oxidized plastoquinone.

Keywords ζ-Carotene desaturase characterization · Carotene Z isomers · CrtQa relict desaturase specificity for Z substrates · Pathway evolution

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Introduction

Carotenoids are antioxidative pigments protecting against photosensitized reactions and oxy radicals. These properties are due to their conjugated central polyene system. Typically, it is extended from three to up to 11 conjugated double bonds. In archeae and non-photosynthetic bacteria, the final four desaturation steps are catalyzed by a single enzyme CrtI (Misawa et al. 1990) and the double bonds are inserted symmetrically at different positions of the molecule. In addition, the 15-Z double bond of the substrate phytoene is isomerized to all-E during catalysis (Sandmann 2009). In cyanobacteria, a non-homologous phytoene desaturase CrtP has evolved. It catalyzes a two-step desaturation at both sides at corresponding positions of the symmetrical molecule. The next two desaturations are catalyzed by CrtQb that is highly homologous to CrtP and may have evolved by gene duplication (Sandmann 2002). The gene of this ζ-carotene desaturase was first identified in plants (Albrecht et al. 1995) and later in *Synechocystis* PCC 6803 (Breitenbach et al. 1998). It seems to be ubiquitous in all other cyanobacteria (Takaichi and Mochimaru 2007). In contrast to CrtI, both cyanobacterial desaturases not only lack the isomerization function, they also generate Z double bonds during the desaturation reactions. There is one exception among the cyanobacteria, *Gloeobacter violaceus*. This species possesses the ancient CrtI with the same desaturation pathway as in non-photosynthetic bacteria (Tsuchiya et al. 2005; Steiger et al. 2005). It resembles an evolutionary link between carotenogeneses in non-photosynthetic bacteria and cyanobacteria.

Although the first ζ-carotene desaturase gene was cloned from the cyanobacterium *Nostoc* (also called *Anabaena*) PCC 7120 (Linden et al. 1994), it seems to be unique to this cyanobacteria. Functional expression and pathway

complementation confirmed that the product of this *crtQa* gene desaturates ζ-carotene to lycopene. A comparison to CrtQb from *Synechocystis* revealed first differences in substrate and product specificity with respect to the isomerization status of the carotenes (Breitenbach et al. 1998). Only CrtQa has been heterologously expressed and biochemically characterized (Albrecht et al. 1996). Inhibition by diphenylamine, an inhibitor of bacterial phytoene desaturase, supports the relationship of CrtQa with CrtI.

In the present investigation, the existence of a parallel poly-*cis* desaturation pathway from ζ-carotene to lycopene and its contribution to lycopene synthesis were addressed in *Nostoc* PCC 7120. First, we screened for a *crtQb* gene in addition to the known *crtQa* gene. After heterologous expression, CrtQb was functionally analyzed, purified and biochemically characterized. In vitro studies of CrtQb in comparison with CrtQa were carried out to elucidate for both desaturases the specificity of ζ-carotene isomers as substrates and their conversion to specific lycopene isomers.

Experimental procedure

Growth of organisms

All cyanobacteria in Table 1 were from the Pasteur Culture Collection, Paris, France. They were grown in BG11 medium (Rippka et al. 1979) at 28 °C gassed with 1.5 % CO_2-enriched air at a light intensity of 50 μE/cm^2 s. The inhibitor J852 (4-(3-methyl-propoxy)-2-isopropylamino-6-methyl pyrimidine) that interacts with the CrtQb ζ-carotene desaturase (Ridley 1982; Breitenbach et al. 2002) was added in a concentration of 50 μM.

Table 1 Accumulation of ζ-carotene upon inhibition with pyrimidine derivative J852 (50 μM) in various cyanobacteria

Strain	Carotenoids (mg/g dw)	
	ζ-Carotene (%)	Other carotenoids
Synechocystis PCC 6803	1.39 (43)*	1.77
Nostoc PCC 7120	0.13 (6)*	2.14
Nostoc PCC 6719	0.21 (9)	2.08
Nostoc PCC 7119	0.14 (7)	1.91
Nostoc PCC 73102	0.75 (33)	1.52
Nostoc PCC 7937	1.10 (38)	1.80
Nostoc PCC 8110	0.79 (54)	0.68
Nostoc PCC 8114	0.88 (45)	1.96
Nostoc PCC 8307	1.02 (49)	1.06
Nostoc PCC 9313	0.57 (29)	1.40
Anabaena PCC 7108	1.65 (51)	1.60
Anabaena PCC 9404	1.36 (36)	2.42

* ζ-carotene is below detection in untreated cultures

Gene complementation experiments were carried out in *Escherichia coli* DH5α grown for 48 h in LB medium at 28 °C with added antibiotics ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) according to their plasmids.

Plasmids

Searches in the genome of *Nostoc* PCC 7120 at Kazusa Cyanobase (listed there as *Anabaena* PCC 7120) were carried out with the similarity search tool BLAST P 2.2.10 (Altschul et al. 1997). Gene *alr1832* homologous to cyanobacterial phytoene desaturase genes (Takaichi et al. 2005) was used to construct plasmid pACCRT-EBP$_{7120}$. After PCR amplification of the entire reading frame (forward primer ATGCGAGTAGCGATCGCTGGCG, and reverse primer TCAGGCAGTTGCAGCATTCGTTG) and cloning into T-overhang vector pMON38201, the insert was cut out with the restriction enzymes *Bam*HI and *Pst*I and subcloned into pUC8-1 (Hanna et al. 1984) resulting in pUC8-1crtQb$_{7120}$. Plasmid pACCRT-EB carrying the genes for a geranylgeranyl pyrophosphate synthase and a phytoene synthase (Misawa et al. 1995) was linearized by *Bam*HI digestion and the ends blunted. Then, the cassette with the *lacZ* promoter *crtQb*$_{7120}$ and the *lacZ* terminator was cut out from pUC8-1crtQb$_{7120}$ with *Pvu*II and blunt end ligated into pACCRT-EB.

Gene alr2382 found in the Kazusa data base homologous to other cyanobacterial ζ-carotene desaturase genes *crtQb* was used to construct the expression vector pQE32crtQb$_{7120}$. The reading frame was PCR amplified with forward primer ATGCGCGTTGCAATCGTAGGAGC and reverse primer CTATTTTTTAATATTATCTAGGATGACTTT, sub-cloned into the T-overhang vector pMON38201 (Borovkov and Rivkin 1997), cut out with *Bam*HI/*Pst*I and ligated into the *Bam*HI/*Pst*I sites of plasmid pPQE32 (Qiagen, Hilden, Germany). Plasmids pZDS1B expressing *crtQa* (Linden et al. 1993) and pACCRT-EBI for the synthesis of all-E lycopene and minor Z isomers (Misawa et al. 1995) were previously described.

Enzyme purification and in vitro assay

Cofactor requirement and FAD content of the enzyme were carried out with purified CrtQb. For this purpose, cells of *E. coli* expressing JM101/pQE32crtQb7120 suspended in 200 mM potassium phosphate buffer at pH 7.8 containing 200 mM NaCl were broken in three rounds in a French Press at 95 MPa. The supernatant after centrifugation (10 min, 40,000 g) was incubated for 2 h with Ni–NTA resin (Sigma). After washing of the column first with 200 mM potassium phosphate buffer at pH 7, 8 and

then with the same buffer containing 10 mM imidazole, the desaturase was eluted with 200 mM potassium phosphate buffer at pH 7,8 containing 200 mM imidazole and the solution desalted on a PD10 column (Pharmacia). FAD content was determined by the absorbance at 450 nm, and protein quantification in the same samples was carried out with the Roti-Quant (Roth, Karlsruhe, Germany) protein reagent.

The ζ-carotene desaturase assays contained either 250 μg of purified CrtQb (for cofactor determination) or supernatant after French press treatment and centrifugation (as above) of *E. coli* expressing either CrtQa or CrtQb equivalent to 7 mg protein. Other components were 2 mg of soy bean phosphatidylcholine, 0.02 % butylated hydroxytoluene, 10 μM decylplastoquinone (all from Sigma) and 4 μg of ζ-carotene in a total volume of 1 ml 200 mM potassium phosphate buffer at pH 7,8. After 3 h of incubation in darkness at 26 °C under vigorous shaking at 180 rpm, the reaction was terminated by addition of 3 ml of acetone, the carotenoids partitioned into diethyl ether/petroleum ether (b.p. 40–60 °C) (1:1, v/v) and analyzed by HPLC.

Carotenoid analysis and preparation

Carotenoid extracts from freeze-dried cyanobacterial or *E. coli* cells were obtained by heating in methanol containing 6 % KOH (60 °C, 20 min) and partitioning into 10 % diethyl ether/petroleum ether (b.p. 40–60 °C). The carotenoids from the upper phase were used directly for the analysis. Carotenoids were separated and quantified by HPLC on a non-end capped polymeric 3 μ C30 column (YMC, Wilmington, NC, USA) with a two step gradient of methanol/methyl-tert-butyl ether/water (56:40:4 for 30 min, then 26:70:4, by vol.) at a flow of 1 ml/min according to Breitenbach and Sandmann (2005). Spectra were recorded online with a Kontron DAD 440 diode array detector (Kontron Instruments, Neufahrn, Germany). Peaks were identified by the same retention times as the reference compounds together with their spectra. Authentic carotenoid standards for HPLC were those previously described (Breitenbach and Sandmann 2005). This includes 7,9,7′,9′-Z lycopene from expression of pACCRT-EBP in combination with pQE30zds in *E. coli* or from the tomato variety Tangella (Clough and Pattenden 1983). The ζ-carotene isomers were preparatively separated by HPLC in the system described above.

Results

BLAST search in the genome data base of *Nostoc* PCC 7120 indicated the presence of an ubiquitous cyanobacterial *crtQb* ζ-carotene desaturase gene in addition to the formerly identified *crtQa* gene (Linden et al. 1994). This indicates that both types of ζ-carotene desaturase genes exist in *Nostoc* PCC 7120 simultaneously. CrtQb is susceptible to the inhibitor J852 (Breitenbach et al. 2002). Therefore, this inhibitor can be used as a tool to screen other cyanobacteria for the existence of a functional CrtQa. *Nostoc* PCC 7120 containing both genes and *Synechocystis* PCC 6803 with CrtQb exclusively were used for comparison. In the presence of sublethal concentrations of J852 (50 μM), *Synechocystis* accumulates ζ-carotene to 43 % of total carotenoids (Table 1). In contrast, the same inhibitory conditions for *Nostoc* PCC 7120 lead to ζ-carotene marginal accumulation of only 6 %. Eight other *Nostoc* strains and two *Anabaena* strains were screened for the existence of CrtQa with low ζ-carotene accumulation as an indicator. Among all the strains tested, only *Nostoc* PCC 6719 and PCC 7119 show a ζ-carotene content below 10 % similar to *Nostoc* PCC 7120. The others exhibited ζ-carotene accumulation of more than 20 % resembling more or less the situation of *Synechocystis*.

The carotenoid desaturation pathway of *Nostoc* PCC 7120 was reconstructed step by step in *E. coli*. Fig. 1a shows the isomeric composition of the ζ-carotene produced by *Nostoc* phytoene desaturase. The dominant ζ-carotene isomers were 9,15,9′-Z (highest), 9,9′-Z, and 9-Z. In two parallel experiments, their formation was coupled to ζ-carotene desaturation by either CrtQa (Fig. 1b) or CrtQb (Fig. 1c). HPLC separation was carried out in comparison with standards of all-E lycopene (plus 9-Z, 5-Z and 13-Z) provided by the desaturase CrtI (Misawa et al. 1995) (trace D) and a 7,9,7,′,9′-Z (= prolycopene) standard (trace E) from ripe Tangella tomato mutant (Clough and Pattenden 1983). The ζ-carotene and the neurosporene isomers were identified by retention time and spectra using the standard carotenoids from Breitenbach and Sandmann (2005).

Both ζ-carotene desaturases CrtQa and CrtQb converted the ζ-carotene isomers produced by the phytoene desaturase. However, the isomeric product patterns differed. CrtQa mainly synthesized L2 identified as all-E, L1, 9-Z and L3, 5-Z lycopene (Fig. 1b). These isomers were also formed by CrtQb but as minor products in addition to the dominating lycopene isomer was L4, which is 7,9,7,′,9′-Z (Fig. 1c). In addition, two neurosporene isomers, N1 and N2 were present as CrtQb products.

Several years ago, CrtQa has been purified and biochemically characterized (Albrecht et al. 1996) and is available for in vitro substrate conversion studies in contrast to CrtQb. Therefore, this ζ-carotene desaturase was expressed in *E. coli* and purified by affinity chromatography to a purity of >90 %. Several cofactors were tested as hydrogen acceptors for the desaturation reaction (Fig. 2a). A basic reaction was observed in the control (no addition) and with FAD, ubiquinone and oxidized nicotine nucleotides. A

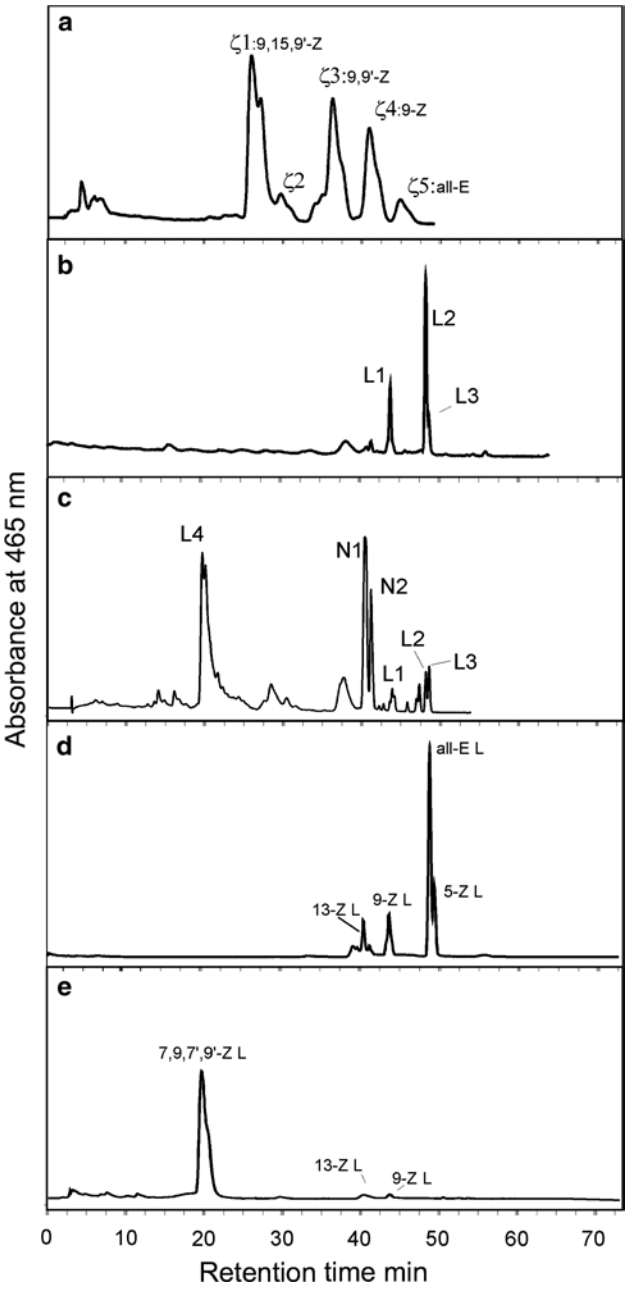

Fig. 1 Reconstruction of carotene desaturation of *Nostoc* PCC 7120 in *Escherichia coli*. HPLC separation of carotenoid extracts from DH5α/pACCRT-EB/pQE32crtP$_{7120}$ (**a**) leading to ζ-carotene isomers, DH5α/pACCRT-EB-P$_{7120}$/pZDS1B with an addition of CrtQa (**b**), DH5α/pACCRT-EB-crtP$_{7120}$/pQE32crtQb$_{7120}$, like A, but with an additional CrtQb (**c**), lycopene standard from DH5α/pACCRT-EBI (**d**) and 7,9,7′,9′-Z (= prolycopene) from tomato variety Tangella (**e**). Carotene isomers: *L* lycopene, *L1* 9-Z, *L2* all-E, *L3* 5-Z, *L4* 7,9,7′,9′-Z, *N* neurosporene, *N1* and *N2* non-specified isomers, *ζ* ζ-carotene isomers as indicated

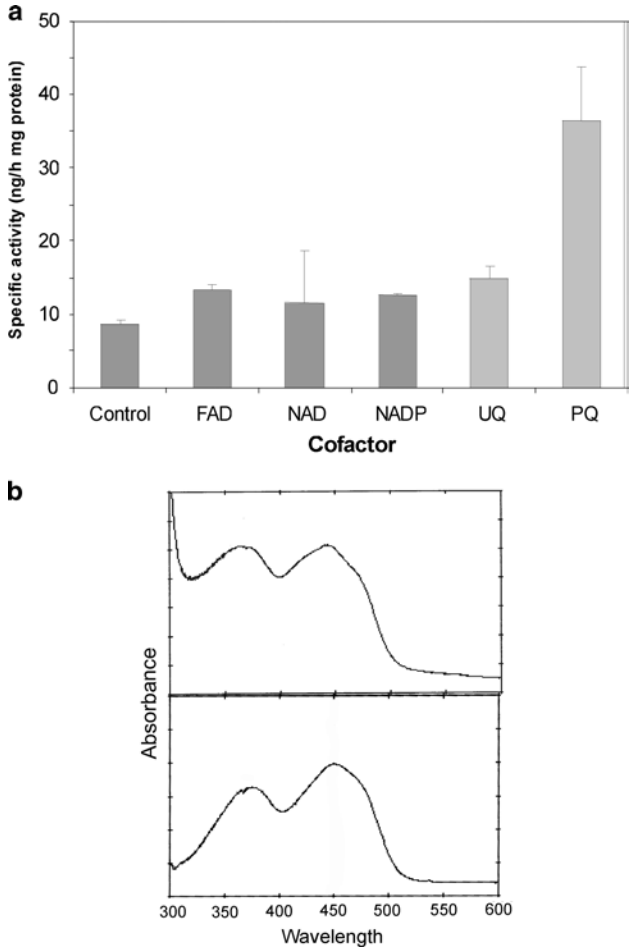

Fig. 2 Dependence of CrtQb$_{7120}$ of *Nostoc* PCC 7120 for oxidized cofactors (1 mM) (**a**) and its absorbance spectrum (**b**, *top*) compared to FAD (**b**, *bottom*). *UQ* ubiquinone, *PC* plastoquinone

significant increased product formation was obtained only with plastoquinone which can be regarded as the cofactor of CrtQb ζ-carotene desaturase in *Nostoc* PCC 7120. The purified CrtQb enzyme showed a yellow color. The spectrum is shown at the top of the Fig. 2b. It fully resembles the spectrum of FAD at the bottom. Quantification led to a 1:1 ratio of FAD per CrtQb enzyme.

In order to determine the ability of both ζ-carotene desaturases to convert different ζ-carotene isomers, in vitro reaction with isomer isolates was carried out. Each contained more than 70 % of a dominant isomer. The enriched substrates applied were all-E, 9-Z and 9,9′-Z ζ-carotene. Their composition is shown by inserts in Figs. 3 and 4. For CrtQa, the major product from all-E ζ-carotene was all-E neurosporene as intermediate and all-E lycopene as the end product of the reaction (Fig. 3a). The small peak of 9-Z neurosporene may originate from residual 9-Z ζ-carotene. 9-Z ζ-Carotene (Fig. 3b) was converted into 9-Z neurosporene (standard in Fig. 4d) in the first step and desaturated further to all-E lycopene. Only in the reaction with 9,9′-Z ζ-carotene, 7,9,7,′,9′-Z lycopene was formed (Fig. 3c). The presence of the low amounts of all-E lycopene here may be caused by spontaneous isomerization of Z isomers during the assay.

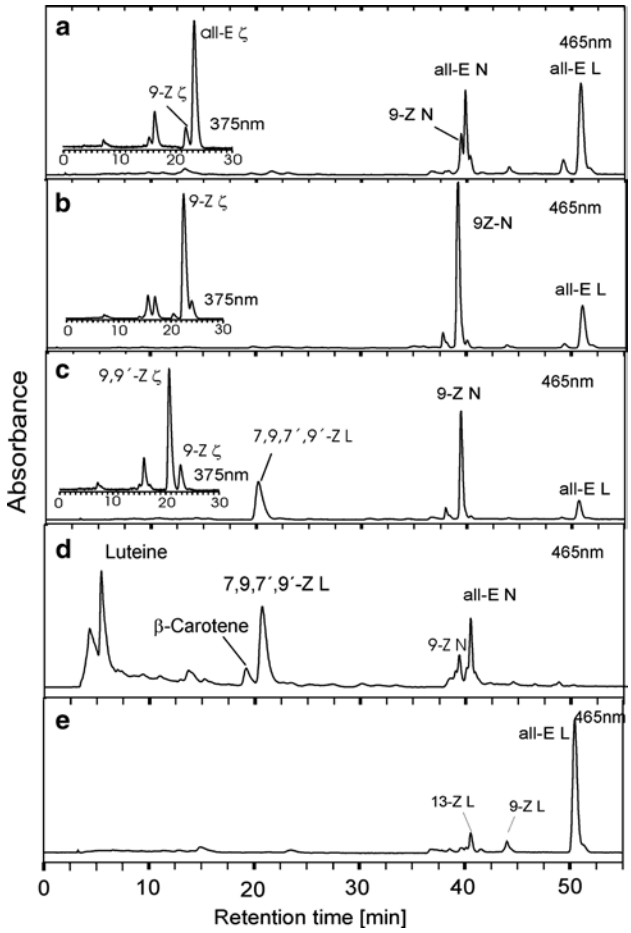

Fig. 3 HPLC analysis of in vitro desaturation products of $CrtQa_{7120}$ of *Nostoc* PCC 7120 with different ζ-carotene isomers as substrates (**a**–**c**). The corresponding ζ-carotene isomer composition is shown at the left side. Reference compounds were lycopene isomer 7,9,7′,9′-Z together with neurosporene isomers in an extract from a premature Tangella tomato fruit (**d**) and all-E lycopene plus Z isomers obtained in *E. coli* by the expression of pACCRT-EBI (**e**). *L* lycopene isomers, *N* neurosporene isomers, *ζ* ζ-carotene isomers

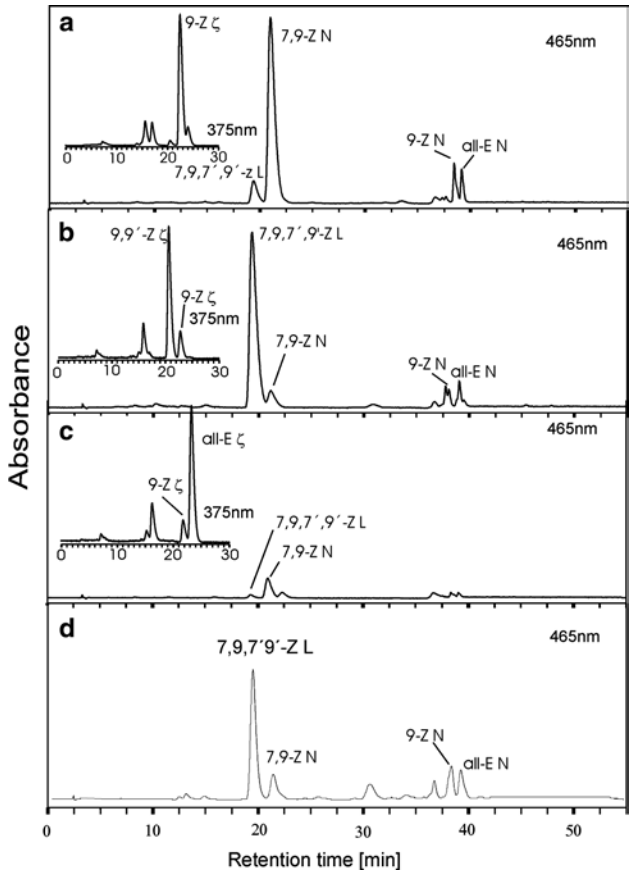

Fig. 4 HPLC analysis of in vitro desaturation products of $CrtQb_{7120}$ of *Nostoc* PCC 7120 with different ζ-carotene isomers as substrates (**a**–**c**). The corresponding ζ-carotene isomer composition is shown at the left side as inserts. Reference compounds 7,9,7′,9′-Z lycopene and neurosporene isomers were obtained by expression of pACCRT-EBP in combination with pQE30zds (**d**). *L* lycopene isomers, *N* neurosporene isomers, *ζ* ζ-carotene isomers

Neurosporene and lycopene reaction products were identified with the references chromatographed in traces D and E.

The ζ-carotene desaturase CrtQb converted 9-Z ζ-carotene efficiently into 7,9-Z neurosporene but stopped at this stage without substantial lycopene formation (Fig. 4a). The ζ-carotene isomer 9,9′-Z is metabolized to 7,9,7′,9′-Z lycopene (Fig. 4b). Small accompanying amounts of 7,9-Z neurosporene may result from residual 9-Z ζ-carotene in the 9,9′-Z ζ-carotene fraction. Application of all-E ζ-carotene to CrtQb yielded only trace amounts of 7,9-Z neurosporene and 7,9,7′,9′-Z lycopene (Fig. 4c). In analogy to trace B, it can be assumed that they originate from the small amount of 9-Z and 9,9′-Z ζ-carotene present and that all-E ζ-carotene cannot be converted by CrtQb. The reference compounds of all important CrtQb reaction products (trace D) were generated by gene combination in *E. coli*.

The specific activity for the conversion of the ζ-carotene isomers 9,9′-Z, 9-Z and all-E were determined for both ζ-carotene desaturases from *Nostoc* PCC 7120 (Fig. 5a). Similar values with 9,9′-Z ζ-carotene indicate that this isomer is a suitable substrate for both enzymes, which both catalyze its conversion into 7,9,7′,9′-Z lycopene. Upon application of 9-Z ζ-carotene, CrtQa shows higher specific activity and the activity for CrtQb is in the same range as for 9-9′-Z ζ-carotene. However, the end products of both enzymatic reactions differ (Figs. 3 and 4). Similarly to 9-Z ζ-carotene, the all-E isomer is efficiently converted by CrtQa, but only poorly by CrtQb.

Discussion

Nostoc PCC 7120 was the only cyanobacterium which in addition to the typical cyanobacterial ζ-carotene desaturase

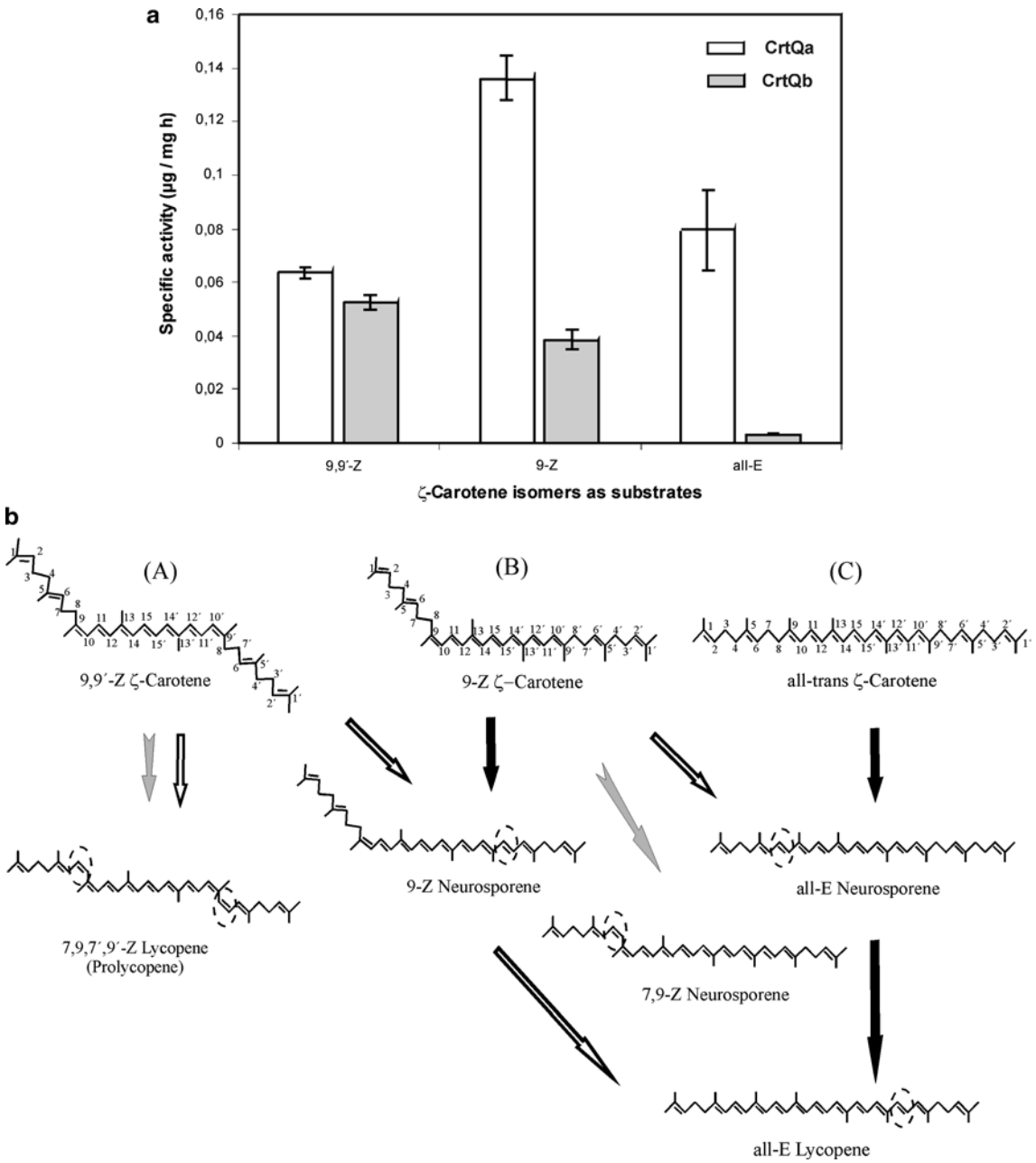

Fig. 5 Part **a**: Specific activities of ζ-carotene desaturase $CrtQa_{7120}$ and $CrtQb_{7120}$ from *Nostoc* PCC 7120 and conversion of ζ-carotene isomers (enriched by at least more than 70 %) produced by the phytoene desaturase $CrtP_{7120}$. **b**: Isomer specificity of both desaturases. *Tailed arrows* represent the exclusive reactions by $CrtQb_{7120}$ **b**. *Bold arrows* indicate the prominent reaction of $CrtQa_{7120}$ and open arrows reactions of $CrtQa_{7120}$ with lower activities. Newly formed double bonds are *circled*

CrtQb, the unrelated ζ-carotene desaturase CrtQa was detected (Sandmann 2002). By screening by an inhibitor approach in which CrtQb activity was blocked, two other *Nostoc* strains were detected in which a ζ-carotene desaturase additional to CrtQb is active (Table 1). The existence of an active CrtQa seems not to be unique among *Nostoc* species. However, with the same procedure, we found no indication for the existence of a CrtQa desaturase in the tested closely related *Anabaena* species. In the reconstituted carotenoid desaturation pathway in *E. coli* with a combination of carotene desaturases from *Nostoc* PCC 7120, the functionality of both ζ-carotene desaturases was confirmed and a substantially different isomeric product pattern demonstrated (Fig. 1).

After heterologous expression of CrtQb, the enzyme was purified and characterized. The CrtQb desaturase contains

a bound FAD and depends on plastoquinone as a hydrogen acceptor (Fig. 2). In both respects, it resembled the plant ζ-carotene desaturase (Hugueney et al. 1992; Breitenbach et al. 1999). However, none of these properties were reported for the CrtQa ζ-carotene desaturase (Albrecht et al. 1996). The isomer specificity as substrate was analyzed in vitro for both ζ-carotene desaturases (Figs. 3 and 4) by the application of several Z ζ-carotene isomers generated by the phytoene desaturase CrtP from *Nostoc* PCC 7120. This included the dominant 9,9′-Z ζ-carotene that is spontaneously formed from 9,15,9′-Z by Z to E isomerization of the C-15 double bond (Breitenbach and Sandmann 2005). The 9,9′-Z isomers were accepted by CrtQa and CrtQb as substrates. CrtQb formed almost exclusively 7,9,7′,9′-Z lycopene presumably via 7,9,7′-Z neurosporene, whereas CrtQa synthesized some of this tetra-*cis* lycopene, but even more 9-Z neurosporene and all-E lycopene. It seems to be more specific for the all-E half of the ζ-carotene molecule and the reactions indicated in Fig. 5b by bold arrows. Nevertheless, CrtQa still possesses some Z to E isomerase function which is an inherited feature from the related desaturase CrtI. In addition to 9,9′-Z ζ-carotene, the CrtP desaturase of *Nostoc* PCC 7120 produced substantial amounts of 9-Z and all-Z ζ-carotene. The latter can be metabolized exclusively by CrtQa to all-E lycopene, which is the final isomeric form for subsequent cyclization. The 9-Z ζ-carotene is utilized by both desaturases. However, the products of the reactions differed. In the case of CrtQa, 9-Z neurosporene was metabolized further to all-E lycopene, but for CrtQb, the reaction yielded 7,9-Z that seems to be a dead end in the desaturation pathway (Fig. 5a) without an isomerization to 9-Z or all-E.

The event in evolution leading to the replacement of the four-step CrtI desaturase by two novel two-step desaturases which utilize plastoquinone as cofactor (Breitenbach et al. 2001, Fig. 2a) allows the coupling of carotene biosynthesis to photosynthetic electron transport via the plastoquinone pool (Sandmann 2009). In *Nostoc* PCC 7120 (and two other *Nostoc* strains, Table 1), the ancient phytoene desaturase remained as CrtQa, a relict now restricted to ζ-carotene desaturation but retaining some of its isomerization function. With the catalytic activities outlined in Fig. 5b, CrtQa bypasses CrtQb function by utilizing ζ-carotene isomers formed by CrtP from *Nostoc* PCC 7120, which are poorly metabolized by CrtQb. This is an advantage to avoid dead ends in the poly-*cis* desaturation pathway.

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