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Purification in an Active State and Properties of the 3-Step Phytoene Desaturase from *Rhodobacter capsulatus* Overexpressed in *Escherichia coli*[1]

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The phytoene desaturase gene from *Rhodobacter capsulatus* was expressed in *Escherichia coli* and the resulting protein was purified. The purification steps involved were ammonium sulfate precipitation and ion exchange chromatography, leading to a homogenous protein of 57 kDa with high specific enzymatic activity. The purified enzyme was characterized with respect to substrate specificity and product formation. In addition to phytoene, the intermediates, phytofluene and ζ-carotene, were both converted to neurosporene, the end product of the reaction. Furthermore, 1,2-epoxy phytoene was a suitable substrate whereas the C30 diapophytoene was not. The K_m values for phytoene and ζ-carotene were determined to be 33.3 and 16.6 μM, respectively. The desaturation reaction is dependent on the cofactor FAD. Oxidized nicotine nucleotides or ATP had no positive effect. The K_m value for FAD was 4.9 μM. Inhibition of the desaturation reaction was observed with diphenylamine.

Key words: ζ-carotene, neurosporene, overexpression of protein, phytoene desaturase, *Rhodobacter capsulatus*.

Carotenoids comprise one of the most abundant classes of pigments found in bacteria, fungi and higher plants. Although there are several hundred different kinds of structures, all the C40 carotenoids originate from phytoene, which is the first compound in the carotenoid biosynthetic pathway. This colorless carotene is converted into colored carotenoids through the introduction of several double bonds (*1*). Phytoene desaturase enzymes from different organisms catalyze the formation of different numbers of double bonds, thus leading to different reaction products (*2*). Phytoene desaturases from cyanobacteria, algae and higher plants convert phytoene into ζ-carotene through a 2-step desaturation process, whereas functionally equivalent enzymes from bacteria and fungi in general catalyze a 4-step desaturation process leading to the maximally desaturated lycopene. A few bacteria, including members of the *Rhodobacter* genus, contain a 3-step phytoene desaturase which converts phytoene into neurosporene (*3*). Comparison of all the desaturases from different sources revealed strong similarities between all the bacterial and fungal enzymes but little conservation in the 2-step desaturases typical of organisms with oxygenic photosynthesis (*4*).

Homogenous active 4-step and 2-step phytoene desaturases have been purified from the bacterium, *Erwinia* (*5*), the cyanobacterium, *Synechococcus* (*6*), and *Capsicum* (*7*). The last enzyme was purified directly from chromoplasts, whereas the other two were obtained after overexpression of the corresponding genes in *Escherichia coli*. Although the gene for the 3-step phytoene desaturase from *Rhodobacter* species has been cloned (*8-10*), purification of the corresponding active enzyme has not been reported. In this paper we present a purification approach for the *R. capsulatus* phytoene desaturase which avoids the use of detergents, and results in a homogenous highly active protein suitable for enzyme kinetic and inhibitor studies.

EXPERIMENTAL PROCEDURES

Plasmids and Organisms—Plasmid pGB721 contains the phytoene desaturase gene, *crtI*, from *R. capsulatus* (*10*); pACCRT-EB contains the *crtE* and *crtB* genes to produce phytoene; pACCRT-EBP contains the *crtE* and *crtB* genes, and the *pds* gene to produce ζ-carotene (*11*); and pUG10 contains the *crtM* gene from *Staphylococcus aureus* to produce diapophytoene (*12*). *E. coli* was grown in LB medium (*13*). BL 21 (DE 3) cells containing pGB721 were grown overnight in the presence of 0.1 mg·ml^{-1} ampicillin and 100 μM IPTG at 37°C. The JM101 cells containing a plasmid, pACCRT-EB or pACCRT-EBP, were grown for two days in the presence of 34 μg·ml^{-1} chloramphenicol at 28°C. The JM101 cells containing pUG10 were grown for two days in the presence of 0.1 mg·ml^{-1} ampicillin at 28°C. Transformation was performed according to standard procedure (*13*). *Phycomyces blakesleeanus* mutant S442 (*14*) was grown in a medium according to Than *et al.* (*15*) for three days at 28°C under constant shaking. It was used as the source for phytofluene. The asymmetric ζ-carotene (7,8,11,12-tetrahydrolycopene) was isolated from diphe-

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nylamine-treated *Rhodospirillum rubrum* cells according to Davies (*16*) by Dr. S. Takaichi, Kawasaki. 1,2-Epoxy phytoene was from a former preparation (*17*).

Pigment Extraction and Purification—Lyophilized cells (bacteria or fungi) were extracted with methanol containing 6% KOH for 20 min at 60°C and then partitioned in 10% ether in petroleum ether. The pigments were further purified by column chromatography on alumina (grade I) according to Britton (*18*).

Isolation and Purification of Phytoene Desaturase—Cells were harvested by centrifugation at 4,000 × *g* for 10 min at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl buffer, pH 8, containing 1 mM DTT. The cells were disrupted twice with a French Press at a pressure of 20 MPa. The resulting homogenate was treated with DNAse (2 $\mu g \cdot ml^{-1}$) for 10 min at room temperature. After centrifugation, the resulting supernatant was precipitated with ammonium sulphate (0-60% saturation) and the proteins were collected by centrifugation (40,000 × *g* for 20 min). The pellet was resuspended in 5 ml buffer (as above) and then desalted on a Sephadex G25 column. The protein was purified by DEAE ion exchange chromatography and eluted with a linear NaCl gradient, from 0-0.5 M. The purified phytoene desaturase was eluted at 25 mM NaCl and used directly for the *in-vitro* assay. The purification process was monitored by SDS-polyacrylamide gel electrophoresis (*19*). Proteins were stained with Coomassie Brilliant Blue. The gels were scanned with a flatbed scanner and the relative amount of phytoene desaturase was estimated with densitometric software. The total protein concentration in all fractions was measured by the method of Petersen (*20*).

In-Vitro Assay—A lipid emulsion, designed to enhance the accessibility to the substrate, was prepared as follows: 0.1 g phosphatidylcholine (SIGMA) was diluted in 2 ml chloroform and partitioned twice against 5 ml buffer (50 mM Tris, pH 8). The organic phase was dried for 5 h under a nitrogen stream. One milliliter of buffer was added and the mixture was sonicated for 20 min for dispersion of the lipids. The emulsion was stored at 4°C in 50 μl aliquots. The *in-vitro* assay mixture consisted 150 μl of this lipid preparation, 2 μg phytoene in 10 μl methanol, 500 μl purified phytoene desaturase (typically 350 μg protein), FAD (0.5 mM), $MnCl_2$ (4 mM), and $MgCl_2$ (6 mM), in 50 mM Tris-HCl buffer, pH 8, containing 1 mM DTT, in a total volume of 660 μl. In studies evaluating the effects of cofactors on desaturation, the cofactors were all applied at a concentration of 1 mM. The assay mixture was incubated for 4 h at 30°C with gentle shaking. This incubation period was still in the temporal linear range, and was therefore used for determination of specific activity and K_m values. The reaction was terminated by the addition of 2.5 ml methanol and pigments were extracted immediately by partitioning twice against 5 ml of 10% ether in petroleum ether. The pigments were dried under nitrogen and stored at −20°C. The extracted pigments were then dissolved in 20 μl acetone and separated and quantified by HPLC. Separation was carried out on a Spherisorb ODS1 column with acetonitrile/methanol/2-propanol (85 : 10 : 5, v/v/v) (*2*). Spectra were recorded on-line with a Kontron DAD 440 photodiode array detector. The amounts of reaction products were calculated by integrating the corresponding HPLC peaks and calibration with authentic standards. Lineweaver-Burk plots were drawn and statistically evaluated with the FigP program from Biosoft.

RESULTS

Protein Purification—The isolation and purification of active membrane-bound carotenogenic enzymes poses unique challenges. We observed that the supernatant fraction of an *E. coli* strain expressing *R. capsulatus* phytoene desaturase contained a considerable amount of enzyme (Fig. 1, lane 5). To determine whether or not this solubilized enzyme was active, we used the supernatant obtained on French press treatment as the starting material for the purification. The first step, ammonium sulfate precipitation at 60% saturation, only resulted in slight enrichment of the phytoene desaturase (Fig. 1, lane 6). The ammonium sulfate precipitate, which easily dissolved in buffer, was desalted on Sephadex G25 and then applied to

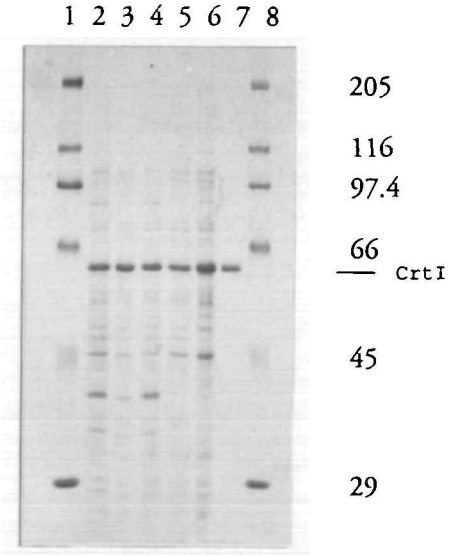

Fig. 1. **SDS-polyacrylamide gel electrophoresis of proteins, to monitor the purification of the phytoene desaturase from *Rhodobacter capsulatus* after its expression in *E. coli*.** Lanes 1 and 8: molecular weight markers; lane 2: whole bacterial cells; lane 3: cell homogenate; lane 4: pellet fraction after centrifugation; lane 5: supernatant; lane 6: 60% ammonium sulphate precipitate; and lane 7: DEAE fraction with about 25 mM NaCl.

TABLE I. **Purification of the recombinant phytoene desaturase from *Rhodobacter*.**

Step	Volume (ml)	Protein (mg)	CrtI protein (mg)	Relative CrtI content (%)	Specific activity ($\mu g/mg \cdot h$)	Recovery (%)
Cells	40	220	66	30	0.08	100
Supernatant	36	82.8	41.4	50		62.3
Ammonium sulphate precipitate	5	40	21.2	53		32.1
Sephadex G25	7	39	20.7	53		31.3
DEAE fraction	2	3.5	3.5	100	17.5	5.3

a DEAE-cellulose column. Proteins were eluted with a gradient of increasing salt concentration. The peak of phytoene desaturase was eluted quite early at about 25 mM NaCl. The purified phytoene desaturase in this fraction is homogenous, as judged by the single band at 57 kDa (Fig. 1, lane 7). The results of the purification procedure, shown in Table I, indicate that typically the process yielded 3.5 mg of purified protein with a 5.3% recovery, and that the specific activity of phytoene desaturase increased 218-fold from 0.08 to 17.5 μg carotenes·mg^{-1} protein·h^{-1}.

Substrate Specificity—The purified enzyme was used to study the substrate specificity of the reaction. Figure 2 shows the HPLC traces of the separated carotenes after the enzymatic reaction. In experiment A, phytoene (peaks 4 and 4′) was converted into phytofluene (peaks 3 and 3′) and ζ-carotene (peaks 2, 2′, and 2″) as intermediates, and neurosporene (peaks 1, 1′, and 1″) as the end product (Fig. 2). Two different isomers were found for phytoflene, and three for ζ-carotene and neurosporene. For all these carotenes the peak with the lowest retention time represents the all-*trans* isomer. All products were identified by co-chromatography with authentic standards of the corresponding all-*trans* isomers and from their absorbance maxima (333, 349, and 358 nm for phytofluene, 379, 398, and 422 nm for ζ-carotene, and 417, 441, and 470 nm for

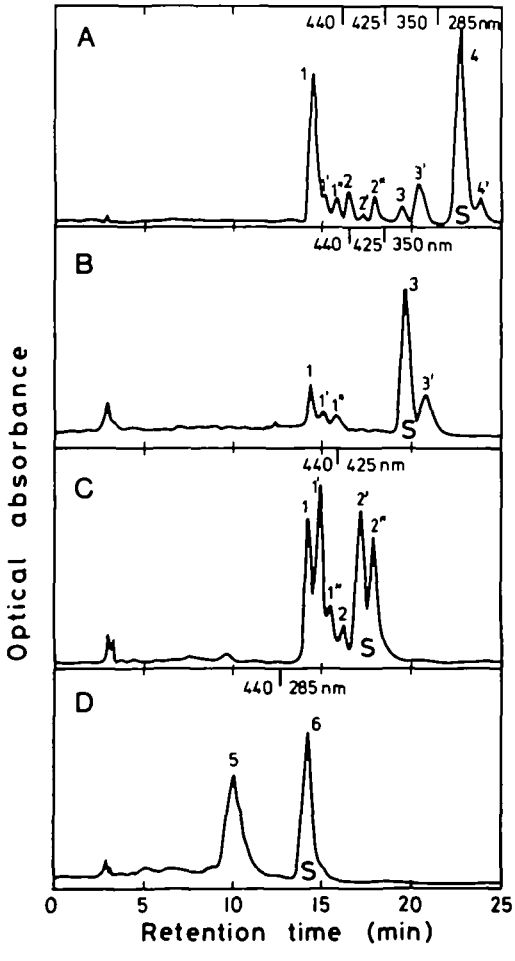

Fig. 2. **Conversion of different substrates by the purified *R. capsulatus* phytoene desaturase.** The substrate carotenes tested (marked by S) were: A, phytoene; B, phytofluene; C, ζ-carotene; and D, 1,2-epoxy phytoene. The reaction products shown in the HPLC diagram were identified as neuroporene (1), ζ-carotene (2), phytofluene (3), and epoxy neuroporene (5).

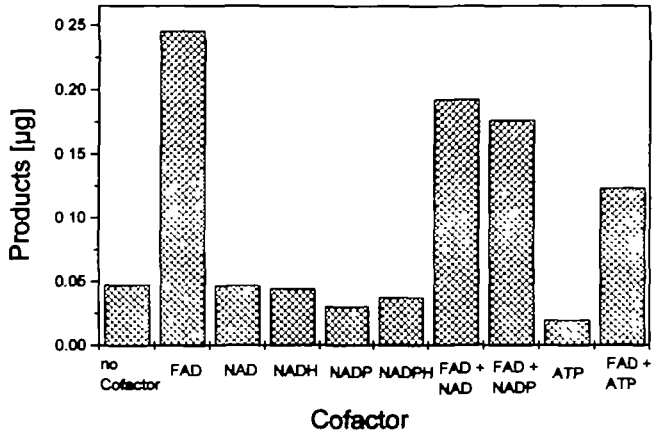

Fig. 3. **Cofactor requirement of the purified *R. capsulatus* phytoene desaturase.** Conversion of phytoene (2 μM) was determined in the presence of the indicated compounds (final concentration, 1 mM). Values are means of three determinations.

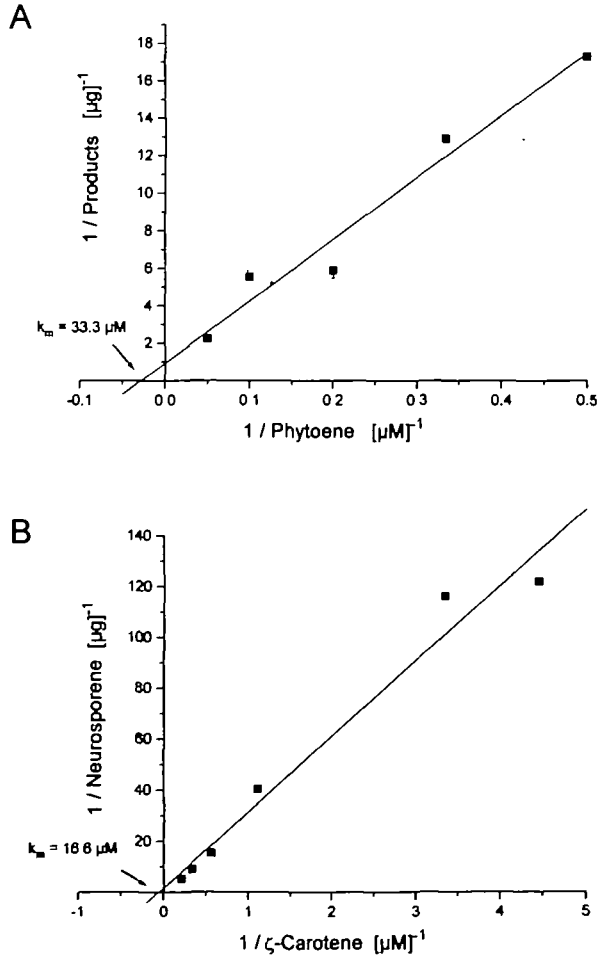

Fig. 4. **Lineweaver-Burk plots to determine the K_m values of the purified phytoene desaturase for phytoene (A) and ζ-carotene (B).** The concentration of the coenzyme FAD was 0.5 mM.

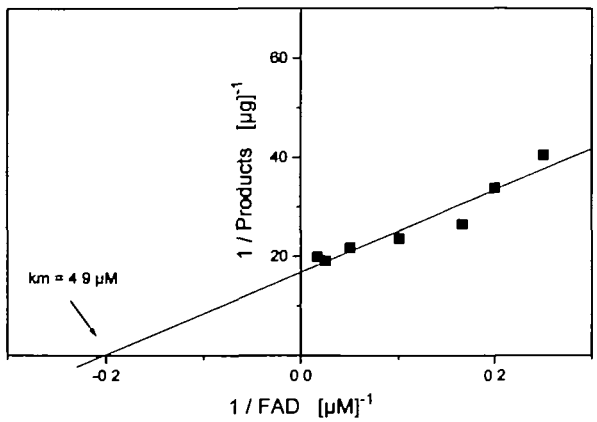

Fig. 5. **Lineweaver-Burk plot to determine the K_m value for the coenzyme, FAD.** The substrate in the reaction mixture was phytoene, at a concentration of 7 μM.

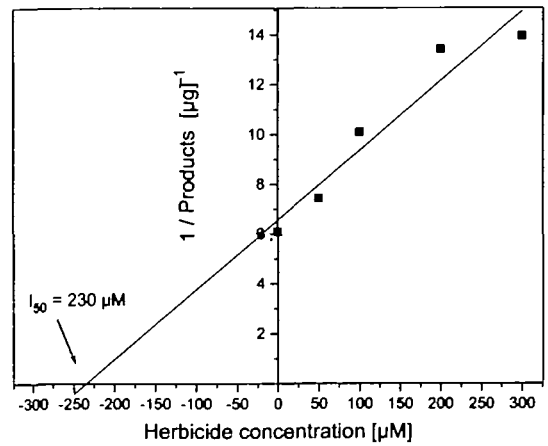

Fig. 6. **Dixon plot to determine the I_{50} value for inhibition of the phytoene desaturase from *Rhodobacter* by diphenylamine (DPA) with phytoene (7 μM) as substrate.**

neurosporene). The proportion of all-*trans versus cis* isomers was about 20% for phytofluene, 46% for ζ-carotene, and 72% for neurosporene. The chromatographic behavior of the ζ-carotene isomers is more in favor of symmetrical than asymmetrical assignment of the conjugated double bond system because none of the isomers co-chromatographed with 7,8,11,12-tetrahydrolycopene. Furthermore, the spectra of our products resembled that of symmetrical ζ-carotene rather than that of 7,8,11,12-tetrahydrolycopene, the latter exhibiting absorbance maxima at 375, 395, and 420 nm.

Both intermediates, phytofluene (trace B) and ζ-carotene (trace C), could also be used as substrates (indicated by S) to yield the subsequent reaction products. In addition, we investigated whether or not 1,2-epoxy phytoene (*16*) could also serve as a substrate for phytoene desaturase. Figure 2D shows that this phytoene epoxide (compound 6) is fully desaturated to the corresponding neurosporene derivative (compound 5). In addition, we also used as substrates the C30 diapophytoene, which was not converted, and the 9,9′-di-*cis* pro-ζ-carotene, which yielded the expected 7,9,9′-*polycis* pro-neurosporene (absorbance maxima at 405, 433, and 462 nm; data not shown).

Cofactor Studies and Enzyme Kinetics—Desaturation of carotenes occurs through a dehydrogenation reaction (*1*). Therefore, we carried out cofactor studies with oxidized dinucleotides and also in combination with ATP (Fig. 3). A low basal reaction was found in the absence of all coenzymes. However, upon the addition of FAD the activity was stimulated by a factor of five. When the combination of FAD with either NAD, NADP, or ATP were examined, the stimulation was less pronounced. All these nicotine dinucleotides, either reduced or oxidized, and also ATP had no stimulatory effect on the desaturase activity when applied alone.

Enzyme kinetic studies were carried out with either phytoene or ζ-carotene as a substrate for the desaturation reaction and also the cofactor, FAD. Figure 4 shows Lineweaver-Burk plots of reciprocal values of the applied phytoene (A) and ζ-carotene (B) concentrations *versus* the amounts of synthesized carotene products. Both plots gave straight lines (with correlation coefficients of >0.97), which are appropriate for determining the K_m value from the intersection with the abscissa. The K_m values obtained were 33.3 μM for phytoene and 16.6 μM for ζ-carotene. The corresponding V_{max} values related to the amount of enzyme in the assays were 0.169 nmol·h^{-1}·mg^{-1} (protein) and 0.086 nmol·h^{-1}·mg^{-1}, respectively. A similar experiment was carried out in which the FAD concentration was varied. The correlation coefficient of the double-reciprocal plot was 0.98 (Fig. 5). From the latter the K_m value for FAD was determined to be 4.9 μM and V_{max} related to the enzyme concentration to be 0.007 nmol·h^{-1}·mg^{-1}. Since diphenylamine (DPA) inhibits the formation of neurosporene in an *E. coli* strain transformed with a plasmid containing the *Rhodobacter* phytoene desaturase gene (*2*), we tested this inhibitor with the purified enzyme. Figure 6 shows a Dixon plot of various inhibitor concentrations against inverse product formation. The intersection with the abscissa (*21*) indicates an I_{50} value of 230 μM for the inhibition.

DISCUSSION

We have developed a successful strategy for a two-step purification of homogenous *R. capsulatus* phytoene desaturase expressed in *E. coli*. The apparent molecular weight of the enzyme, as determined by SDS polyacrylamide gel electrophoresis (57 kDa), is consistent with the value calculated from the gene sequence (58 kDa; Ref. *10*). Upon purification, the specific activity of the enzyme increased 218-fold (Table I). This high level of activity made it possible to employ a new *in vitro* test which, unlike the one previously used (*3*), avoids the use of radioactive substrates. The new assay is based on an earlier membrane-reconstitution technique involving *R. capsulatus* mutants (*22*). The defined soybean lipid environment used here allowed very efficient access to carotene substrates of the enzyme. Only with this assay procedure was it possible to study the potential of the purified phytoene desaturase to convert different carotene substrates. Enzymological studies with different carotene desaturases indicated a dehydrogenation mechanism in which protons and electrons are transferred to an appropriate acceptor (*1*). In case of the bacterial phytoene desaturase from *Erwinia*, FAD is involved as a coenzyme in the reaction (*5*), whereas the

phytoene desaturase from *Synechococcus* is dependent on either NAD or NADP (*6*). The results in Fig. 3 clearly show that only FAD participates in the reaction of the *R. capsulatus* phytoene desaturase. Contrary to the results obtained in an *in vitro* assay using a crude enzyme extract (*8*), we did not observe any inhibition of the reaction by FAD or stimulation by ATP. These differences could be due to interference by other enzymes in the crude enzyme system used by Lang *et al.* (*8*). For the desaturation of phytoene in *Narcissus* chromoplasts the requirement of a redox system has been emphasized (*23*). As our assay mixture contains only the purified phytoene desaturase together with the substrate carotene, and FAD as an essential cofactor, we can exclude the participation of further redox components in the case of the *Rhodobacter* enzyme.

In our studies involving different carotene substrates, phytoene was converted to the end product, neurosporene, with phytofluene and ζ-carotene as intermediates (including additional *cis* isomers), as previously shown *in vitro* (*3*). As similar *cis* isomers can also be found in *E. coli* cells in which the phytoene desaturase genes including the one from *Rhodobacter* are expressed (*11*), we assume that their formation is part of the catalytic mechanism. Although in some photosynthetic bacteria, like *R. rubrum* (*16*) and *Rhodopseudomonas globiformis* (*24*), the asymmetric ζ-carotene, 7,8,11,12-tetrahydrolycopene, was definitely identified, the ζ-carotene accumulating in a previous *in vitro* reaction closely resembled the symmetrical ζ-carotene by its spectrum (*22*). The same result was obtained in our study. It should be pointed out, that in addition to phytofluene, the symmetrical ζ-carotene was accepted by phytoene desaturase as a substrate (Fig. 2). The affinity for ζ-carotene, as judged from the K_m values for the two substrates, was more than twice as high as for phytoene (Fig. 4), perhaps explaining why the accumulation of ζ-carotene is not observed in wild type cells of *R. capsulatus* (*25*). Our first attempts to elucidate the structural requirements for a carotene, apart from the natural substrate or the intermediate, to interact with the *Rhodobacter* phytoene desaturase revealed that the 1,2-epoxy group has no affect on the acceptance of phytoene by the enzyme and that the truncated C30 diapophytoene, in which the central phytoene structure for the introduction of further double bonds is retained, cannot be converted. We conclude that the substrate must either resemble half the molecule of phytoene or both halves of the structure of ζ-carotene. For a carotene substrate of crtI type phytoene desaturases, the requirement of a minimum recognition site containing a double bond at one side and a dienoic group at the other side to the position where the new double bond is introduced was proposed (*1*). The lower K_m value, which means higher affinity for ζ-carotene *versus* phytoene, indicates that, in addition to the proposed model, an extended conjugation system next to the newly formed double bond has a strong influence on substrate binding. Furthermore, the occurrence of isomerization of the double bond from *trans* to *cis* seems not to be very crucial. This can be concluded from the desaturation reaction shown in Fig. 2C, where a ζ-carotene mixture of mainly two different *cis* isomers was used as the substrate, and the domination of *cis* neurosporene isomers as reaction products.

It is still a puzzle why, unlike the phytoene desaturase from *Erwinia* which can continue with the subsequent desaturation of neurosporene to lycopene, the catalytic function of the 3-step desaturase stops at the level of neurosporene. The method presented here may allow the purification of high amounts of active phytoene desaturase to obtain protein NMR and crystallographic data which will contribute to our understanding of the unique catalytic properties of this enzyme from *R. capsulatus*.

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