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Structural and kinetics properties of a mutated phytoene desaturase from *Rubrivivax gelatinosus* with modified product specificity

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ABSTRACT

The phytoene desaturase CrtI from *Rubrivivax gelatinosus* catalyzes simultaneously a three- and four-step desaturation producing both neurosporene and lycopene. These carotenes are intermediates for the synthesis of spheroidene and spirilloxanthin, respectively. Two different mutation libraries for the *crtI* gene from *R. gelatinosus* were constructed to screen for modified enzymes which synthesize almost exclusively either neurosporene or lycopene. The resulting mutants carried between one and four amino acid exchanges and at least one of them affected the secondary protein structure by shortening or extending one of the helices. A prominent amino acid which was exchanged in the neurosporene or lycopene-forming desaturase was leucine 208. Enzyme kinetic studies were carried out with the L208 modified desaturase and the specificities for phytoene and neurosporene as substrates determined. Higher and lower values correlate well with the higher or lower potential for the synthesis of lycopene from neurosporene. TopPred analysis of the mutations of L208 indicated that the location is in a highly hydrophobic membrane-integrated region which is a good candidate for the substrate-binding site of the desaturase.

Introduction

The prominent structural feature of carotenoids is their conjugated polyene system. It determines their colors and their antioxidative properties [1]. Typically, double bonds are inserted into phytoene in sequential reactions. In bacteria, archeae and fungi, desaturation is catalyzed by CrtI type enzymes [2]. A single enzyme can form up to five double-bonds by a hydride-proton transfer mechanism [3]. In most bacteria, CrtI phytoene desaturase catalyzes a 4-step desaturation converting phytoene into lycopene with FAD as hydrogen acceptor [4]. Within the purple bacteria, several genera like *Rhodospirillum* belong to this type. However, *Rhodobacter* represents another group with 3-step desaturases. This enzyme also converts phytoene but stops at neurosporene as the end product [5]. The formation of either lycopene or neurosporene determines the operation of either a spirilloxanthin or a spheroidene paythway [6]. *Rubrivivax gelatinosus* is the only bacterium known to synthesize neurosporene and lycopene as desaturation products, simultaneously [7,8]. As a result of these parallel 3-step and 4-step desaturations, we find in this purple bacterium a dual pathway with a spheroidene and a spirilloxanthin branch (Fig. 1). A phylogenetic tree of CrtI from purple bacteria shows a cluster separation of the 3-step desaturases from the 4-step desaturases with CrtI from *R. gelatinosus* in between [2]. In a phylogenetic view, one can regard the carotenogenic pathway in *R. gelatinosus* as a missing link between the spirilloxanthin-type and spheroidene-type purple bacteria. In a previous publication, *in vitro* and *in vivo* analysis of this phytoene desaturase demonstrated that high enzyme concentrations or low phytoene supply favors the formation of lycopene [9]. Under these conditions, other 3-step phytoene desaturases can be forced *in vitro* to lycopene formation. These results were explained by a dynamic competition between phytoene and its desaturation products including neurosporene as substrates for subsequent desaturation reactions by the same enzyme. They all compete for the substrate-binding site of phytoene desaturase in *R. gelatinosus*. Above a certain concentration of the desaturase from *R. gelatinosus*, even the formation of 3,4,3′,4′-tetradehydrolycopene resulting from a 6-step desaturation of phytoene is possible. Furthermore, mutated phytoene desaturases from *R. gelatinosus* with increased lycopene formation have been generated: in one of them, the resulting increased lycopene formation correlated to higher phytoene desaturase expression levels. However, for another high lycopene-forming mutants phytoene synthase concentration is not the dominating factor for lycopene synthesis. Alternatively, a change in structural properties determines altered substrate affinities of phytoene desaturase for individual carotenes. This structural influence on the type of product to be formed is another important feature for the understanding of the function and evolution of CrtI.

In the present publication, we generated several CrtI mutants with altered structural properties which significantly affect the substrate affinities for individual carotenes. They resulted in CrtI

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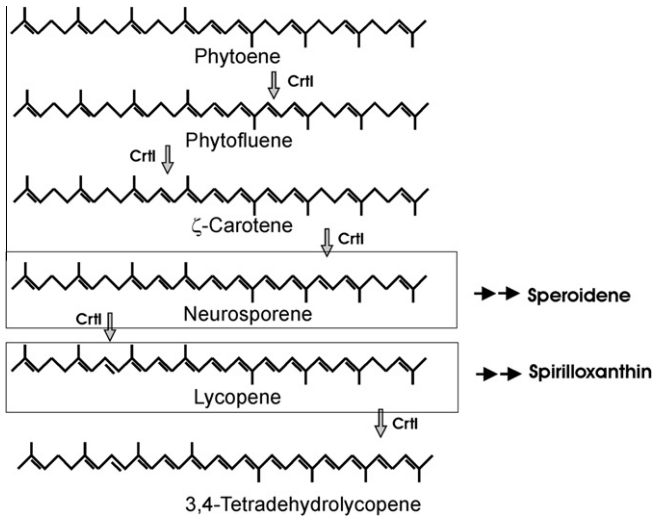

Fig. 1. Reaction sequence of desaturations in *Rubrivivax gelatinosus* catalyzed by a single phytoene desaturase $CrtI_{Rg}$. The resulting products which are further converted to spheroidene and spirilloxanthin in a parallel pathways are boxed.

vatiants with either enhanced or decreased activities for conversion of neurosporene to lycopene. In all mutated desaturase, the sequence alterations had an influence on the secondary protein structure.

Experimental procedures

Strain and cultivation

Escherichia coli DH5α was used as host for molecular genetic manipulations, synthesis of phytoene and neurosporene and the expression of the CrtI proteins. It was cultivated in LB medium [10] at 37 °C with added antibiotics according to the plasmids carried by *E. coli*, 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 and the cells finally grown to the late logarithmic phase for a total of 24 h.

Plasmids and random mutagenesis by error prone PCR

Plasmids used were pACCRT-EB [11] for complementation experiments in combination with other CrtI plasmids or alone to generate phytoene in *E. coli*, as well as pACCRT-EBI_{Rc} [5] for the synthesis of neurosporene. Plasmid pUC$crtI_{Rg}$ [9] was the source of *crtI* from *R. gelatinosus*. Empty pUC8-2 [12] and pPEU originating from pPQE30 by exchanging the *lacZ*-type promoter by the promoter in front of the *crt* operon in *Erwinia uredovora* [9] were used as cloning vectors for both mutant libraries.

Error prone PCR was carried out with the Mn-inosit triphosphate method according to Xu et al. [13] in two successive steps. The first PCR round used pUC$crtI_{Rg}$ as template DNA and 1.5 mM $MgCl_2$ plus 40 μM $MnCl_2$ and 200 μM dNTP mix over 20 cycles. In the second round (25 cycles), $MnCl_2$ was replaced by 50 μM inosit triphosphate. The primers used were those binding within pUC8-2, M13fw-21 (tgtaaacgagggccagt) and M13rev (caggaaacagctatgacc) at both sides of the multi cloning region. Finally, $crtI_{Rg}$ resulting from this error prone PCR was digested with *Sac*I/*Hin*dIII and cloned into the corresponding pPEU sites or digested with *Eco*RI/*Hin*dIII and cloned into pUC8-2. After co-transformation of the resulting pUC$crtI_{Rg}$ and pPEU$crtI_{Rg}$ libraries with pACCRT-EB in *E. coli* DH5α, colonies were visually screened for color changes. All genetic manipulations were carried out according to Sambrook et al. [10].

In vitro reactions

For the determination of enzyme kinetic parameters, cells transformed with mutated CrtI 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), the carotenes phytoene or neurosporene in 20 μl acetone in concentrations varied from 2 to 50 μM in a total volume of 0.5 ml. 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. Protein content of the supernatant was determined with the Bio-Rad protein assay. The relative amounts of the phytoene desaturases in the supernatant were estimated after separation on the SDS polyacrylamid gels after electrophoresis by staining with Coomassie brilliant blue and scanning using a densitometric software. From these values, the concentrations of phytoene desaturase were calculated.

Carotenoid analysis

Carotenoids were extracted from freeze-dried *E. coli* transformants 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 quantified by HPLC according to Steiger et al. [7].

Software

The program TopPred allows the prediction of the topology of a transmembrane protein (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) [14,15]. The program Predator was used to model the secondary structures of CrtI proteins from their sequences (http://bioweb.pasteur.fr/seqanal/interfaces/predator-simple.html) [16,17].

Results

Random mutagenesis of $CrtI_{Rg}$ was carried out by error prone PCR in which misrecognition of dNTP by Taq DNA polymerase is initiated by Mn^{2+} and mispairing by dITP [13]. The conditions used exhibited an average mutation rate of 1 to 2 per clone. Since the expression levels of $CrtI_{Rg}$ determines the formation of lycopene, two different libraries were constructed for the screening of $CrtI_{Rg}$ with either lower or with higher synthesis capacity for lycopene (i.e. variations in the fourth step of desaturation, see Fig. 1). The first goal was obtained by ligation into pUC8 with a strong promoter and the second by ligation into pPEU with low promoter activity. Both libraries were co-transformed with pACCRT-EB which mediates the formation of phytoene, the substrate for $CrtI_{Rg}$. In general, the pUC-$crtI_{Rg}$ library produced deep-red colonies with a few yellow-pinkish one. From them, colonies with slightly varying pigmentation were picked and four of them analysed for carotenoid composition (Fig. 2). In EP08, EP16, and EP22 neurosporene is the dominating carotenoid in contrast to the control (Fig. 2A). Lycopene formation was strongly decreased and the synthesis of tretradehydrolycopene was stalled in all mutants or found only as a trace in EP08. In EP21, ζ-carotene and phytofluene which are both early intermediates of the desaturation pathway and which are absent in WT are the main carotene products.

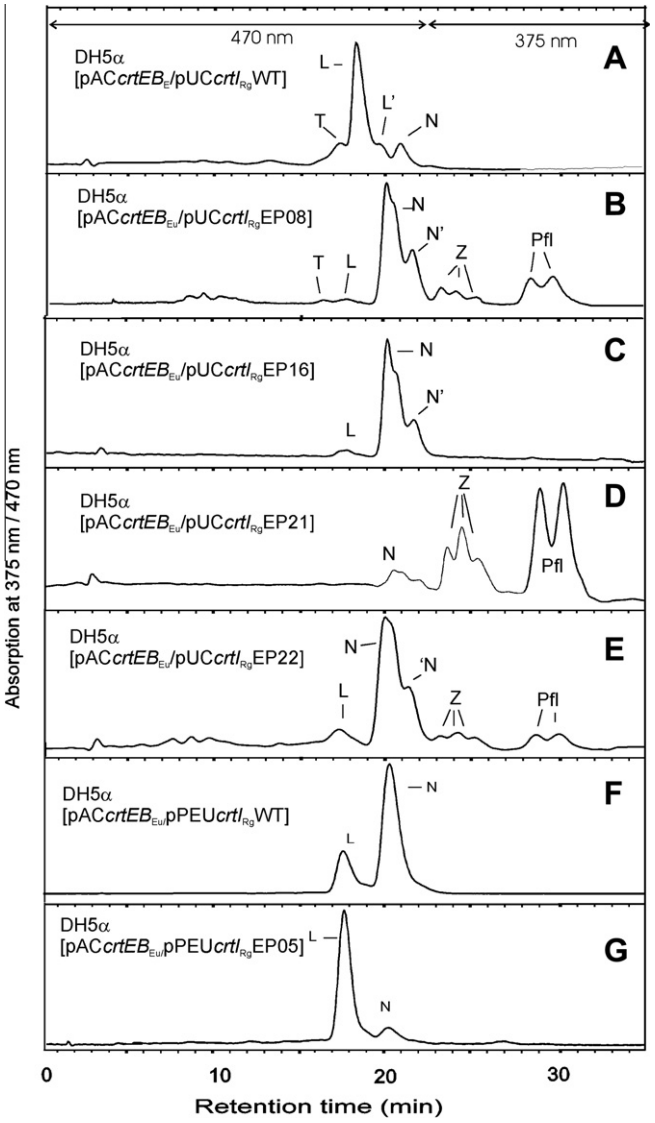

Fig. 2. HPLC separation of carotenoids formed by pathway complementation with plasmids pACcrtEB and CrtI$_{Rg}$ wild type (WT) and mutants EP08, EP16, EP21 and EP22 from the pUC8 and EP05 from the pPEU library. L, lycopene; N, neurosporene; Pfl, phytofluene; T, tetradehydrolycopene; Z, ζ-carotene; N′ and L′ are corresponding *cis* isomers.

Upon complementation, the pPEUcrtI$_{Rg}$ library exhibited mainly pink colonies with very few red ones. The pink colonies including the WT control (Fig. 2F) contained neurosporene and lycopene in a ratio of about 3:1. In all red colonies, lycopene was the dominating carotene and was accompanied by only small amounts of neurosporene (Fig. 2G).

Properties of the five mutated CrtI$_{Rg}$ are compared in Table 1. Sequence analysis showed modifications in all mutants. They include base exchanges, codon modification resulting in at least one amino acid exchanges. The mutants EP08, EP16, and EP21 in which desaturation was almost completely stopped at the stage of neurosporene, carried between one or four amino acid exchanges. Furthermore, at least one amino acid modification had an influence on the protein structure. Mutant EP22, the one with decreased but still substantial potential for lycopene synthesis compared to WT, carries two amino acid exchanges both with relevance on the secondary protein structure. Mutant EP05 screened from the pPEU library exhibited enhanced lycopene cyclization. Here, we found a modification of L208F. This is the same amino acid which was exchanged in EP08 from leucine to proline. In contrast to a series of previously analysed CrtI mutants from *R. gelatinosus* which exhibited higher expression levels of phytoene desaturase [9], the amounts of expressed CrtI$_{Rg}$ in the selected mutations in Table 1 were very similar when compared on polyacrylamide SDS gels (data not shown). The relevance of the mutations on CrtI$_{Rg}$ structure is shown in Fig. 3. Compared to the non-mutated CrtI$_{Rg}$, EP05 and EP08 possess a helix shortened by one or four amino acid, respectively. In EP21 a helix is shortened and a beta-sheath close by extended. An extension of another helix by two amino acids is observed in EP16. The strongest effect on CrtI$_{Rg}$ structure was in EP22. Two different helices were shortened by one or two amino acids. In addition, a beta-sheath of four amino acids was completely abolished.

EP05 and EP08 which both exhibit a mutation of L208 towards either increased formation of lycopene or decreased formation, were chosen for the determination of their enzyme kinetic properties in comparison with wild type CrtI$_{Rg}$. The desaturases were heterologously expressed in *E. coli* and their catalytic activities determined with varying concentrations of phytoene and neurosporene. K_m and V_{max} values were determined and specificity values K_m/V_{max} [18] calculated (Table 2). These values represent relative turnover numbers and are useful for the direct comparison of catalytic activity for a given substrate. V_{max} values for phytoene were more or less unaffected in both mutants and for neurosporene in EP08. The most striking differences were the K_m values

Table 1
Modifications of phytoene desaturase mutants from *Rubrivivax gelatinosus* and ratios for the formation of lycopene vs neursoporene (L:N) upon complementation in *Escherichia coli*.

Mutant	Base exchange	Codon modification	Amino acid exchange	L:N ratio
CrtI$_{Rg}$WT	–	–	–	88:12[a]
CrtI$_{Rg}$EP08	T623C	CUC>CCC	L208P*	3:97[a]
CrtI$_{Rg}$EP16	A131G	UAC>UGC	Y44C	3:97[a]
	A158G	GAC>GGC	D53G	
	C400T, C401T	CCG>UUG	P134L*	
	T184C	GUG>GCG	V395A	
CrtI$_{Rg}$EP21	C767T	ACG>AUG	T256M	0:100[a]
	A1064G	GAC>GGC	D355G	
	T1270C	CUG>CCG	L424P*	
CrtI$_{Rg}$EP22	T458C	CUG>CCG	L153P*	12:88[a]
	T833C	CUG>CCG	L278P*	
CrtI$_{Rg}$WT	–	–	–	26:74[b]
CrtI$_{Rg}$EP05	C622T	CUC>TUC	L208F*	89:11[b]

* Amino acid exchange influencing the secondary protein structure.
[a] Complementation of CrtI in pUC8 (DH5α/pACCrtEB$_{EU}$/pUCCrtI$_{Rg}$).
[b] Complementation of CrtI in pPEU (DH5α/pACCrtEB$_{EU}$/pPEUCrtI$_{Rg}$).

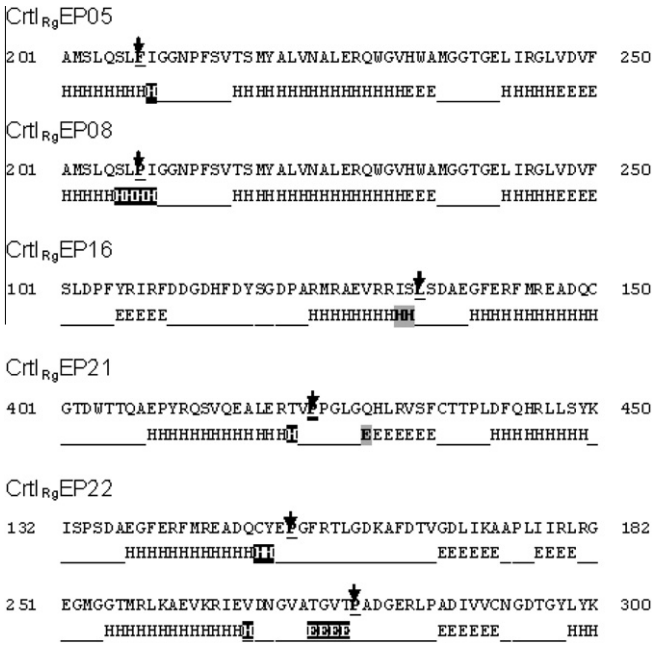

Fig. 3. Helical (H) and beta-sheath regions (E) of phytoene desaturase CrtI$_{Rg}$ modified in the mutants. Modified amino acids are indicated by an arrow, shortened structural regions are marked in black, extended regions in grey.

Table 2
Kinetic factors of phytoen desaturase CrtI$_{Rg}$ from different mutants of *Rubrivivax gelatinosus* for conversion of phytoene (P) and neurosporene (N).

Mutant	K_m value (μM)	V_{max} value (nmol mg^{-1} h^{-1})	V_{max}/K_m
Wild type			
P	14.8	5.2	35.1×10^2
N	33.1	0.6	1.8×10^2
EP05			
P	4.8	5.2	108×10^2
N	65.8	3.3	5.0×10^2
EP08			
P	17.1	5.4	30.2×10^2
N	41.7	0.3	0.7×10^2

which were increased for neurosporene in both mutants and additionally for phytoene EP05. The resulting K_m/V_{max} ratios indicate that in EP05 the specificities for phytoene and neurosporene are increased and that EP08 exhibits a lower preference for neurosporene. This result correlates well with the higher or lower potential for the synthesis of lycopene from neurosporene.

Discussion

CrtI-type phytoene desaturases recognize a trienoic region at one side and a double bond next to the carbon atoms between which the new double bond is integrated into the substrate carotene [18]. Therefore, these desaturases posses the general potential to insert up to six double bonds into phytoene. However, affinity for individual intermediates may vary considerably allowing only three, four or five desaturation steps [2].

R. gelatinosus is the only bacterium in which neurosporene is the major desaturation product together with further desaturated lycopene (Fig. 1). Typically in the sequence of desaturation, the K_m value for the substrate of the last desaturation step is significantly lower than for the previous one [5]. This ensures that only one final CrtI product is formed. However in the case of CrtI$_{Rg}$, the affinity for neurosporene conversion is poorer than for phytoene conversion (Table 2). This explains the formation of two desaturation products.

Mutants have been generated for functional modification of CrtI from *Rhodobacter spheroides* [19] and *R. gelatinosus* [9]. The latter mutants all exhibited much stronger expression of CrtI. It is a special feature of CrtI that high enzyme concentrations favour the formation of additional desaturation steps. In this work, two different libraries were employed for the screening of a series of mutants which all affect the secondary structure of CrtI$_{Rg}$ (Table 1) with a negative effect on the desaturation of phytofluene, ζ-carotene or neurosporene (Fig. 2). In addition, a mutant with extremely high lycopene formation was obtained. The enzyme kinetic properties of the latter and of one mutated CrtI$_{Rg}$ in which lycopene synthesis was almost completely suppressed demonstrated that the structural modifications affected the substrate specificity for neurosporene desaturation in one way or another (Table 2). TopPred analysis of the mutations of L208 in EP05 and EP08 indicated that the location is in a highly hydrophobic membrane-integrated region. It can be assumed that this conserved region of the desaturase could be near the active site where the very lipohilic substrate carotenes are bound. Mutations of *R. spheroides* CrtI generated by a combination of random and subsequent directed mutagenesis changed a neursoprene-forming to a lycopene-forming enzyme. In these mutants, the product distribution is determined by amino acid modification in the N-terminal region where also most of the modifying mutations of CrtI$_{Rg}$ are located [20]. Based on structural analysis of CrtI$_{Rg}$, a model for the CrtI-related desaturases with four membrane-spanning regions is presented in Fig. 4. It includes the conserved N-terminal dinucleotide binding site [21] and the important amino acid L208 which influences the kinetic properties for carotene conversion (Table 2) and the degree of desaturation (Table 1) is marked.

It can be assumed that the neurosporene-based pathway evolved from a lycopene pathway in *R. gelatinosus* (and in other purple bacteria) after a *crtA* ketolase gene was acquired by lateral gene transfer [22]. The final pathway product keto speroidene resulting from neurosporene (Fig. 1) posses 11 conjugated double bonds which are regarded as a minimum for efficient quenching of singlet oxygen [23]. In contrast, non-ketolated spirilloxanthin derived from lycopene carries 13 conjugated double bonds. Since CrtI$_{Rg}$ synthesizes both carotenes, it is a missing link between lycopene and neurosporene-producing desaturases. Mutation of a single amino acid in different ways was capable of either completing the evolution to an almost exclusive neurosporene- or reverting CrtI$_{Rg}$ to the original lycopene-forming enzyme.

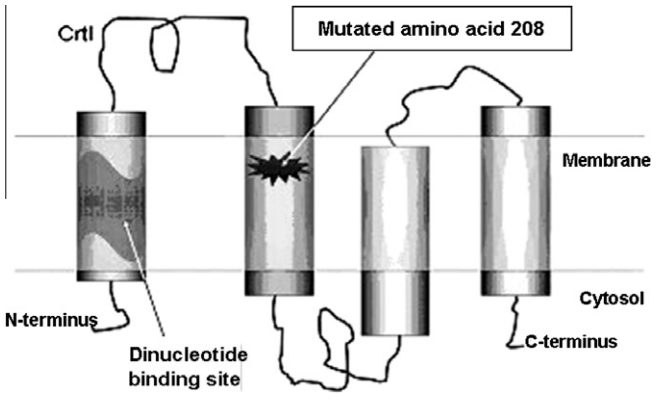

Fig. 4. Model of phytoene desaturase CrtI including the membrane-spanning regions and location of amino acid 208 which determines product specificity in the enzyme from *Rubrivivax gelatinosus*.

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