Substrate specificity of the expressed carotenoid 3,4-desaturase from *Rubrivivax gelatinosus* reveals the detailed reaction sequence to spheroidene and spirilloxanthin

Sabine STEIGER*, Chantal ASTIER† and Gerhard SANDMANN*¹

*Biosynthesis Group, Botanical Institute, J. W. Goethe Universität, P.O. Box 111930, D-60054 Frankfurt, Germany, and

†Centre de Génétique Moléculaire du CNRS (UPR 9061) associé à l'Université Pierre et Marie Curie, Bâtiment 24, Avenue de la Terrasse, 91198 Gif sur Yvette Cedex, France

Carotenoid biosynthesis in the photosynthetic bacterium *Rubrivivax gelatinosus* leads to the formation of hydroxyspheroidene and spirilloxanthin as the products of a branched pathway. In this study we investigated the role of the desaturase encoded by *crtD* which catalyses the introduction of C-3,4 double bonds into acyclic carotenoids. The desaturase was expressed in *Escherichia coli*, and the activity and the substrate specificity of the enzyme were evaluated *in vitro* by application of structurally different carotenoids. The results indicate that the enzyme is a 3,4-desaturase that converts 1-hydroxy carotenoids. The 3,4-desaturation reaction can only occur with mono-1-hydroxy caro-

tenoids at a ψ -end group or with 1,1'-dihydroxy derivatives carrying a 3',4'-double bond. In addition, 1-HO- ζ -carotene could also be converted by the desaturase. Enzyme kinetic studies showed a substrate preference of 1-HO-neurosporene over 1-HO-lycopene. Consequences from the biochemical data for the reaction sequence of hydroxyspheroidene and spirilloxanthin formation and the interconnection of both branches are discussed.

Key words: acyclic 1-hydroxy carotenoid 3,4-desaturase, *crtD* gene, enzyme kinetics, *in vitro* assay, spirilloxanthin biosynthesis.

INTRODUCTION

The purple photosynthetic bacteria belonging to the Rhodospirillaceae synthesize acyclic C₄₀ carotenoids with C-3,4 conjugated double bonds, which are modified by hydroxy or methoxy groups at position C-1 and/or C-1'. The initial part of all the pathways includes the synthesis and desaturation of phytoene [1]. In *Rhodobacter capsulatus* a single enzyme mediates the desaturation to neurosporene [2], which is then converted by subsequent hydration, desaturation and methylation reactions into hydroxyspheroidene under anaerobic conditions or into the ketocarotenoid spheroidenone in the presence of oxygen [3,4].

In *Rubrivivax gelatinosus* a second branch leads to the biosynthesis of the maximally desaturated carotenoid spirilloxanthin, in addition to the spheroidene pathway [5]. It is not clear whether spirilloxanthin formation in *R. gelatinosus* proceeds exclusively via lycopene and its 1- and 1'-hydroxy derivatives or also via spheroidene, which involves hydration of neurosporene. The route of spirilloxanthin biosynthesis has been established in different species of the Rhodospirillaceae, mainly in *Rhodospirillum rubrum* where spirilloxanthin is found as the major carotenoid, based on the identification of intermediates [3,6] and inhibitor studies [7,8]. The creation of mutant strains by gene inactivation resulting in the accumulation of intermediates achieved further progress [9,10]. It is generally accepted that lycopene is an important precursor. It is converted into spirilloxanthin by a number of consecutive reactions that operate first at one end of the symmetrical lycopene molecule and then at the other [7]. Lycopene was detected in small amounts as a precursor in deletion mutants of *R. gelatinosus* [9] as well as in the light-harvesting complexes of this bacterium [11]. Nevertheless, an alternative spirilloxanthin pathway which by-passes the formation of 1-HO-lycopene and proceeds via the neurosporene route to spheroidene, as proposed by Eimhjellen and Liaaen-

Jensen [6], may exist. In order to establish the precise reaction sequence leading to spirilloxanthin formation in *R. gelatinosus* enzymic studies are indispensable. Only the determination of the substrate specificities of the key enzymes involved in the *R. gelatinosus* spirilloxanthin branch using all putative intermediates will disclose the spirilloxanthin pathway. Several genes coding for the enzymes participating in the carotenoid biosynthesis pathways in the Rhodospirillaceae have been identified [9,12,13]. However, the characterization of the enzymes involved has only just started. Only two of the carotenogenic enzymes, a phytoene desaturase from *Rh. capsulatus* [14] and the 3,4-desaturase of *Rh. sphaeroides*, have been functionally characterized [15].

In this article we report the expression of the *R. gelatinosus crtD* gene in *Escherichia coli* to produce a functional enzyme. After production of putative substrate carotenoids by combination of different carotenogenic genes, detailed studies of the reactions involved in carotenoid biosynthesis were carried out. The definite reaction sequence of hydroxyspheroidene and spirilloxanthin formation in *R. gelatinosus* and the role of the 3,4-desaturase, *CrtD*, in this pathway could be established.

EXPERIMENTAL

Cultivation

Cells of wild-type *R. gelatinosus* and a mutant, SID2, lacking the *crtD* gene [9], were grown photoheterotrophically at 32 °C in a malate medium [16] for 4 days. *E. coli* JM101 cells containing plasmids with different combinations of carotenogenic genes were used for the production of marker carotenoids and various substrates for enzyme reactions. The plasmids are listed in Table 1, including the references where the constructions were described. Transformants were cultivated for 2 days at 28 °C in

¹ To whom correspondence should be addressed (e-mail sandmann@em.uni-frankfurt.de).

Table 1 Plasmid combinations in *E. coli* JM101 used for the production of substrate carotenoids

Abbreviations used: amp, ampicillin; chl, chloramphenicol; kan, kanamycin; tet, tetracycline. Assignment of genes: *crtE*, geranylgeranyl pyrophosphate synthase; *crtB*, phytoene synthase; *crtI_{R.c.}*, phytoene desaturase from *Rhodobacter capsulatus*; *crtI_{E.u.}*, phytoene desaturase from *Erwinia uredovora*; *crtC_{R.g.}*, carotene hydratase from *R. gelatinosus*; *crtC_{R.c.}*, carotene hydratase from *Rhodob. capsulatus*; *crtD_{R.g.}*, carotenoid 3,4-desaturase from *R. gelatinosus*; *al1*, phytoene desaturase from *Neurospora crassa*; *crtP*, phytoene desaturase; *crtY*, lycopene cyclase; *crtF*, 1-HO-carotenoid methylase.

Plasmid	Resistance	Gene	Carotenoid formed
pACcrtEBI _{R.c.} [24] + pRKcrtC _{R.c.} [15]	chl, tet	<i>crtE</i> , <i>crtB</i> , <i>crtI_{R.c.}</i> , <i>crtC_{R.g.}</i>	1-HO-Neurosporene, 1,1'-(HO) ₂ -neurosporene
pACcrtEBI _{E.u.} [24] + pRKcrtC _{R.c.}	chl, tet	<i>crtE</i> , <i>crtB</i> , <i>crtI_{E.u.}</i> , <i>crtC_{R.c.}</i>	1-HO-Lycopene, 1,1'-(HO) ₂ -lycopene
pACcrtEBI _{R.c.} + pRKcrtC _{R.c.} + pUCcrtF [15]	chl, tet, amp	<i>crtE</i> , <i>crtB</i> , <i>crtI_{R.c.}</i> , <i>crtC_{R.g.}</i> , <i>crtF</i>	1-(CH ₃ O)-Neurosporene
pACcrtEBI _{E.u.} + pSO50 [9]	chl, tet	<i>crtE</i> , <i>crtB</i> , <i>crtI_{E.u.}</i> , <i>crtC_{R.g.}</i> , <i>crtD_{R.g.}</i>	1,1'-(HO) ₂ -3,4-Didehydrolycopene
pACcrtEBal1 [15] + pRKcrtC _{R.c.} + pSO53	chl, tet, kan	<i>crtE</i> , <i>crtB</i> , <i>al1</i> , <i>crtC_{R.c.}</i> , <i>crtD_{R.g.}</i>	1'-HO-3,4-Didehydrolycopene
pACcrtEBP [24] + pRKcrtC _{R.c.}	chl, tet	<i>crtE</i> , <i>crtB</i> , <i>crtP</i> , <i>crtC_{R.c.}</i>	1-HO-ζ-Carotene, 1,1'-(HO) ₂ -ζ-carotene
pACCAR16Δcrtx [25] + pRKcrtC _{R.c.}	chl, tet	<i>crtE</i> , <i>crtB</i> , <i>crtI_{E.u.}</i> , <i>crtY</i> , <i>crtC_{R.c.}</i>	1-HO-γ-Carotene
PACcrtEBI _{R.c.} + pSO50 + pUCcrtF	chl, kan, amp	<i>crtE</i> , <i>crtB</i> , <i>crtI_{R.c.}</i> , <i>crtC_{R.g.}</i> , <i>crtD_{R.g.}</i> , <i>crtF</i>	1'-HO-Spheroidene
PACcrtEBI _{R.c.} + pSO50	chl, kan	<i>crtE</i> , <i>crtB</i> , <i>crtI_{R.c.}</i> , <i>crtC_{R.g.}</i> , <i>crtD_{R.g.}</i>	Demethylspheroidene, 1-HO-demethylspheroidene
PACcrtEBI _{R.c.} + pRKcrtC _{R.c.} + pSO53 + pUCcrtF	chl, tet, kan, amp	<i>crtE</i> , <i>crtB</i> , <i>crtI_{R.c.}</i> , <i>crtC_{R.c.}</i> , <i>crtD_{R.g.}</i> , <i>crtF</i>	1-CH ₃ O-1'-HO-3,4-Didehydrolycopene
PaCrtEBI _{E.u.} + pRKcrtC _{R.c.} + pSO50 + pUCcrtF	chl, tet, kan, amp	<i>crtE</i> , <i>crtB</i> , <i>crtI_{E.u.}</i> , <i>crtC_{R.g.}</i> , <i>crtD_{R.g.}</i> , <i>crtF</i>	1-CH ₃ O-3,4-Didehydrolycopene

Luria–Bertani broth [17] in the presence of chloramphenicol (35 µg/ml), ampicillin (100 µg/ml), kanamycin (25 µg/ml) or tetracycline (25 µg/ml) according to the plasmids used.

Protein expression and *in vitro* enzyme reaction

E. coli strain JM101 was the host for the plasmid pSO53, which carries the desaturase gene *crtD* from *R. gelatinosus*. The pSO53 plasmid was constructed from the pSO50 plasmid. The pSO50 plasmid contained a 3-kb *SacI* fragment encoding *crtD*–*C* genes under the control of the *lac* promoter [18]. The downstream fragment from the *Bam*HI site to the end was deleted to keep only the *crtD* gene. Cultures were grown in Luria–Bertani broth [17] containing kanamycin (25 µg/ml) at 37 °C until an attenuation (D_{600}) of 0.1 was reached. Protein expression was induced by addition of isopropyl β-D-galactoside to a final concentration of 0.4 mM and incubation at 28 °C overnight.

The cells were harvested by centrifugation at 6000 *g* for 15 min at 4 °C, resuspended 1:20 in 50 mM potassium phosphate buffer, pH 7.8, and broken by two passages through a French pressure cell at 95 MPa. The homogenate was incubated with DNase (10 µg/ml) on ice for 10 min. After centrifugation at 40000 *g* for 20 min at 4 °C the supernatant (500 µl; approx. 6 µg of desaturase) was used for enzyme assays *in vitro* in a final volume of 1 ml. Purified carotenoids (3 µg) were added in lipid suspension (100 µg of soya-bean lipid/ml of buffer). To determine the K_m values carotenoid concentrations from 2 to 50 µM were used. Assays were incubated for 18 h under shaking at 28 °C in the dark.

The amount of the desaturase was estimated after SDS/PAGE on 15 % gels [19] after staining the polypeptides with Coomassie Brilliant Blue. Total protein was determined using the method of Bradford [20].

Carotenoid extraction and HPLC analysis

Carotenoids were extracted from freeze-dried *R. gelatinosus* cells and *E. coli* transformants by addition of methanol containing 6% KOH and heating for 20 min at 60 °C. Extracts were partitioned against 10% ether in petrol. The upper phase was collected and evaporated. Separation of the carotenoids was carried out by chromatography on aluminium oxide as described previously [15]. If necessary they were purified further by HPLC.

The HPLC system used for purification and for separation of substrates from products after the enzymic reactions consisted of a Nucleosil C₁₈ 3-µm column (Macherey-Nagel, Düren, Germany) with acetonitrile/methanol/2-propanol (75:17.5:7.5, by vol.) as eluent. Carotenoids were identified and quantified with authentic standards, by their retention times and their absorbance spectra, which were recorded from the elution peaks using a Kontron diode-array detector model 440 [15].

RESULTS

Comparison of the carotenoid content (Figure 1) of the *R. gelatinosus* wild-type cells and the mutant cells lacking *crtD* give a first indication of the role of the desaturase in the carotenoid

Figure 1 HPLC separation of the carotenoids of *Rubrivivax gelatinosus* wild-type (WT) and the mutant lacking a functional *crtD* gene

Peaks correspond to: 1, 1'-HO-spheroidene; 2, spirilloxanthin; 3, 1-CH₃O-3,4-didehydrolycopene; 4, demethylspheroidene; 5, spheroidene; 6, 1,1'-(HO)₂-neurosporene; 7, 1-CH₃O-1'-HO-lycopene; 8, 1-CH₃O-1'-HO-neurosporene; 9, 1-HO-neurosporene; 10, 1-CH₃O-lycopene; 11, 1-CH₃O-neurosporene; 12, neurosporene.

Figure 2 Substrate specificity and catalytic reactions of the carotenoid 3,4-desaturase, CrtD

Percentage conversions of 3 μ g of the corresponding substrate with enzyme extracts containing 6 μ g of desaturase for 18 h are shown above the arrows. (A) Substrates with the highest conversion of 40–50% under the reactions indicated in the Experimental section. (B) Conversion of 20–40%. (C) Carotenoids that were not converted by CrtD.

Table 2 Kinetic parameters for the most efficient substrates of CrtD from *R. gelatinosus*

K_m and V_{max} values are the means of at least three determinations obtained from double-reciprocal plots of the reaction rates versus the substrate concentrations with 5–8 data points and a correlation coefficient for linearity of > 0.98 . The S.D. values for both parameters were in the range of 14–24% of the mean values.

Substrate	K_m (μM)	V_{max} (nmol $\cdot$ mg of protein $^{-1} \cdot$ h $^{-1}$)	V_{max}/K_m ($\times 10^{-2}$)
1-HO-Neurosporene	25	1.35	5.4
1,1'-(HO) $_2$ -Neurosporene	19	0.58	3.0
1-HO-Lycopene	40	0.69	1.7

pathway. The major carotenoids in the wild-type *R. gelatinosus* grown under anaerobic conditions were spheroidene (Figure 1, peak 5) and 1'-HO-spheroidene (peak 1), with absorption maxima at 430, 455 and 470 nm. Another product was spirilloxanthin (peak 2), a carotenoid with absorption maxima at 470, 495 and 515 nm. The intermediates were identified as 1-CH $_3$ O-3,4-didehydrolycopene (peak 3) and demethylspheroidene (peak 4). The *R. gelatinosus* mutant showed a different carotenoid pattern. The neurosporene-related carotenoids 1-HO-neurosporene (peak 9), 1,1'-(HO) $_2$ -neurosporene (peak 6), 1-CH $_3$ O-1'-HO-neurosporene (peak 8) and 1-CH $_3$ O-neurosporene (peak 11), with their typical absorption spectra of 410, 440 and 470 nm, were accumulated. In addition the lycopene derivatives 1-CH $_3$ O-1'-HO-lycopene (peak 7) and 1-CH $_3$ O-lycopene (peak 10), with typical absorption maxima of 440, 470 and 490 nm, were enriched.

Determination of the specificity of the carotenoid 3,4-desaturase CrtD for a range of substrates that may be alternatively involved as intermediates is the ultimate procedure for the study of the formation of spirilloxanthin in *R. gelatinosus*. For enzymic investigations, the functional expression of the 3,4-desaturase was optimized and monitored by SDS/PAGE. The yield of this protein was 3–5% of the total *E. coli* protein. After disruption of the *E. coli* cells under high pressure and centrifugation, the protein was enriched in the supernatant, and could be used without additional solubilization for enzymic characterization.

Structurally different carotenoids, which can all be considered putative substrates and which may play a possible role as intermediates in the carotenoid pathway of *R. gelatinosus*, were used for characterization of the enzyme *in vitro*. Figure 2 shows the carotenoid substrate specificity for 1-hydroxy derivatives and the catalytic reactions of the 3,4-desaturase. Among the carotenoids tested, 1-HO-neurosporene, 1-HO-lycopene and 1,1'-(HO) $_2$ -neurosporene were the most efficient substrates for the 3,4-desaturase. Under our conditions, 47, 33 and 39%, respectively, of these substrate carotenoids were converted into demethylspheroidene, 1-HO-3,4-didehydrolycopene and 1'-HO-demethylspheroidene.

As 1-HO-neurosporene, 1-HO-lycopene and 1,1'-(HO) $_2$ -neurosporene are putative branching products for the carotenoid pathway in *R. gelatinosus*, enzyme kinetic studies were performed using these substrates. K_m values were determined to be 25 μM for 1-HO-neurosporene, 40 μM for 1-HO-lycopene and 19 μM for 1,1'-(HO) $_2$ -neurosporene. The corresponding V_{max} values were 1.35 nmol $\cdot$ h $^{-1} \cdot$ mg $^{-1}$ for 1-HO-neurosporene, 0.69 nmol $\cdot$ h $^{-1} \cdot$ mg $^{-1}$ for 1-HO-lycopene and 0.58 nmol $\cdot$ h $^{-1} \cdot$ mg $^{-1}$ for 1,1'-(HO) $_2$ -neurosporene, respectively (Table 2).

To determine the structural characteristics recognized by the desaturase, different carotenoids were used in the assay *in vitro*.

Not only were the 3,4-didehydrolycopene derivatives 1,1'-(HO) $_2$ -3,4-didehydrolycopene, 1-CH $_3$ O-1'-HO-3,4-didehydrolycopene and 1'-HO-3,4-didehydrolycopene converted by 38, 37 and 28%, respectively, by the enzyme but also were 1-HO- ξ -carotene and 1-HO- γ -carotene, by 15% each. In contrast, the dihydroxy derivatives 1,1'-(HO) $_2$ -lycopene and 1,1'-(HO) $_2$ - ξ -carotene and the neurosporene derivatives 1-CH $_3$ O-neurosporene, 1'-HO-demethylspheroidene and demethylspheroidene were not recognized as substrates by the 3,4-desaturase. For each enzyme preparation 1-HO-neurosporene was used as a positive control to ensure enzyme activity in the range of 42–48% conversion.

DISCUSSION

Substantial progress to elucidate the individual steps of carotenoid biosynthetic pathways can be made by the development of enzyme assays *in vitro* [21]. We followed this strategy for the carotenoid 3,4-desaturase CrtD from *R. gelatinosus*. Thus it was possible to express from the encoding gene a soluble form of this protein which due to its high activity was suitable for enzyme kinetic studies. The characterization of the enzymic properties of CrtD showed that this desaturase is able to convert structurally different substrates participating simultaneously in the biosynthesis of hydroxyspheroidene as well as of spirilloxanthin. Furthermore, deletions of a single *crtD* gene in *R. gelatinosus* resulted in the accumulation of intermediates of both branches, all lacking the 3,4-double bond (Figure 1).

The results of Figure 2 indicate that generally a hydroxy group at position C-1 is required for the 3,4-desaturation reaction. But other structural features of the substrate molecule also determine acceptance by the enzyme. Substrate molecules derived from 1-HO-lycopene are converted, but only if no additional hydroxy group is located on the other end of the molecule in position C-1'. Thus, 1-HO-lycopene is converted into 1-HO-3,4-didehydrolycopene as well as 1-HO- γ -carotene, and 1'-HO-3,4-didehydrolycopene into 1'-HO-torulene and 1'-HO-tetra-didehydrolycopene, whereas 1,1'-(HO) $_2$ -lycopene is not a substrate. However, conversion of dihydroxylycopene derivatives occurs only if one of the hydroxy or corresponding methoxy groups is accompanied by an additional double bond in position C-3,4, as in 1,1'-(HO) $_2$ -3,4-didehydrolycopene or 1-CH $_3$ O-1'-HO-3,4-didehydrolycopene. Unlike lycopene derivatives, 1-HO-neurosporenes are desaturated at position C-3,4 regardless of the presence or absence of a hydroxy group at the C-1' end of the molecule. However, desaturation occurs only at the half of the molecule that resembles lycopene, as shown by the conversion of 1-HO-neurosporene into demethylspheroidene, and of 1,1'-(HO) $_2$ -neurosporene into 1-HO-demethylspheroidene. When the 1-hydroxy group is replaced by a methoxy group, 3,4-desaturation is inhibited. Thus the C-3,4 desaturase from *R. gelatinosus* is functional as a 1-hydroxy carotenoid desaturase but not as a 1-methoxy carotenoid desaturase, as shown previously for the 1-HO-neurosporene desaturase from *Rh. sphaeroides* [15].

Taking the results presented in Figure 2 the individual steps of the reaction sequence to hydroxyspheroidene and also to spirilloxanthin in *R. gelatinosus* are outlined in Scheme 1. The general reaction sequence involving CrtD is hydration of the isopropylidene end group of lycopene or neurosporene, followed by a dehydrogenation to introduce a C-3,4 double bond at the hydroxylated end and a final methylation of the hydroxy group. In case of hydroxyspheroidene synthesis the desaturation of hydroxyneurosporene leads to demethylspheroidene, followed by the methylation of the hydroxy group to spheroidene and addition of water to the double bond at C-1',2'. The results

Scheme 1 Individual reactions involved in the biosynthesis of 1'-hydroxyspheroidene and spirilloxanthin in *R. gelatinosus*

exclude that the desaturation step occurs before the transfer of the methyl group. This finding is in contrast with the postulation by Davies [7] for *Rh. sphaeroides* that in spheroidene biosynthesis 3,4-dihydrospheroidene is an intermediate rather than demethylspheroidene, but confirms the pathway established by Liaaen-Jensen et al. [3].

The initial reaction for the biosynthesis of spirilloxanthin is the formation of 1-HO-lycopene. This direct route proceeds via 1-HO-didehydrolycopene in *R. gelatinosus* (Scheme 1). From 1-HO-didehydrolycopene onwards, the pathway branches by either formation of the 1'-hydroxy group or methylation of the 1-hydroxy group, yielding 1,1'-(HO)₂-didehydrolycopene or 1-CH₃O-3,4-didehydrolycopene, respectively. After establishment of the 1'-hydroxy group of the latter carotenoid, they are further desaturated by CrtD and metabolized to spirilloxanthin by methylation of the hydroxy group. Obviously, the generalization [7] that one side of the molecule should be processed completely before the other side is substituted is not absolutely strict for the

spirilloxanthin pathway in *R. gelatinosus* and can be modified slightly in such a way that, after one 3,4-desaturation step, introduction of the second hydroxy group at C-1' may occur before the hydroxy group at C-1 is methylated. This reaction sequence can also be concluded for spirilloxanthin synthesis in *Rhodosp. rubrum* because 3,4,3',4'-tetrahydrosirilloxanthin was detected in a mutant with an inactivated *crtD* gene [10].

The formation of 1,1'-(HO)₂-lycopene, which is catalysed by the *crtC*-encoded hydratase from *R. gelatinosus* (S. Steiger and G. Sandmann, unpublished work), leads to a dead end of the spirilloxanthin biosynthetic branch. One of the expected hydroxy and methoxy products, 1-CH₃O-1'-HO-lycopene was found only in the mutant with the deleted *crtD* gene (Figure 1).

Our investigation gave no evidence for an additional reaction sequence to spirilloxanthin via the spheroidene branch. 1'-HO-Demethylspheroidene, demethylspheroidene and 1'-HO-spheroidene were not converted by CrtD. However, for a final conclusion that both branches operate independently, one has to

wait for the availability of the *crtI* gene from *R. gelatinosus* and use it to evaluate whether the corresponding enzyme is able to desaturate the 7',8' position of 1'-HO-spheroidene.

When the neurosporene-derived spheroidene pathway of *R. gelatinosus* is compared with the lycopene-derived spirilloxanthin branch, it is obvious that the decisive difference is at the level of the phytoene desaturation. In addition to CrtI, the subsequent enzymes of the pathway have also adapted to simultaneous spheroidene or spirilloxanthin synthesis. This is the case for CrtD from *R. gelatinosus* but enzyme kinetic studies revealed a higher affinity for 1-HO-neurosporene than for 1-HO-lycopene (Table 2). Furthermore, $V_{\max}/K_m$ ratios were determined for different substrates. These specificity constants indicate the substrate preference of an enzyme [22,23]. The > 3-fold-higher value for 1-HO-neurosporene over 1-HO-lycopene demonstrates that this substrate is much better converted by CrtD.

The present investigation provided a better understanding of the existing and possible enzymic reactions involved in the hydroxyspheroidene and spirilloxanthin pathways of *R. gelatinosus* and of the separation of both metabolic branches. When other enzymes from this bacterium, especially its phytoene desaturase CrtI and hydratase CrtC, are available and accessible to enzymic studies, the detailed overall picture of hydroxyspheroidene and spirilloxanthin synthesis will be completed.

This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

- Goodwin, T. W. (1980) The Biochemistry of the Carotenoids, vol. 1, Chapman and Hall, London
- Linden, H., Misawa, N., Chamovitz, D., Pecker, I., Hirschberg, J. and Sandmann, G. (1991) Functional complementation in *Escherichia coli* of different phytoene desaturase genes and analysis of accumulated carotenes. *Z. Naturforsch.* **46c**, 1045–1051
- Liaaen-Jensen, S., Cohen-Bazire, G. and Stanier, R. Y. (1961) Biosynthesis of carotenoids in purple bacteria: a re-evaluation based on consideration of chemical structure. *Nature (London)* **192**, 1168–1172
- Scolnik, P., Walker, A. M. A. and Marrs, B. L. (1980) Biosynthesis of carotenoid derivatives derived from neurosporene in *Rhodospseudomonas capsulata*. *J. Biol. Chem.* **255**, 2427–2432
- Schmidt, K. (1978) Biosynthesis of carotenoids. In *The Photosynthetic Bacteria* (Clayton, R. K. and Sistrom, W. R., eds), pp. 729–750, Plenum Press, New York
- Eimhjellen, K. E. and Liaaen-Jensen, S. (1964) The biosynthesis of carotenoids in *Pseudomonas gelatinosa*. *Biochim. Biophys. Acta* **82**, 21–40
- Davies, B. H. (1970) Alternative pathways of spirilloxanthin biosynthesis in *Rhodospirillum rubrum*. *Biochem. J.* **116**, 101–110
- Singh, R. K., Ben-Aziz, A., Britton, G. and Goodwin, T. W. (1973) Biosynthesis of spheroidene and hydroxyspheroidene in *Rhodospseudomonas* species: experiments with nicotine as inhibitor. *Biochem. J.* **132**, 649–652
- Ouchane, S., Picaud, M., Verotte, C., Reiss-Husson, F. and Astier, C. (1997) Pleiotropic effects of *puf* interposon mutagenesis on carotenoid biosynthesis in *Rubrivivax gelatinosus*. *J. Biol. Chem.* **272**, 1670–1676
- Komori, M., Ghosh, R., Takaichi, S., Hu, Y., Mizoguchi, T., Koyama, Y. and Kuki, M. (1998) A null lesion in the rhodopin 3,4-desaturase of *Rhodospirillum rubrum* unmarks a cryptic branch of the carotenoid biosynthetic pathway. *Biochemistry* **37**, 8987–8994
- Takaichi, S. and Shimada, K. (1999) Pigment composition of two pigment-protein complexes derived from anaerobically and semi-aerobically grown *Rubrivivax gelatinosus*, and identification of a new keto-carotenoid. *Plant Cell Physiol.* **40**, 613–617
- Armstrong, G. A. (1994) Eubacteria show their true colors: genetics of carotenoid pigment biosynthesis from microbes to plants. *J. Bacteriol.* **176**, 4795–4802
- Gari, E., Toledo, J. C., Gibert, I. and Barbe, J. (1996) Nucleotide sequence of the methoxyneurosporene dehydrogenase gene from *Rhodobacter sphaeroides*: comparison with other bacterial carotenoid dehydrogenases. *FEMS Microbiol. Lett.* **93**, 103–108
- Raisig, A., Bartley, G. P., Scolnik, P. and Sandmann, G. (1996) Purification in an active state and properties of the 3-step phytoene desaturase from *Rhodobacter capsulatus* overexpressed in *Escherichia coli*. *J. Biochem.* **119**, 559–564
- Albrecht, M., Ruther, A. and Sandmann, G. (1997) Purification and biochemical characterization of a hydroxynurosporene desaturase in the biosynthetic pathway of the carotenoid spheroidene in *Rhodobacter sphaeroides*. *J. Bacteriol.* **179**, 7462–7467
- Agalidis, I., Rivas, E. and Reiss-Husson, F. (1990) Reaction center light harvesting B875 complexes from *Rhodocyclus gelatinosus*: characterization and identification of quinones. *Photosynth. Res.* **23**, 249–255
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor
- Ouchane, S., Picaud, M., Verotte, C. and Astier, C. (1997) Photooxidative stress stimulates illegitimate recombination and mutability in carotenoid-less mutants of *Rubrivivax gelatinosus*. *EMBO J.* **16**, 4777–4787
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- Sandmann, G. (1997) High level expression of carotenogenic genes for enzyme purification and biochemical characterization. *Pure Appl. Chem.* **69**, 2163–2168
- Wu, W., Lui, Y., Riedel, B., Wissing, J., Fisch, A. and Carman, G. M. (1996) Purification and characterization of diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 1869–1876
- Ferri, S. R. and Toguri, T. (1997) Substrate specificity modification of the stromal glycerol-3-phosphate acyltransferase. *Arch. Biochem. Biophys.* **337**, 202–208
- Linden, H., Misawa, N., Saito, T. and Sandmann, G. (1994) A novel carotenoid biosynthesis gene coding for ζ -carotene desaturase: functional expression, sequence and phylogenetic origin. *Plant Mol. Biol.* **24**, 369–379
- Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Daito, T., Ohtani, T. and Miki, W. (1995) Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *J. Bacteriol.* **177**, 6575–6584

Received 11 January 2000/12 April 2000; accepted 11 May 2000