Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 461 (2007) 235–241
ABB
www.elsevier.com/locate/yabbi

Kinetic variations determine the product pattern of phytoene desaturase from *Rubrivivax gelatinosus*

Per Stickforth, Gerhard Sandmann *

Molecular Biosciences 213, J.W. Goethe Universität, P.O. Box 111932, D-60054 Frankfurt, Germany

Received 22 December 2006, and in revised form 22 February 2007
Available online 21 March 2007

Abstract

In bacteria and fungi, the degree of carotenoid desaturation is determined by a single enzyme, the CrtI-type phytoene desaturase. In different organisms, this enzyme can carry out either three, four or even five desaturation steps. The purple bacterium *Rubrivivax gelatinosus* is the only known species in which reaction products of a 3-step and a 4-step desaturation (i.e. neurosporene and lycopene derivatives) accumulate simultaneously. The properties of this phytoene desaturation to catalyze neurosporene or lycopene were analyzed by heterologous complementations in *Escherichia coli* and by in vitro studies. They demonstrated that high enzyme concentrations or low phytoene supply favor the formation of lycopene. Under these conditions, CrtI from *Rhodobacter spheroides* can be forced in vitro to lycopene formation although this carotene is not synthesized in this species. All results can be explained by a model based on the competition between phytoene and neurosporene for the substrate binding site of phytoene desaturase. Mutations in CrtI from *Rvi. gelatinosus* have been generated resulting in increased lycopene formation in *Escherichia coli*. This modification in catalysis is due to increased amounts of CrtI protein.

Keywords: Mutation of phytoene desaturase; Neurosporene; Lycopene; Product specificity of phytoene desaturase; *Rhodobacter spheroides*

Carotenoid pigments are synthesized by archea, bacteria, fungi and photoautotrophic eukaryotes. Their color is dependent on the polyene structure within the carotenoid molecule [1]. The number of conjugated double bonds is a typical characteristic which determines the spectroscopic properties [2]. Starting from the basic structure of phytoene with three conjugated double bonds, a maximum of six conjugated double bonds can be inserted in a C40 carotene by a single enzyme, a phytoene desaturase of the CrtI type [3]. In this desaturation sequence, the first two double bonds are formed symmetrically at positions C-11,12 and C-11′,12′ of phytoene. All following double bonds are introduced more peripheral at adjacent positions of the carotene molecule extending the conjugated polyene structure (Fig. 1). In archea, bacteria and fungi, all desaturase steps are catalyzed by a single enzyme, the product of the related genes *crtI* and *al-1* [4]. This enzyme has been functionally produced in *E. coli* using the genes from *Erwinia uredovora* [5], *Rhodobacter capsulatus* [6] and *Neurospora crassa* [7]. Although highly homologous, these three desaturases of the CrtI-type differ in their properties. The cofactor for H acceptance in the dehydrogenase reaction is FAD for the bacterial phytoene desaturases and NAD in case of the desaturase from *N. crassa*. Depending on the organism, the enzymes can catalyze either a 3-step, 4-step or 5-step desaturation reaction (Fig. 1). The purple photosynthetic bacterium *Rubrivivax gelatinosus* is the only known organism that synthesizes neurosporene as well as lycopene as carotene metabolites resulting from insertions of three and four double bonds into phytoene, respectively [8]. This leads to the formation of a spheroidene and a spirilloxanthin branch within its carotenoid biosynthetic pathway. To date, little is known about the factors which determine the number of double bonds inserted into phytoene or

* Corresponding author. Fax: +49 69 798 24822.
E-mail address: sandmann@bio.uni-frankfurt.de (G. Sandmann).

0003-9861/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2007.02.035

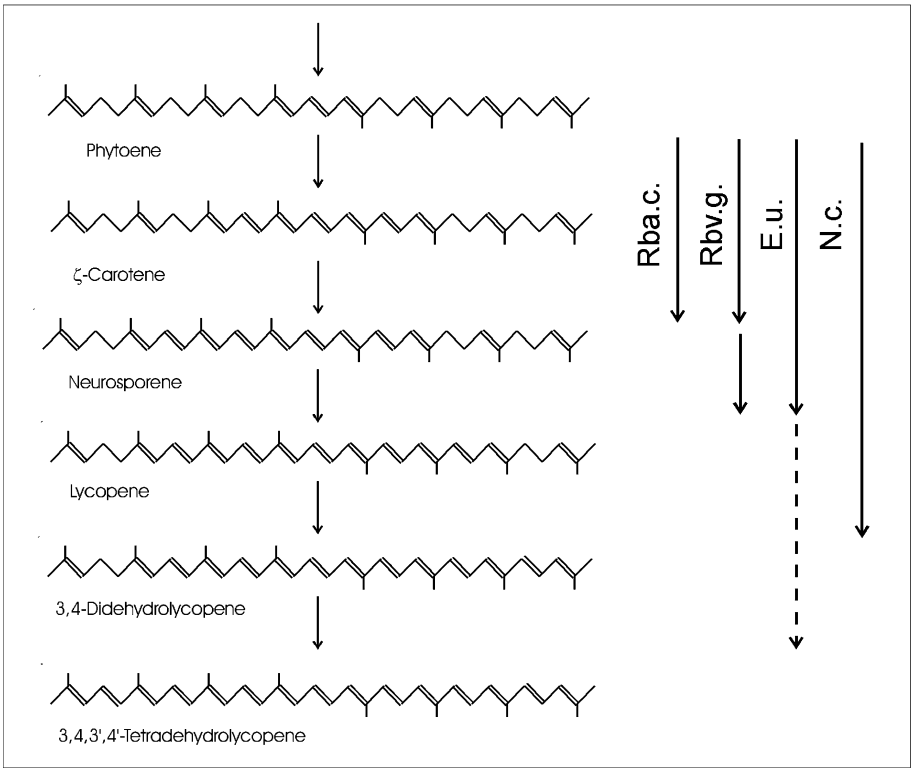

Fig. 1. Desaturation reactions and products of CrtI-related phytoene desaturases. The arrows indicate the different desaturation sequences catalyzed by the phytoene desaturases from *Rhobacter capsulatus* (Rba.c.), *Rubrivivax gelatinosus* (Rvi.g.), *Erwinia uredovora* (E.u.) and *Neurospora crassa* (N.c.). Dotted arrow indicates additional reaction steps of *E. uredovora crtI* expressed in *Escherichia coli*.

about the structural features of the enzyme that may specify the degree of desaturation of the reaction product. It was suggested that the number of desaturation steps may depend on the degree of oligomerization [9,10] but experimental evidence is missing.

Upon heterologous expression of *crtI* from *E. uredovora* encoding for a phytoene desaturase that exclusively forms lycopene in *E. uredovora* as judged from the carotenoid composition [11], a substantial amount of 3,4,3′,4′-tetradehydrolycopene with six double bonds inserted into phytoene accumulated [3]. This indicates that kinetic interaction may alter the product specificity. Therefore in this study, we produced phytoene desaturase from *Rvi. gelatinosus* which in vivo catalyzes three and four desaturation steps. Formation of carotene products with different degrees of desaturation under variable reaction conditions were analyzed and product pattern compared to the 3-step desaturase from *Rba. capsulatus*. We could also generate mutations in *crtI* which resulted in a higher formation of lycopene compared to the wild-type enzyme of *Rvi. gelatinosus*.

Experimental procedures

Strains and cultivation

Escherichia coli DH5α was used for plasmid amplification, heterologous phytoene synthesis and formation of CrtI proteins. It was grown in LB medium [12] at 37 °C with added antibiotics according to plasmids, ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). For induction of protein synthesis, 0.5 mM of IPTG was added after the optical density at 600 nm had reached a value of 0.3. Then, the cells were grown for 24 h to the late logarithmic phase. Mutator strain *E. coli* XL-1 red [13] was used for mutagenesis of $crtI_{Rg}$. In the experiments presented in Table 1, all cultures were grown to an OD_{600} of 1.

Plasmids and genetic manipulations

DNA for gene amplification was isolated from cells of *Rvi. gelatinosus* and *Rhodobacter spheroides* using the Plasmid Miniprep Kit I from peQLab Biotechnologie according to the supplier's instructions. Cloning vectors were pTrc99a [14] and pUC8-2 [15]. A new vector pPEU was constructed starting from pPQE30 by exchanging the *lacZ*-type promoter by the promoter in front of the *crt* operon in *E. uredovora* [11]. A 100-bp fragment of the promoter region was amplified from pACCRT-EBI [16] generating a *Xho*I at the 5′-end and a *Eco*RI site at the 3′-end with primers EuProm-fwd-(*Xho*I) 5′-taccgctcgagctgccaa-3′ and EuProm-end-(*Eco*RI) 5′-gaattctcgggctgtccttat-3′. The *lacZ* promoter was removed from pPQE32 by *Xho*I/*Eco*RI digestion and the *E. uredovora* promoter ligated into the same sites. Plasmid pACCRT-EB [16] was used in the complementation experiments in combination with other plasmids or alone to generate phytoene in *E. coli*. The *crtI* genes from *Rvi. gelatinosus* and *Rba. spheroides* were amplified by PCR with primers crtI-Rvi-fwd (*Sac*I) 5′-aggacgagctcgacggatgc-3′, crtI-Rvi-end (*Hin*dIII) 5′-gctcccaagctttcagcg-3′, and the pair crtI-Rsph-fwd (*Sph*I) 5′-gcatgctgatgccctcgatctcg-3′ and crtI-Rsph-end (*Sac*I) 5′-gagctctcattccgcggcaag-3′, respectively. The products were sub-cloned into the T-overhang *Xcm*I-digested vector pMON38201 [17]. The gene $crtI_{Rg}$ was cut out by *Sac*I/*Hin*dIII and ligated into pPQE32 and pPEU opened with the same restriction enzymes or with *Eco*RI/*Hin*dIII for cloning into pTrc99a or pUC8-2. The gene $crtI_{Rc}$ was cut out

Table 1
Characteristics of mutations in the phytoene desaturase gene $crtI_{Rg}$ from *Rvi. gelatinosus,* carotenoid, DNA and $CrtI_{Rg}$ concentration

Mutant	Exchange of nucleotides[a]	Exchange of amino acids	Lycopene[b] (μg/mg) (%)		$CrtI_{Rg}$ (μg/mg dry weight)	DNA[c] (μg/mg dry weight)
Wild-type	—	—	0.1	7	4.6	0.9
r4802	A670G	N224S	2.3	79	9.7	3.5
r4805	G475A	G159S	0.9	21	5.3	1.1
	T885C	Neutral				
	A1109G	Y370C				

[a] In the codon region.
[b] Sum of lycopene plus neurosporene.
[c] Plasmid pPEUcrtI$_{Rg}$ in *E. coli* DH5α.

by *Sac*I/*Hin*dIII and ligated into pPQE32. All genetic manipulations were carried out according to Sambrook et al. [12].

Mutagenesis of $crtI_{Rg}$ in E. coli XL1-red

Competent *E. coli* XL1-red cells were transformed with pTrccrtIRg and incubated over night on LB agar plates. Then, all cells were washed off and further grown in liquid medium for 48 and 54 h. From aliquots of the culture, plasmids were isolated digested with *Sac*I/*Hin*dIII and ligated into the *Sac*I/*Hin*dIII cut plasmid pPEU32. After co-transformation of the resulting pPEU32crtIRg library with pACCRT-EB in *E. coli* DH5α, colonies were visually screen for reddish pigmentation.

In vitro reactions

Cells were suspended in 100 mM phosphate buffer pH 8, broken in a French pressure cell at 20 MPa and the homogenate centrifuged at 40,000*g* for 20 min at 4 °C. Of the supernatant, 400 μl were used for the assay together with 400 μg of emulsified L-α-phosphatidylcholine, FAD (0.2 mM), 5.44 μg phytoene in 20 μl acetone (final concentration 20 μM) in a total volume of 0.5 ml. The amounts of supernatant or phytoene were varied in some of the experiments as indicated there. After incubation for 5 h in the dark at 28 °C, the reaction was terminated by addition of 3 ml of acetone and heating for 15 min at 55 °C. Total protein content of the supernatant were determined with the Bio-Rad protein assay. The relative amounts of the phytoene desaturases in the supernatant were estimated after protein separation on the SDS–polyacrylamid gels by electrophoresis after staining with Coomassie Brilliant Blue and scanning the protein pattern with a densitometric software. From the relative density of the phytoene desaturase band and the total protein loaded per lane, the concentrations of pyhtoene desaturase were calculated [18].

Carotenoid analysis

Carotenoids were extracted from freeze-dried *E. coli*/pACCRT-EB cells as a source of phytoene or other transformants with *crtI*-related plasmids by heating for 15 min at 55 °C in methanol containing 6% KOH. The extracts and also the heated enzyme incubations were partitioned against 10% ether in petrol and the carotenoids in the upper phase separated and quantitated by HPLC according to Steiger et al. [8].

Results

The phytoene desaturase gene $crtI_{Rg}$ was cloned in frame into different vectors which differ in their strength of protein synthesis. Plasmids pUCcrtI$_{Rg}$, pTrccrtI$_{Rg}$ and pPEUcrtI$_{Rg}$ were co-expressed in *E. coli* with pACCRT-EB, a plasmid which mediates phytoene synthesis

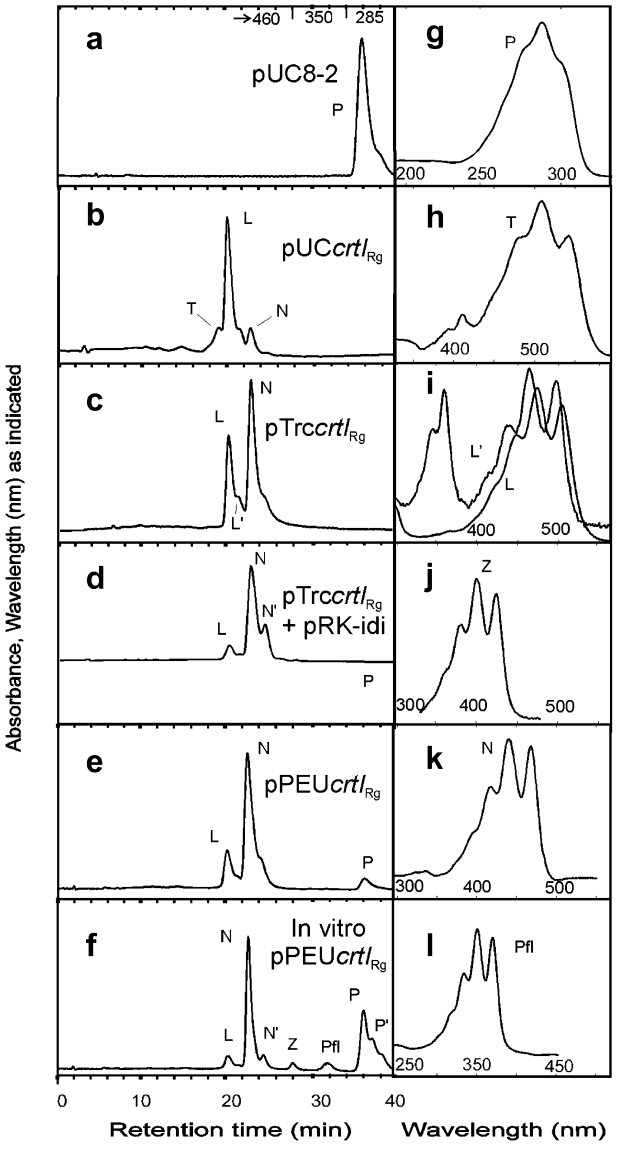

Fig. 2. Formation of desaturation products from phytoene by complementation of $crtI_{Rg}$ in *E. coli* carrying plasmids of different expression strength (a–e) and in vitro by the isolated enzyme (f). Plasmid combinations are indicated in the panel. (g–l) The spectra of the carotenoids in the HPLC diagrams. Carotenoids are labeled as L, lycopene; N, neurosporene; P, phytoene; Pfl, phytofluene; T, tetradehydrolycopene; Z, ζ-carotene.

(Fig. 2b, c and e, control a with an empty pUC8-2 vector instead). All transformants contain variable ratios of lycopene and neurosporene. The spectra of the individual carotenes are shown (Fig. 2g–l). Upon complementation with pUCcrtI$_{Rg}$, lycopene dominates over neurosporene with 88% of the reaction products and even small amounts of 3,4,3′,4′-tetradehydrolycopene was formed by CrtI$_{Rg}$ (Fig. 2b). Compared to pUC vectors optimized by deletion of the copy number controlling *rop* gene [19], plasmid pTrc99A with the origin of replication from pBR322 ([14] and references therein) produces a lower number of plasmid copies. With pTrccrtI$_{Rg}$ the relative amount of lycopene was definitely lower with 39% (Fig. 2c). With pPEUcrtI$_{Rg}$ plasmid with a weak promoter from the *E. uredovora* gene crt cluster, the percentage of lycopene as desaturation product was the lowest with 29% (Fig. 2e). In this complementation some residual phytoene is detectable in contrast to the others. This indicates a low phytoene desaturase activity. In complementation of Fig. 2d, *E. coli* was transformed with plasmid pRK-idi expressing an isopentenyl pyrophosphate isomerase in addition to pTrccrtI$_{Rg}$. This leads to enhanced formation of carotenoids [20]. Under these conditions of high formation of phytoene and its further metabolization, the accumulation of lycopene was much lower than with pTrccrtI$_{Rg}$ alone.

The results with the different *crtI$_{Rg}$* plasmids in *E. coli* gave a first indication that the number of double bonds inserted into phytoene may depend on the enzyme concentration. Since it is not possible to quantitate the amounts of active heterologous desaturase in the different transformants and discriminate it from the protein sequestered in inclusion bodies, this assumption was further investigated directly in vitro with the isolated phytoene desaturase. Fig. 2f shows an enzyme assay with CrtI$_{Rg}$ produced from pPEUcrtI$_{Rg}$ in *E. coli* under standard conditions (20 μM phytoene, 200 μg/ml phytoene desaturase). The active enzyme converts phytoene mainly to neurosporene and to some lycopene. In addition, the intermediates ζ-carotene and phytofluene can be detected. In this assay, the CrtI$_{Rg}$ concentration was varied from 19 to 446 μg/ml (Fig. 3a and b). As expected, this led to an increase of the amount of desaturated phytoene. However, the composition of the more desaturated products shifted to higher amounts of lycopene (Fig. 3a). A significant correlation was observed between the relative lycopene content formed by the reaction and the amount of enzyme applied (Fig. 3b). The same experiment was carried out with CrtI from *Rba. spheroides* (Fig. 3c). This enzyme catalyzed in vivo the formation of neurosporene exclusively as indicated by the products of its carotenoid biosynthetic pathway [21]. In the in vitro reaction, CrtI$_{Rs}$ is able to form small amounts of lycopene in addition to neurosporene. In contrast to CrtI$_{Rg}$ which synthesizes lycopene even at low enzyme concentrations, CrtI$_{Rs}$ has to overcome a threshold of 50 μg/ml before lycopene is detectable. But then, the relationship between relative amounts of lycopene and enzyme concentration is linear.

Since the same enzyme catalyzes the desaturation of phytoene and all carotene intermediates including the conversion of neurosporene to lycopene, product composition was determined depending on the phytoene concentrations in the assay. At the lowest phytoene concentration of 2 μM, almost a 1:1 ratio of neurosporene and lycopene formation was observed (Fig. 4). With increasing substrate concentrations, more reaction products were formed but absolute amounts of lycopene decreased. With up to 50 μM of phytoene, the relative amount of lycopene was below 5% (Fig. 4b). We also increased the concentration of the cofactor FAD from 0.2 to 0.5 mM which had no effect on the product composition in the experiments of Figs. 3 and 4 indicating that different lycopene to neurosporene rations are not caused by FAD limitation.

Mutants were generated by random mutagenesis of plasmid pTrccrtI$_{Rg}$ in *E. coli* XL-1 red and cloning of the mutant crtI$_{Rg}$ library into pPEU. The low lycopene yield of the wild type enzyme with this plasmid allows a color screening for CrtI$_{Rg}$ mutations that produce more lycopene. Several mutants with a more reddish color were obtained and three different ones were further analyzed (Table 1). They include r4802 with a single amino acid exchange of asparagines 224 to serine, and r4805 with three nucleotides exchanged resulting in two amino acid substitutions at two different regions of the protein. HPLC analysis of carotenoids formed by the complemented mutated CrtI$_{Rg}$ demonstrates that in each case the lycopene content is higher than in the wild type (Fig. 5). Both mutants are able to produce a range of lycopene amounts from 9- to 23-fold higher than the wild-type enzyme (Table 1). In r4802, the share of the reaction product lycopene was up to 79% versus 7% of the wild-type. The lycopene ratio of the other mutant was in between. For mutant r4802, lycopene ratios and amounts correspond well with the increased concentrations of CrtI$_{Rg}$ in the cells and also with a higher amount of plasmid pPEUcrtI$_{Rg}$ as indicated by the more than 4× increased DNA value compared to the wild-type.

Discussion

CrtI type phytoene desaturases catalyze in situ up to 3, 4 or 5 desaturation steps depending on the individual organism. In general, the number of desaturations may be determined either by structural features of the enzyme especially at the catalytic site, oligomerization of the protein [9,10] or by kinetic factors like competition between phytoene and its desaturation product as substrates [6]. In contrast to other phytoene desaturases, the enzyme in *Rvi. gelatinosus* is the only known one which generates two products, neurosporene and lycopene as starting points for two different branches of the carotenoid biosynthetic pathway [8]. Synthesis of CrtI$_{Rg}$ by different vectors with high and lower copy numbers or strong and weak promoters resulted in variable ratios of lycopene and neurosporene formation (Fig. 2). The highest amount of CrtI$_{Rg}$ is produced with

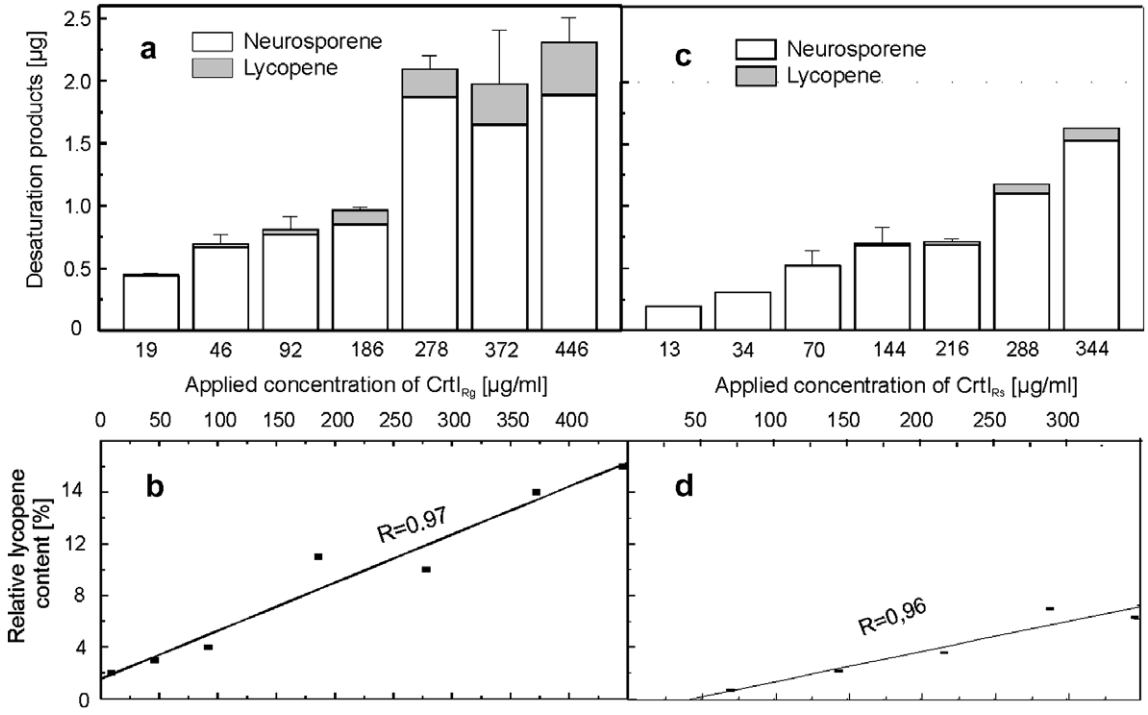

Fig. 3. In vitro formation of lycopene and neurosporene depending on the concentration of phytoene desaturase *crtI*$_{Rg}$ from *Rvi. gelatinosus* (a) or *Rba. spheroides crtI*$_{Rs}$ (c) and linear relationship between relative lycopene content and CrtI$_{Rg}$ (b) or CrtI$_{Rs}$ concentration (d). The phytoene concentration in the assay was 20 µM. The values of enzymatic carotene formation are the means of three determinations and are given with the standard deviation.

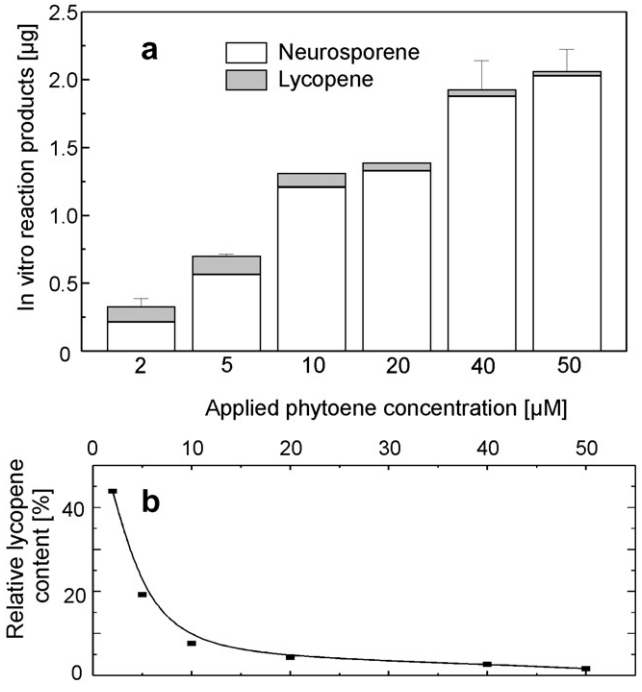

Fig. 4. Formation of desaturation products neurosporene and lycopene at varied substrate concentrations (a) and relationship between lycopene content and applied phytoene concentration (b). The phytoene desaturase concentration in the assay was 220 µg/ml. The values of enzymatic carotene formation are the means of three determinations and are given with the standard deviation.

pUC vectors (10–15% of total *E. coli* protein) with high copy number and a strong promoter. This transformant synthesized the highest lycopene amount. The lowest CrtI$_{Rg}$ concentrations (5–8%) were found with pPEU carrying a weak promoter. In this transformant lycopene formation was minor. These results are the first indication that the additional desaturation step from neurosporene to lycopene (Fig. 1) is favored by high enzyme concentrations. Above a certain level of CrtI$_{Rg}$, even the formation of 3,4,3′,4′-tetradehydrolycopene (Fig. 1) resulting from a 6-step desaturation of phytoene is possible (Fig. 2a). The synthesis of this highly desaturated carotene has been observed before when *crtI* from *E. uredovora* was expressed in *E. coli* in a pUC18 vector [3].

The dependence of lycopene formation on the concentration of CrtI$_{Rg}$ in the transformants can be established in vitro (Fig. 3a). Even at low enzyme concentrations, CrtI$_{Rg}$ can convert small amounts of neurosporene to lycopene. With increased enzyme concentration, a proportion of lycopene higher than in *Rvi. gelatinosus* cells [8] could be obtained. This potential of lycopene formation could also be observed for the desaturase from *Rba. spheroides* although this purple bacterium is unable to synthesize lycopene [21]. However, the enzyme concentration had to be much higher than for CrtI$_{Rg}$. Two mutants of CrtI$_{Rg}$ were obtained which synthesized increased lycopene amounts (Fig. 5). Both differed in the exchanged amino acids

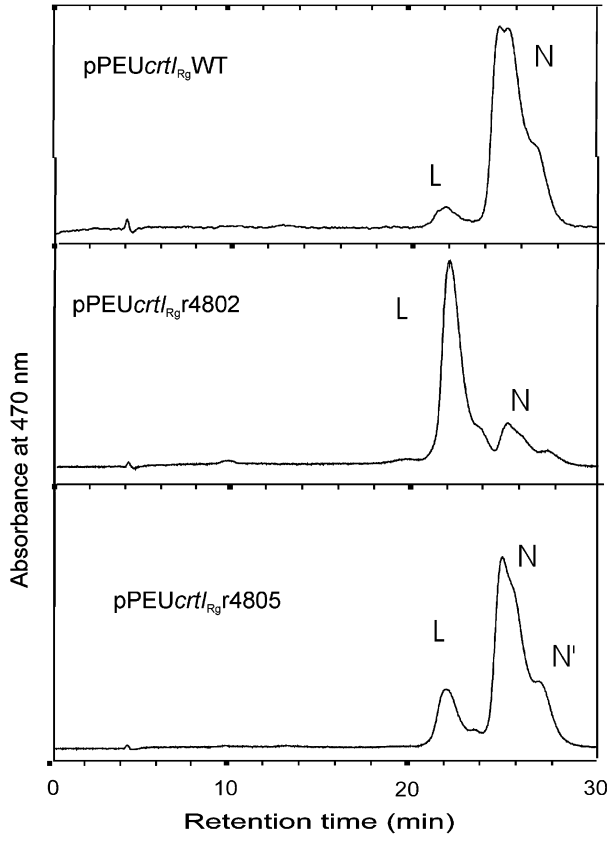

Fig. 5. Complementation of mutated phytoene desaturase $crtI_{Rg}$ in *E. coli* with a phytoene background and HPLC analysis of reaction products.

(Table 1). For at least one of them r4802, this modified catalytic property can be attributed to higher phytoene desaturase concentrations which also coincides with increase amount of plasmid DNA. At the moment, we do not have a rational explanation of how a modification of the *crtI* sequence influences the replication of the plasmid.

In one of the complementation experiments, pTrccrtI$_{Rg}$ was co-transformed in *E. coli* with a phytoene background together with pRK-idi (Fig. 2d). This plasmid mediates an increased influx of prenyl pyrophosphates into the carotenogenic pathway [20]. This means that higher amounts of phytoene are synthesized and more carotenoid end products accumulate. When pRK-idi was present, pTrccrtI$_{Rg}$-mediated lycopene formation was lower than without pRK-idi. Again the in vitro experiments demonstrated directly that low amounts of phytoene, which is the first substrate in the multiple reactions of CrtI, favor the yield of lycopene in a 4-step reaction (Fig. 4).

The combined influence of high enzyme and low phytoene concentration on neurosporene to lycopene desaturation can be explained by a competition between phytoene and neurosporene for the substrate binding sites of CrtI$_{Rg}$. At low enzyme concentrations, they are preferentially occupied by phytoene yielding neuroporene as the dominating desaturation product. When phytoene concentrations are low or a higher number of enzyme molecules are present, neuroporene has a better chance to be accepted as a substrate. These results demonstrate that the kinetics of phytoene desaturase is an important factor for the action of this enzyme. However, structural properties determining i.e. the substrate affinities have an additional influence on lycopene conversion to neurosporene. They drive the degree of this dynamic competition for the carotenes phytoene and neurosporene. For example in *Rba. capsulatus,* affinity for ζ-carotene is higher than for phytoene and neurosporene is a poorly accepted substrate [6]. Therefore, only in the presence of very high enzyme concentrations in the in vitro reaction of the *Rhodobacter* desaturase (Fig. 3c), sufficient enzyme molecules are available for neurosporene binding and conversion in competition to phytoene. This extreme differences in the affinities for neurosporene of the phytoene desaturases is reflected by a threshold of enzyme concentration that has to be overcome before lycopene formation is observed (Fig. 3c). By the phytoene desaturase from *Rvi. gelatinosus*, neurosporene is much better accepted as compared to the *Rhodobacter* enzyme. Nevertheless, phytoene is still the preferred substrate for the desaturase as indicated by their k_m values of 15 and 30 μM for phytoene and neurosporene, respectively (unpublished results). In the two mutants characterized in Table 1, improved neuropsorene to lycopene conversion is caused by different effects. In r4802, the amino acid exchange led to higher production of phytoene desaturase. This is not the case for mutant r4805 where a structural modification by exchange of two amino acids may lead in a modification of the protein resulting in a better acceptance of neurosporene as substrate for CrtI$_{Rg}$.

Looking at the in situ situation in purple bacteria, additional factors may influence the formation of lycopene and its reaction products. In *Rvi. gelatinosus* with an inactivated endogenous desaturase replaced by the 4-step desaturase from *E. uredovora,* less than half of the carotenoids are lycopene-derived [22]. An important feature is the interaction of neurosporene and lycopene with the hydratase CrtC, which catalyses addition of water to a terminal double bond in both carotenes. The indirect influence of this enzyme on neuroporene conversion to lycopene is demonstrated by replacement of the endogenous 3-step CrtI in *Rba spheroides* by the 4-step desaturase from *E. uredovora*. Although the biosynthetic capacity for lycopene formation is high in this transformant, it forms relatively low amounts of lycopene and their products [23]. However, when *crtC* is additionally inactivated and the hydration reaction prevented, lycopene is the dominating carotene formed [24]. In this transformant, the heterologous phytoene desaturase no longer competes with CrtC for neuroporene desaturation.

Acknowledgment

This work was supported by the Deutsche Forschungsgemeinschaft by a grant to G.S.

References

[1] T.W. Goodwin, The Biochemistry of the Carotenoids, second ed., Chapman and Hall, London, New York, 1980.
[2] G. Britton, FASEB J. 9 (1995) 1551–1558.
[3] H. Linden, N. Misawa, D. Chamovitz, I. Pecker, J. Hirschberg, G. Sandmann, Z. Naturforsch. 46c (1991) 1045–1051.
[4] G. Sandmann, Physiol. Plantarum 116 (2002) 431–440.
[5] P.D. Fraser, N. Misawa, H. Linden, S. Yamano, K. Kobayashi, G. Sandmann, J. Biol. Chem. 267 (1992) 19891–19895.
[6] A. Raisig, G. Bartley, P. Scolnik, G. Sandmann, J. Biochem. 119 (1996) 559–564.
[7] A. Hausmann, G. Sandmann, Fungal Genet. Biol. 30 (2000) 147–153.
[8] S. Steiger, C. Astier, G. Sandmann, Biochem. J. 349 (2000) 635–640.
[9] G.E. Bartley, T.J. Schmidhauser, C. Yanofsky, P.A. Scolnik, J. Biol. Chem. 265 (1990) 16020–16024.
[10] I. Pecker, D. Chamovitz, H. Linden, G. Sandmann, J. Hirschberg, Proc. Natl. Acad. Sci. USA 89 (1992) 4962–4966.
[11] N. Misawa, M. Nakagawa, H. Kobayashi, S. Yamano, K. Nakamura, K. Harashima, J. Bacteriol. 172 (1990) 6704–6712.
[12] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning, a Laboratory Manual, second ed., Cold Spring Harbour Laboratory Press, New York, 1989.
[13] A. Greener, M. Callahan, B. Jerpseth, Methods Mol. Biol. 57 (1996) 375–385.
[14] E. Amann, B. Ochs, K.J. Abel, Gene 69 (1988) 301–315.
[15] Z. Hanna, C. Fregeau, G. Prefontaine, R. Brousseau, Gene 30 (1984) 247–250.
[16] N. Misawa, Y. Satomi, K. Kondo, A. Yokoyama, S. Kajiwara, T. Saito, T. Ohtani, W. Miki, J. Bacteriol. 177 (1995) 6575–6584.
[17] A.Y. Borovkov, M.I. Rivkin, BioTechniques 22 (1997) 812–814.
[18] F. Badenhop, S. Steiger, M. Sandmann, G. Sandmann, FEMS Microbiol. Lett. 222 (2003) 237–242.
[19] G. Cesareni, M.A. Muesing, B. Polisky, Proc. Natl. Acad. Sci. USA 79 (1982) 6313–6317.
[20] M. Albrecht, N. Misawa, G. Sandmann, Biotechnol. Lett. 21 (1999) 791–795.
[21] K. Schmidt, in: R.K. Clayton, W.R. Sistrom (Eds.), The Photosynthetic Bacteria, Plenum Press, New York, 1978, pp. 729–759.
[22] J. Harada, K.V. Nagashima, S. Takaichi, N. Misawa, K. Matsuura, K. Shimada, Plant Cell Physiol. 42 (2001) 1112–1118.
[23] C.N. Hunter, B.S. Hundle, J.E. Hearst, H.P. Lang, A.T. Gardiner, S. Takaichi, R.J. Cogdell, J. Bacteriol. 176 (1994) 3692–3697.
[24] G. Garcia-Asua, R.J. Cogdell, C.N. Hunter, Mol. Microbiol. 44 (2002) 233–244.