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Purification and Biochemical Characterization of a Hydroxyneurosporene Desaturase Involved in the Biosynthetic Pathway of the Carotenoid Spheroidene in *Rhodobacter sphaeroides*

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Hydroxyneurosporene desaturase is involved in the carotenoid biosynthetic pathway of *Rhodobacter* species. The gene encoding this enzyme was expressed in *Escherichia coli*, purified, and biochemically characterized. The resulting protein contained an N-terminal six-histidine extension which derived from the cloning vector; this allowed for a one-step purification of the enzyme to homogeneity after solubilization with Nonidet P-40. The hydrogen acceptor in the C-3,4 desaturation reaction was molecular oxygen. NAD^+, $NADP^+$, and flavin adenine dinucleotide had no influence on enzymatic activity. Different acyclic 1-hydroxycarotenoids were tested as substrates. Very good conversion was achieved with 1-hydroxyneurosporene and 1-hydroxylycopene, whereas 1-hydroxy-γ-carotene and 1,1′-dihydroxylycopene were much less effective. From 1′-hydroxy-3,4-didehydrolycopene only trace amounts of product were obtained, and 1-methoxyneurosporene was not converted by purified hydroxyneurosporene desaturase. A K_m of 13.4 μM was determined for 1-hydroxyneurosporene.

Carotenoids are essential for the growth of organisms carrying out oxygenic or anoxygenic photosynthesis (12). The carotenoids found in photoautotrophic bacteria are either cyclic (e.g., in cyanobacteria) or acyclic C_{40} derivatives. The latter structures are typical for *Rhodospirillaceae* (10). In the genus *Rhodobacter*, the end products of the carotenoid biosynthetic pathway are spheroidene and spheroidenone. The synthesis of carotenoids is transcriptionally regulated in *Rhodobacter* species by molecular oxygen (11). Two genes in the biosynthetic pathway, *crtB* and *crtI*, are known to form an oxygen-inducible transcriptional operon (14). The biochemical pathway leading to these carotenoids proceeds via the synthesis of phytoene, its subsequent three-step desaturation to neurosporene, and addition of water to the peripheric C-1,2 double bond. The following steps to spheroidene may proceed alternatively. Either an additional double bond is introduced at position C-3,4 before the hydroxy group is methylated, or O-methylation occurs first and is followed by the C-3,4 desaturation step. Both alternatives have been proposed (2, 22, 27), but the reaction sequence has not been determined using purified enzyme.

The first carotenogenic genes with defined functions were cloned as a gene cluster from *Rhodobacter capsulatus* (2). The gene cluster included two different but structurally related desaturase genes, *crtI* and *crtD*, encoding a phytoene desaturase and hydroxyneurosporene desaturase (HND), respectively. A homologous *crtD* gene was also cloned from *Rhodobacter sphaeroides* (9). The gene for phytoene desaturase was recently expressed in *Escherichia coli*; the enzyme was purified to homogeneity, and its biochemical properties were determined (20). The results showed that a single enzyme catalyzes the three desaturation steps from phytoene to neurosporene. Other enzymes from the carotenoid biosynthetic pathway of *Rhodobacter* species have not yet been characterized with respect to properties and reaction mechanism. Especially for the second desaturase, HND, in the pathway to spheroidene, a biochemical characterization similar to that performed on *crtI* would be very helpful for assigning the precise position of the enzyme in the biosynthetic pathway and for comparing properties with those of the structurally related phytoene desaturase.

In the present work, the *crtD* gene from *R. sphaeroides* was expressed at a high level in *E. coli*, the enzyme was purified to homogeneity, and the reaction catalyzed by this enzyme was charaterized. The cofactor requirements, enzyme kinetic measurements, and substrate specificity of the purified enzyme were determined in the current study. The results indicate that this HND functions in *Rhodobacter* cells as a hydroxyneurosporene desaturase rather than as a methoxyneurosporene desaturase.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. *E. coli* M15 harboring plasmid pREP4 (Qiagen) was the host for the pQEcrtD construct, which contains the HND gene, *crtD*, from *R. sphaeroides* (9), under the control of the *lacZ* promoter, and an N-terminal six-histidine sequence used for affinity purification. The pQEcrtD-overexpressing plasmid was constructed by digestion of pUA144 (8) with the restriction enzyme *Sma*I and blunt-end ligation of the resulting 1.8-kb DNA fragment bearing the *crtD* gene into *Sma*I-digested and dephosphorylated vector pQE30 (Qiagen). For protein overexpression, the culture was cultivated at 37°C in Luria-Bertani broth in the presence of ampicillin (100 μg/ml) and kanamycin (25 μg/ml) until it reached an optical density at 600 nm of 0.6. Then, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the culture was grown overnight at 28°C. For carotenoid production, *E. coli* JM101 was used as a host for the plasmids listed in Table 1. These transformants were cultivated for 2 days at 28°C in Luria-Bertani broth in the presence of ampicillin (100 μg/ml), chloramphenicol (35 μg/ml), and/or tetracycline (25 μg/ml) depending on the plasmids used.

The construction of plasmids pACCRT-EBI_{Eu} (which mediates the formation of lycopene), pACCRT-EBI_{Rc} (used in the synthesis of neurosporene), and pACCRT16ΔcrtX (used in the synthesis of β-carotene) has been described previously (16, 18, 26). Plasmid pRKcrtC, containing the gene for neurosporene hydratase, was constructed by digestion of pFL104 (2) with *Nco*I. The resulting 1.11-kb DNA fragment carrying the *crtC* gene was treated with Klenow enzyme and subsequently blunt-end ligated into the *Bam*HI-digested, Klenow fragment-

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TABLE 1. Plasmids used for transformation of *E. coli* JM101 for synthesis of various substrate carotenoids

Carotenoid(s) produced	Plasmid(s)
Neurosporene	pACCRT-EBI$_{Rc}$
1-HO-neurosporene	pACCRT-EBI$_{Rc}$ + pRKcrtC
1-HO- and 1,1′-(HO)$_2$-lycopene	pACCRT-EBI$_{Eu}$ + pRKcrtC
1′-HO-3,4-didehydrolycopene	pACCRT-EBal1 + pRKcrtC
1′-HO-γ-carotene	pACCRT16ΔcrtX + pRKcrtC
1-Methoxyneurosporene	pACCRT-EBI$_{Rc}$ + pRKcrtC + pUCcrtF

treated, and dephosphorylated vector pRK404 (6). The *crtF* gene for hydroxyneurosporene methylase was amplified by PCR using the plasmid pFL227 (2) as a template, 5′GAGAGGGGATCCTCATGCCG3′ as the start primer, and 5′GCGACAGACTGCAGTGCGAC3′ as the end primer. Start and end primers were modified such that in each of them a new restriction site, *Kpn*I or *Pst*I, respectively, was created and the GTG start codon was replaced by an ATG codon. The amplified PCR fragment was digested with *Kpn*I and *Pst*I and ligated into the *Kpn*I- and *Pst*I-digested pUC18 vector (29), resulting in the plasmid pUCcrtF. The *crtC* and *crtF* genes were both from *R. capsulatus* (2), and they code for the neurosporene hydroxylase and the corresponding hydroxycarotene methylase, respectively. For the construction of pACCRT-EBal1, which mediates the formation of 3,4-dehydrolycopene, the *al1* cDNA sequence (24) was truncated, losing its first three codons, and subcloned into the pUC18 vector. Subsequently, this construct was digested with *Pvu*II and the resulting fragment carrying the *al1* gene with the *lacZ* promoter was ligated into the *Bam*HI-digested, Klenow fragment-treated, and dephosphorylated plasmid pACCRT-EB (15). The recombinant DNA techniques were performed by standard methods (21) or as instructed by the suppliers.

Enzyme purification. The overnight-induced cells (*E. coli* M15/pREP4/pQEcrtD) were harvested by centrifugation at 6,000 × *g* for 15 min at 4°C. The pelleted cells were resuspended in 50 mM Tris-HCl buffer, pH 8.0, and broken by two passages through a French pressure cell at 31 MPa. The resulting homogenate was incubated with DNase (10 μg/ml) for 15 min on ice to reduce the viscosity of the suspension. After centrifugation of the treated homogenate for 15 min at 12,000 × *g*, the pellet was resuspended in 50 mM Tris-HCl, pH 8.0, containing 0.1% Nonidet P-40 and stirred on ice for 1 h. Then, the suspension was centrifuged at 48,000 × *g* for 1 h at 4°C. To the resulting supernatant 1 ml of nickel nitrilotriacetic acid (Ni-NTA; Qiagen) was added. After incubation for 30 min on ice the entire mixture was packed in a column and washed with 10 ml of washing buffer (50 mM Tris-HCl, pH 8.0; 0.1% Nonidet P-40; 10 mM imidazole) to remove unspecifically bound proteins. After stepwise increases in the imidazole concentration, the homogeneous protein was eluted with 2 ml of the same buffer containing 100 mM imidazole. This purified protein was used for in vitro characterization of HND. The purification process was monitored by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 12.5% gels (13). Proteins were stained with Coomassie brilliant blue and scanned with a flatbed scanner, and the relative amount of HND to total protein content was estimated by using densitometric software (20). Protein concentrations were determined by the method of Bradford (3).

In vitro enzyme reaction. The assay mixture (final volume, 0.5 ml) contained 100 μl of the protein fraction (routinely with 3 to 6 μg of purified enzyme) and 2 to 3 μg of the purified carotenoid substrate in 50 mM Tris-HCl buffer, pH 8.0, with 0.1% Nonidet P-40. When the effects of anaerobic conditions were investigated, such conditions were established by adding glucose (2 mM), glucose oxidase (20 U/ml), and catalase (20,000 U/ml) before the reaction vessels were tightly sealed. Incubations were routinely carried out with 2.5 μg of purified HND protein in the dark at 30°C for 4 h, which is within the linear time range. The assays were terminated by adding methanol (2.5 ml). The remaining substrate and the product formed were extracted from the incubation mixture with diethyl ether-petroleum ether (boiling point [bp], 35 to 80°C) (1:9, vol/vol). Analysis of the products was performed by high-performance liquid chromatography (HPLC) on a Nucleosil C_{18} column with a particle diameter of 3 μm eluted isocratically with either eluent A (acetonitrile-methanol-water, 50:40:10 [vol/vol/vol]), eluent B (acetonitrile-methanol-water, 50:44:6 [vol/vol/vol], or eluent C (acetonitrile-methanol-water, 50:48:2 [vol/vol/vol]) at a flow rate of 1 ml/min. The separated carotenoids were detected with a Kontron 440 diode array detector, and spectra were directly recorded on-line. Reaction products were identified by their spectra and by cochromatography with reference compounds which were identified by mass spectrometry. Quantitation was carried out with reference carotenoids, using the extinction coefficients of carotenes with identical chromophores (5).

Isolation of substrate carotenoids. The carotenoids (carotenes and hydroxylated carotenes) were extracted from freeze-dried cells of the *E. coli* transformants listed in Table 1 by adding either methanol containing 6% KOH or acetone (in the case of lycopene-producing transformants) and heating for 20 min at 60°C. The different extracts were partitioned against diethyl ether-petroleum ether (bp, 35 to 60°C) (1:9, vol/vol), and the ether phases were evaporated under a stream of nitrogen. For further purification, the carotenoids were processed as follows: they were resuspended in petroleum ether; loaded on alumina columns of either grade II, III, or IV, depending on the carotenoids to be separated; and eluted with diethyl ether-petroleum ether (bp, 35 to 60°C) mixtures of increasing polarity (4). The purity of the substrate carotenoids was controlled by HPLC in the systems described above.

RESULTS

For the first time, attempts were made to express an HND so that this enzyme could be purified. The pQE30 vector used for integration of the *crtD* gene is responsible for an N-terminal sequence extension encoding an additional six histidine molecules of the expressed polypeptide; this facilitates affinity purification. After IPTG induction, HND, at 58 kDa, was the prominent protein band on SDS-polyacrylamide gels for transformants carrying this expression plasmid (Fig. 1, lane 3). The yield of HND was more than 6% of the total *E. coli* protein (see Table 3). This protein was absent in a transformant lacking the *crtD* gene (Fig. 1, lane 2). Because the solubilization of membrane-bound carotenogenic proteins in an active state is very crucial, a wide range of detergents were tested for their ability to solubilize HND without causing the loss of enzyme activity (Table 2). Eight different detergents were tried, and with five of them an active HND-containing solubilisate was obtained. 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) and Nonidet P-40 gave the best yields of soluble protein (Table 2). Since the HND activity per milliliter of solubilisate was highest for Nonidet P-40, it was used in the subsequent purification steps. After centrifugation, the Nonidet P-40-solubilized fraction contained more of the HND protein than the supernatant did (Fig. 1, lanes 5 and 4, respectively). About 17% of the total HND was recovered in this solubilisate, and a greater than twofold enrichment was achieved (Table 3). The solubilisate was treated with Ni-NTA, and most of the HND was adsorbed (Fig. 1, lane 6). After unspecifically bound proteins were washed off, HND was consecutively eluted with 50 and 100 mM imidazole (lanes 8 and 9). In the 100 mM imidazole fraction, a homogeneous protein was ob-

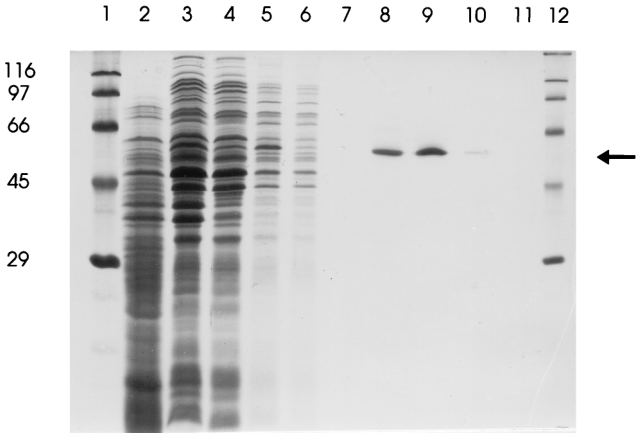

FIG. 1. SDS-polyacrylamide gel electrophoresis of fractions obtained during purification of hydroxyneurosporene desaturase. Lanes: 1 and 12, molecular weight markers (5 and 2 μg per protein band, respectively); 2, transformant without the *crtD* gene (about 500 μg of total protein); 3, homogenate from transformant M15/pREP4/pQE30crtD induced with 0.5 mM IPTG (about 500 μg of total protein); 4, supernatant after centrifugation; 5, solubilisate after treatment with 0.1% Nonidet P-40 from 1 mg of total protein; 6, solubilisate passed through an Ni-NTA column; 7, eluate without imidazole; 8, eluate with 50 mM imidazole; 9, eluate with 100 mM imidazole; 10, eluate with 200 mM imidazole; 11, eluate with 400 mM imidazole. Equal volumes of fractions were applied in lanes 7 to 11, corresponding to 5 μg of purified enzyme in lane 9.

TABLE 2. Solubilization by detergents (at 0.5%) of HND in an active state from *E. coli* membranes[a]

Detergent[b]	Solubilized protein (μg/ml)	Enzyme activity (ng of DMS/ml · h)[c]
Triton X-100	110	0.16
CHAPS	390	0.30
Dodecyl maltoside	200	0.28
SB10	45	0.17
Nonidet P-40	220	0.41

[a] The total concentration of HND in the membrane suspension was adjusted to 1 mg/ml.
[b] Other detergents tested but determined to be unable to solubilize an active enzyme were Tergitol, Tween 40, and Brij 58.
[c] Activity was determined by formation of demethylspheroidene (DMS) from hydroxyneurosporene.

tained. Upon use of this purification procedure, the specific activity of HND increased 285-fold (Table 3).

Desaturation of carotenoids involves the transfer of hydride to an appropriate acceptor (22). For other bacterial carotene desaturases, these were flavin adenine dinucleotide (FAD) (20), NAD^+, $NADP^+$ (7), and molecular oxygen (1). Therefore, dependency on all of these cofactors was tested under aerobic conditions as well as in a reduced-oxygen atmosphere (Table 4). Furthermore, the influence on HND of diphenylamine was assayed. This compound is a specific inhibitor of phytoene desaturases from bacteria and fungi which interacts with the isolated phytoene desaturase from *R. capsulatus* with an I_{50} value of 230 μM (20). None of the cofactors significantly affected the comparably high specific activity of purified HND in the presence of oxygen. When molecular oxygen was depleted by the glucose-glucose oxidase system, less than 10% of the specific activity was retained. The addition of possible electron acceptors did not restore activity. Diphenylamine (up to 100 μM) was not inhibitory to HND.

For a better understanding of the participation of HND in the carotenoid biosynthetic pathway of *Rhodobacter* species, it was important to determine the substrate specificity of HND. Figure 2 shows the results of an HPLC separation of residual carotenoid substrates from their reaction products when several 1-hydroxy derivatives were employed as substrates. All of the carotenoid substrates structurally resemble exactly that half of the hydroxyneurosporene molecule which carries the 1-hydroxy group next to the C-3,4 region to be desaturated by HND (see the structures below [Fig. 3]). All products of the HND reaction except that for the reaction with 1,1′-dihydroxylycopene acquired one additional double bond compared to the substrate. This increases the polarity, which is determined

TABLE 3. Purification of the recombinant *Rhodobacter* HND

Fraction	Volume (ml)[a]	Protein mass (mg)	HND mass (mg)[b]	Recovery (%)	Sp act (μg of DMS/mg · h)[c]
Homogenate	50	530	35 (6.6)	100	0.07
Membranes	25	405	30 (7.5)	86	0.09
Nonidet P-40 soluble[d]	25	30	5.9 (19.7)	17	0.78
Ni-NTA[e]	2	0.03	0.03 (100)	0.1	20.09

[a] From 1 liter of an IPTG-induced *E. coli* culture.
[b] Values in parentheses indicate percentages of total protein in the fractions.
[c] Activity was determined by measuring the formation of demethylspheroidene (DMS) from hydroxyneurosporene.
[d] Soluble fraction collected after treatment with 0.1% Nonidet P-40 for 1 h at 4°C.
[e] Fraction obtained on elution of a nickel-NTA column with 0.1 M imidazole.

TABLE 4. Influence of various cofactors on hydroxyneurosporene desaturase activity

Addition[a]	Sp act (μg of DMS/mg · h)[b]
None (aerobic control)	33.7
NAD^+	29.3
$NADP^+$	26.4
FAD	34.6
100 mM DPA	34.7
None (anaerobic control)	1.6
NAD^+	2.6
$NADP^+$	1.6
FAD	1.6

[a] The cofactor concentration was 1 mM in each case. For anaerobic conditions the oxygen in the incubation mixture was depleted with glucose-glucose oxidase. DPA, diphenylamine.
[b] DMS, demethylspheroidene.

by the very polar hydroxy group in the molecule, only slightly. The HPLC separation had to be optimized for separation of the products from the substrate, which could only be achieved with a 3-μm reversed-phase material and a comparably polar

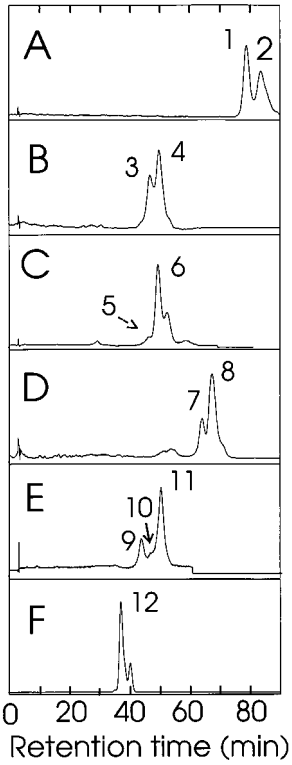

FIG. 2. HPLC separation of carotenoids after in vitro reactions of purified HND with different substrates, i.e., 1-hydroxyneurosporene (detection at 455 nm) (A), 1-hydroxylycopene (detection at 475 nm) (B), 1′-3,4-didehydrolycopene (detection at 485 nm) (C), 1-hydroxy-γ-carotene (detection at 460 nm) (D), 1,1′-dihydroxylycopene (detection at 475 nm) (E), and 1-methoxyneurosporene (detection at 455 nm) (F). HPLC system A was used for the separation of 1,1′-dihydroxylycopene from the reaction products, system C was used when methoxyneurosporene was the substrate, and system B was utilized in all other cases. The numbers of the peaks correspond to the carotenoids as follows: 1, demethylspheroidene; 2, 1-hydroxyneurosporene; 3, 1-hydroxy-3,4-didehydrolycopene; 4, 1-hydroxylycopene; 5, 1′-hydroxy-3,4,3′,4′-tetradehydrolycopene; 6, 1′-hydroxy-3,4-didehydrolycopene; 7, 1′-hydroxy-3′,4′-didehydro-γ-carotene; 8, 1′-hydroxy-γ-carotene; 9, 1,1′-dihydroxy-3,4,3′,4′-tetradehydrolycopene; 10, 1,1′-dihydroxy-3,4-didehydrolycopene; 11, 1,1′-dihydroxylycopene; and 12, 1-methoxyneurosporene.

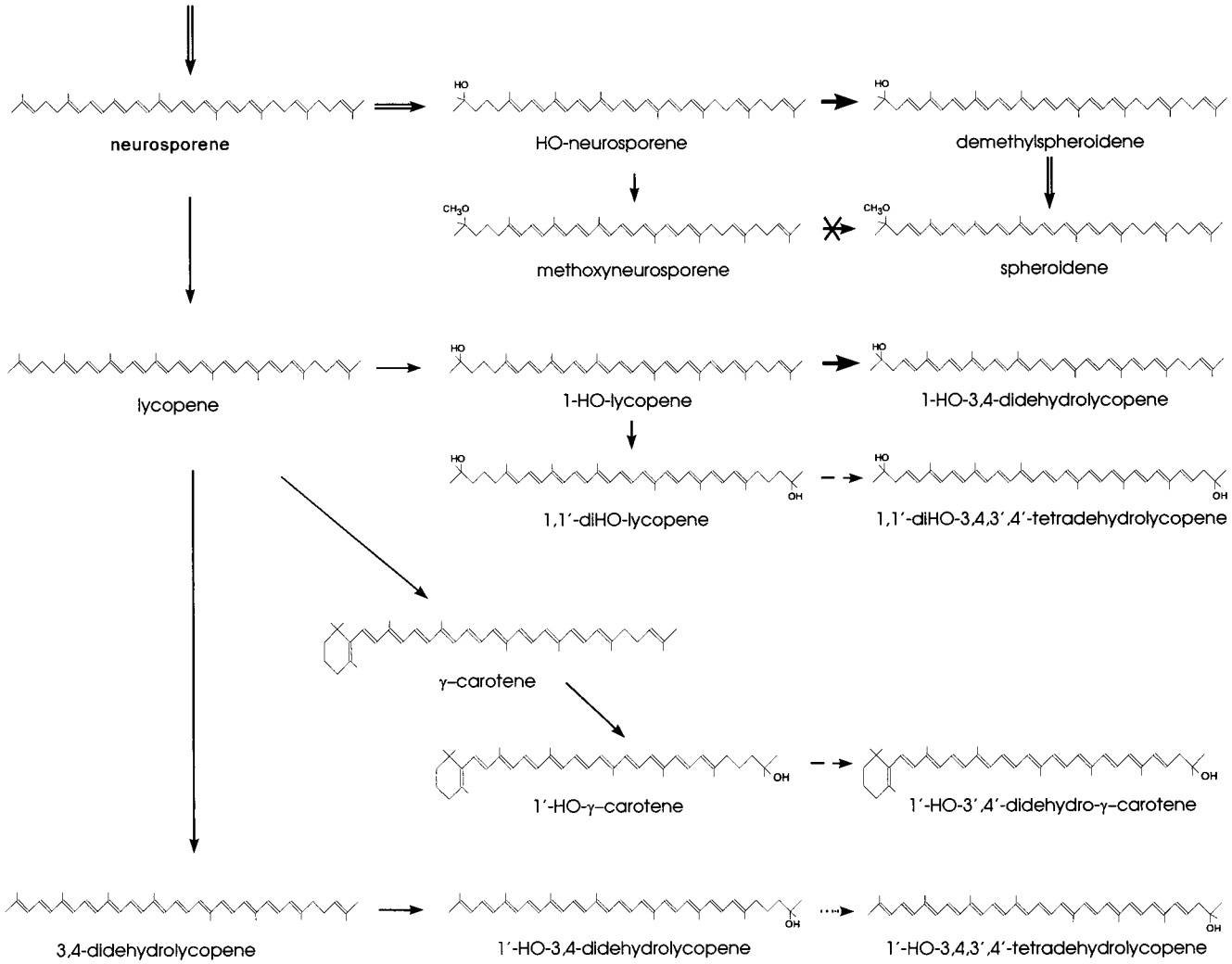

FIG. 3. Synthesis of substrate carotenoids and their conversion by HND from *R. sphaeroides*. All 1-hydroxy carotenoids shown were tested as substrates in the HND reaction. Boldface arrows indicate high-level substrate conversion, dashed arrows refer to low activity, the dotted arrow denotes trace activity, and the crossed-out arrow indicates a reaction which is not catalyzed by HND. Double-lined arrows indicate the sequence of the pathway leading to the formation of spheroidene in *Rhodobacter* species.

mobile phase containing several percent water. The resulting retention times were 2 to 3 min less than those of the corresponding substrates. Due to the insertion of one double bond by HND, the products of the reaction could be identified by their absorbance at wavelengths about 15 nm longer (28). In the case of dihydroxylycopene, an extention of the conjugated system by a maximum of two double bonds is expected. This should result in a chromophore with 13 conjugated double bonds, as in spirilloxanthin, with an absorbance maximum that is 20 nm longer than that of the substrate.

For the experiment shown in Fig. 2A, 1-hydroxyneurosporene (peak 2) was used as the substrate. The resulting demethylspheroidene (peak 1) was identified by its absorbance spectrum, with maxima at 428, 453, and 482 nm. Alternatively, 1-hydroxylycopene (Fig. 2B, peak 4) was another substrate resulting in the formation of 1-hydroxy-3,4-didehydrolycopene (peak 3), with absorbance maxima at 462, 485, and 515 nm. When 1′-hydroxy-3,4-didehydrolycopene (Fig. 2C, peak 6) was applied as the *trans* isomer with a *cis* isomer as the minor compound, the reaction product 1′-hydroxy-3,4,3′,4′-tetradehydrolycopene (peak 5) was formed in only trace amounts. Nevertheless, it is seen as a small shoulder in Fig. 2C and could be identified by its absorbance maxima at 480, 504, and 535 nm. With 1-hydroxy-γ-carotene (Fig. 2D, peak 8) as a substrate, formation of 1′-hydroxy-3′,4′-didehydro-γ-carotene (peak 7; absorbance maxima at 455, 476, and 505 nm) was observed. In the case of 1,1′-dihydroxylycopene (Fig. 2E, peak 11), two reaction products were detectable: mainly, 1,1′-dihydroxy-3,4,3′,4′-tetradehydrolycopene (peak 9; absorbance maxima at 472, 492, and 522 nm), with traces of the intermediate 1,1′-dihydroxy-3,4-didehydrolycopene (peak 10; absorbance maxima at 465, 488, and 520 nm) seen as a shoulder. In contrast, 1-methoxyneurosporene (peak 12), with the *trans* isomer as the major component and the *cis* isomer as the minor component, was not converted to any detectable product, as seen in Fig. 2F.

By the peak heights in Fig. 2 it can be seen that 1-hydroxyneurosporene, 1-hydroxylycopene, and 1′-hydroxy-γ-carotene are converted by HND very well whereas 1′-hydroxy-3,4-didehydrolycopene and 1,1′-dihydroxylycopene are poor substrates for this enzyme. In the case of 1-methoxyneurosporene, no conversion to spheroidene was observed. Neurosporene with-

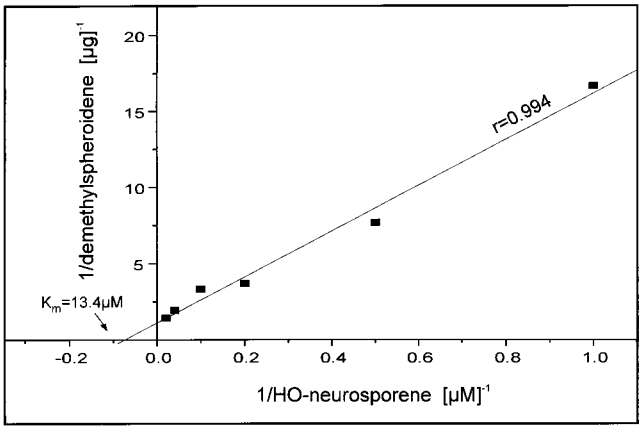

FIG. 4. Double-reciprocal plot of HND activity versus the concentration of hydroxyneurosporene for K_m value determination.

out the 1-hydroxy group is also not converted by HND (data not shown).

Enzyme kinetic studies were carried out to obtain the K_m for hydroxyneurosporene, which is the only natural substrate of HND in *Rhodobacter* cells. A series of assays using various amounts of hydroxyneurosporene were performed. The double-reciprocal Lineweaver-Burk plot of the reaction product formed versus the amount of added hydroxyneurosporene resulted in a straight line with $r = 0.99$ (Fig. 4). From the intercept with the abscissa, the K_m for hydroxyneurosporene as a substrate of HND was calculated to be 13.4 μM.

DISCUSSION

A long time ago, Goodwin (10) suggested the use of purified enzyme systems for a definitive elucidation of the reaction sequence in the carotenoid biosynthetic pathway in *Rhodobacter* species. After purification and determination of the biochemical properties of the phytoene desaturase (20), the next enzyme in the pathway, HND, was expressed (Fig. 1). The protein was obtained in a high yield, around 6% of the total *E. coli* protein (Table 3). In general, enzymes of the carotenoid pathway are membrane bound but their solubilization is hampered by their sensitivity to detergent, which results in the complete loss of activity (20, 26). HND is one of the very few carotenogenic enzymes which can be solubilized by a detergent and remain in an active state (Table 2). The only other carotenoid desaturase with a similar behavior is ζ-carotene desaturase from the cyanobacterium *Anabaena* sp. strain PCC 7120 (1). Due to the six-histidine extension, which originates from the pQE cloning vector, a one-step affinity purification was sufficient to obtain a homogeneous protein with a molecular mass of 59 kDa (Fig. 1). This is consistent with the value (58 kDa) calculated from the deduced amino acid sequence (9). The specific activity of around 20 to 30 μg/mg of protein · h is definitely higher than those of other carotenoid desaturases which are functionally different but exhibit sequence similarities (1, 20). This indicates that most of the purified enzyme is in an active state.

Looking at the cofactor requirement of purified HND, neither NAD^+, $NADP^+$, nor FAD was an effective hydrogen acceptor, even under oxygen-depleted conditions (Table 4). It is rather unlikely that molecular oxygen is the genuine cofactor for hydroxyneurosporene desaturation reactions in *Rhodobacter* cells when they reach their maximum carotenoid content under anaerobic conditions. Consequently, another, as-yet-unknown electron acceptor may exist in *Rhodobacter* species that replaces molecular oxygen during anaerobic growth. In contrast to the *Rhodobacter* phytoene desaturase, the HND is not inhibited by diphenylamine in concentrations up to 100 μM. All the biochemical characteristics shown in Table 4 resemble very closely those of the *Anabaena* ζ-carotene desaturase. In contrast to the C-3,4 HND, the *Anabaena* enzyme is a C-7,8 desaturase. It should be mentioned that HND exhibited the closest relationship to ζ-carotene desaturase based on amino acid sequence (17). The biochemical data obtained in the current study support the assumption that the *Anabaena* ζ-carotene desaturase is phylogenetically related to the *Rhodobacter* HND (23).

For the evaluation of substrate specificity, five different acyclic 1-hydroxy carotenoids were synthesized by *E. coli* transformants (Table 1). Their identities were confirmed by spectral analysis and mass spectroscopy. Starting from a defined substrate, absorbance spectra were sufficient for the identification of the reaction products. The results shown in Fig. 2 demonstrate that 1-hydroxyneurosporene and 1-hydroxylycopene are very efficiently converted to demethylspheroidene and 1-hydroxy-3,4-didehydrolycopene, respectively. As 1-hydroxyneurosporene is the natural substrate in *R. sphaeroides* and *R. capsulatus*, a K_m of 13.4 μM was determined for this carotenoid. This K_m value is in the same range as others, e.g., 9.7 μM for ζ-carotene in the reaction of the *Anabaena* ζ-carotene desaturase (1) and 33.3 μM for phytoene in the *R. capsulatus* phytoene desaturase reaction (20). Although the presence or absence of a double bond at C-7′,8′ had no effect on substrate conversion by HND, other modifications of the ψ end group influenced substrate specificity. For example, the additional hydroxy group at C-1′, i.e., in 1,1′-dihydroxylycopene or the β-ring in 1′-hydroxy-γ-carotene at the side of the molecule which is not desaturated, decreased the acceptance of both carotenoids as substrates by HND (Fig. 2). The C-3′,4′ double bond in 1-hydroxy-3,4-didehydrolycopene even prevents the desaturation of this substrate almost completely.

The ability of purified HND to convert the other possible natural substrate, 1-methoxyneurosporene, to spheroidene was also investigated in the present study. The results clearly demonstrated that this reaction is not catalyzed by HND. Consequently, in *R. sphaeroides*, there is only one possible reaction sequence, from 1-hydroxyneurosporene via 3,4-desaturation and, finally, O-methylation to spheroidene. This is indicated in Fig. 3 by double-lined arrows. There is no evidence for a parallel pathway involving the methylation of 1-hydroxyneurosporene prior to C-3,4 desaturation (2, 22, 27). *Rubrivivax gelatinosus*, which was formerly grouped with *Rhodobacter* species, synthesizes spheroidene and substantial amounts of spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetradehydrolycopene) (25). This is in contrast to *R. capsulatus* and *R. sphaeroides*, in which only trace amounts of the latter carotenoid are detectable. Spirilloxanthin originates from 1,1′-dihydroxylycopene in the same manner as spheroidene originates from 1-hydroxyneurosporene. Apart from a modified phytoene desaturase which should have an increased potential for the formation of lycopene compared to the enzyme from *R. capsulatus* (20), an HND with a more or less equal specificity for 1-hydroxyneurosporene and 1,1′-dihydroxylycopene is required in *Rubrivivax gelatinosus*. The functional differences between these enzymes are sufficient to explain the different carotenoid composition in the latter bacterium. The *crtD* gene from *Rubrivivax gelatinosus* has been cloned very recently (19) and is now available for further biochemical studies.

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