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In Vitro Characterization of Astaxanthin Biosynthetic Enzymes*

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Escherichia coli strains expressing the marine bacteria (*Agrobacterium aurantiacum* and *Alcaligenes* sp. strain PC-1) astaxanthin biosynthetic genes (*crtZ* and *W*), *Haematococcus pluvialis* *bkt*, and *Erwinia uredovora* *crtZ* genes were used for *in vitro* characterization of the respective enzymes. Specific enzyme assays indicated that all of the enzymes are bifunctional, in that the *CrtZ* enzymes formed zeaxanthin from β -carotene via β -cryptoxanthin, as well as astaxanthin from canthaxanthin via phenoxanthin (adonirubin). The *BKT/CrtW* enzymes synthesized canthaxanthin via echinenone from β -carotene and 4-ketozeaxanthin (adonixanthin) with trace amounts of astaxanthin from zeaxanthin. Comparison of maximum catalytic activities as well as selectivity experiments carried out in the presence of both utilizable substrates indicated that the *CrtZ* enzymes from marine bacteria converted canthaxanthin to astaxanthin preferentially, whereas the *Erwinia* *CrtZ* possessed a favorability to the formation of zeaxanthin from β -carotene. The *CrtW/BKT* enzymes were not so defined in their substrate preference, responding readily to fluctuations in substrate levels. Other properties obtained indicated that the enzymes were strictly oxygen-requiring; and a cofactor mixture of 2-oxoglutarate, ascorbic acid, and Fe^{2+} was beneficial to activity. Based on enzymological data, a predicted pathway for astaxanthin biosynthesis is described, and it is proposed that *CrtZ*-like enzymes be termed carotenoid 3,(3')- β -ionone ring hydroxylase and *CrtW/BKT* carotenoid 4,(4')- β -ionone ring oxygenase.

Astaxanthin (3,3'-dihydroxy- β , β -carotene-4,4'-dione) is the most commonly found carotenoid pigment in marine animals (1). It is responsible for the red/pink coloration of crustaceans (1), shellfish (2), and the flesh of salmonoids (3). Despite high endogenous levels, these marine animals do not possess the ability to synthesize astaxanthin or other carotenoid pigments *de novo*. Instead carotenoid pigments must be acquired via their diet (4). Industrially, astaxanthin has been exploited as a feed supplement for cultured fish and shellfish (5–7). Other diverse biological functions of astaxanthin include an involvement in cancer prevention (8), enhancer of immune responses (9), and a free radical quencher (10, 11). It is evident, therefore, that astaxanthin is a molecule with potential both to the pharmaceutical and food industries.

Astaxanthin belongs to the class of compounds known as

xanthophylls. These are carotenoids modified with oxygen-containing functional groups. Xanthophylls are found universally in chloroplast-containing plant tissues. However, the biosynthesis of astaxanthin is limited in a virtually exclusive manner to microorganisms, for example, the yeast *Phaffia rhodozyma* (12), the freshwater alga *Haematococcus pluvialis* (13) and the marine bacteria *Agrobacterium aurantiacum* and *Alcaligenes* sp. strain PC-1 (14). Recently the genes involved in the formation of astaxanthin and its intermediates have been isolated and functionally characterized *in vivo* by complementation, providing the first insight into the biosynthetic route (15). The pathway proposed (Fig. 1) is based on the lack of enzyme specificity surmised from complementation studies (15). The hydroxylation of β -carotene at positions 3 and 3' on the β -ionone ring forming zeaxanthin via β -cryptoxanthin is mediated by the product of the gene designated *crtZ*, which has been isolated from *Erwinia* species (16, 17) and marine bacteria (15). The direct conversion of methylene to keto groups at positions 4 and 4' on the β -ionone ring forming canthaxanthin via echinenone are reactions performed by the gene product encoded by the *crtW* gene from marine bacteria (18) and *bkt* gene of *H. pluvialis* (19). A comparison of the deduced amino acid sequences indicates the existence of 90% identity between the marine bacteria *A. aurantiacum* and *Alcaligenes* PC-1 *CrtZ*, which in turn show 54% identity with the *Erwinia* species. The *CrtW* proteins possess a 75% identity among the marine bacteria species, whereas a 37% identity is evident when compared with the *BKT* gene product of *H. pluvialis*. When combinations of the gene products responsible for the introduction of the hydroxyl moieties at positions 3,3' and formation of keto groups at 4,4' positions on the β -ionone ring are expressed in *Escherichia coli*, astaxanthin as well as substantial quantities of various intermediates are synthesized (15). Because of the limitations of *in vivo* complementation, the bifunctional character of the *CrtZ* and/or *CrtW* type gene products has not been ascertained conclusively. Thus it remains unclear whether *CrtZ* can convert canthaxanthin to astaxanthin via phenoxanthin (adonirubin) or *CrtW* can convert zeaxanthin to astaxanthin via 4-ketozeaxanthin (adonixanthin).

At present no specific *in vitro* assay systems, cofactor requirements, or properties of the enzymes originating from an astaxanthin-forming organism have been reported. Advances in carotenoid enzymology have, however, been hindered by the practical difficulties associated with their assay (20, 21). The heterologous expression of the recently isolated astaxanthin biosynthetic genes in *E. coli* has provided a valuable opportunity to study the respective enzymes. In this article the *in vitro* characterization of the marine bacteria *CrtZ* and *CrtW* enzymes specific to astaxanthin biosynthesis are reported for the first time as well as a comparison with the *Erwinia* *CrtZ* and *Haematococcus* *BKT*.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids pCAR16 Δ crtX and pCAR25 Δ crtX, corresponding to pCAR16 Δ elB and pCAR25 Δ elB (16), contain the *Erwinia ure-*

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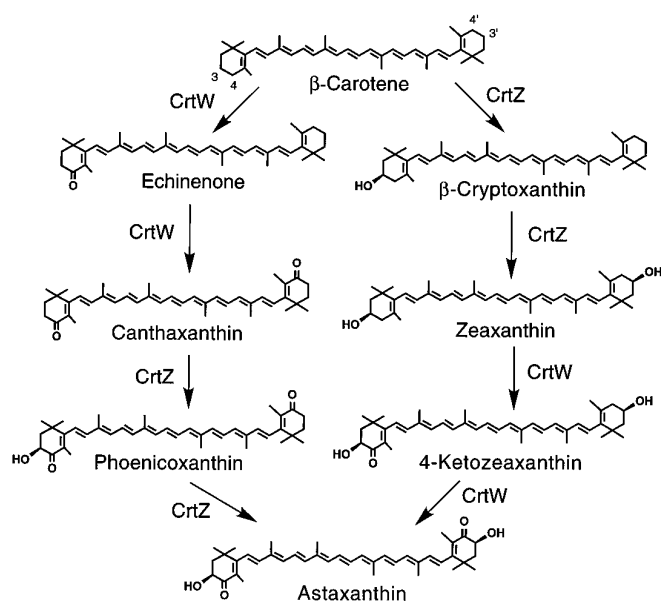


FIG. 1. Postulated astaxanthin biosynthetic pathway deduced from *in vivo* complementation studies (15).

dovora genes for the synthesis of β -carotene and zeaxanthin, respectively. pACCAR16 Δ crtX has been described (18). pCrt-Z containing the *E. ureidovora* crtZ gene was constructed by inserting the *Sph*I(5599)-*Eco*RI(6505) fragment of pCAR25 (16) into the *Eco*RI-*Sph*I site of pUC18. Plasmids pAK916 and pAK96NK, which contain the *A. aurantiacum* crtW and crtZ genes, respectively, were described previously (15). pPC17-3 (18) and pPC13 (15) carried the *Alcaligenes* sp. strain PC-1 crtW and crtZ genes, respectively. Plasmid pUCBKT containing the *H. pluvialis* bkt gene was constructed by polymerase chain reaction using plasmid pHP51 (19) as a template, where ATG at nucleotide position 264 (19) was placed next to the *Hind*III site of pUC19 to form a fusion protein with the amino terminus of β -glucosidase as follows.

264
 —ATG ACC ATG ATT ACG CCA AGC TTG ATG CCA TCC GAG TCG—
 —Met Thr Met Ile Thr Pro Ser Leu Met Pro Ser Glu Ser—

Organisms and Culture Conditions—The *E. coli* strains were grown in 2 $\times$ YT medium (22) containing the appropriate additions as described below. In all cases an initial overnight culture (5 ml) was prepared from a glycerol stock. An aliquot constituting 1% by volume of the fresh culture medium was used to inoculate cultures for induction. These cultures contained 0.1 mM isopropyl 1-thio- β -D-galactopyranoside and the following antibiotics depending on the recombinant strain. Strains used and antibiotic requirements follow. *E. coli* JM109 containing plasmids pCAR16 Δ crtX, pCAR25 Δ crtX, pCrt-Z, pAK916, pAK96NK, pPC17-3, pPC13, and pUCBKT required ampicillin at a final concentration of 150 μ g/ml. *E. coli* JM101 containing pACCAR16 Δ crtX and pAK916, which synthesized canthaxanthin (18), required ampicillin (150 μ g/ml) and chloramphenicol (30 μ g/ml). All *E. coli* strains were grown at 30 $^{\circ}$ C and shaken at 200 rpm. Marine bacteria *A. aurantiacum* and *Alcaligenes* PC-1 were grown in Sakaguchi flasks (Eagleglass, Tokyo) containing 150 ml of marine broth (Difco). Culture vessels were shaken (164 rpm) at 20 $^{\circ}$ C for 4 days under diffuse light.

Preparation of Cell Extracts—Recombinant *E. coli* extracts were prepared from cells induced for 4 or 6 h. Cells were collected by centrifugation at 6,000 $\times$ g. Pelleted cells were placed on ice and resuspended in 100 mM Tris-HCl, pH 8.0, containing 1 mM dithiothreitol, protease mixture (0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin) as well as 5% glycerol v/v. The suspension was passed through a French press cell at an internal pressure of 500 p.s.i. DNase (50 μ g) was added to the broken extract, and the mixture was incubated on ice for 30 min. The crude extract was centrifuged at 10,000 $\times$ g to remove cell debris. When determining cofactor requirements the supernatant was desalted with a PD10 column (Pharmacia Biotech Inc.) in 100 mM Tris-HCl, pH 8.0, containing 1 mM dithiothreitol.

Incubation Conditions—Incubations were carried out in a total volume of 800 μ l. Typically 400 μ l of the substrate extract of *E. coli*

producing β -carotene, canthaxanthin, or zeaxanthin was added (the precise volume varied depending on the quantity of substrate supplied). An equal volume of the *E. coli* extract to be analyzed was also added. The remaining 200 μ l was buffered with 0.4 M Tris-HCl, pH 8.0, containing 1 mM dithiothreitol, 0.1% v/v Tween 60, 3 mM ATP, 0.5 mM FeSO₄, 0.5 mM 2-oxoglutarate, 5 mM ascorbic acid. The presence of 1 mg/ml catalase was optional. The mixture was incubated by shaking in the dark for 4 h at 30 $^{\circ}$ C. When performing incubations with both utilizable substrates the mixture could be boiled for 30 s to remove any endogenous activities. Incubations were terminated by the addition of methanol and stored at -70° C under an atmosphere of nitrogen. Anaerobic incubations were performed in Thunberg tubes (23). Extracts and incubation mixture were degassed under vacuum then purged with nitrogen gas prior to sealing. Control incubations were performed simultaneously in all experiments. They were identical apart from the presence of an *E. coli* extract from a non-crtZ or crtW expressing strain, as well as incubations containing heat-denatured (boiled 1 min) enzyme preparations.

Extraction and Analytical Methods—Carotenoid standards were extracted and purified from recombinant *E. coli* strains and *A. aurantiacum* using general carotenoid methodology as described (24). Products and substrates from the *in vitro* incubations were extracted with 10% (v/v) diethyl ether in petroleum ether 40–60 $^{\circ}$ C (3 volumes). After mixing, a partition was formed by centrifugation at 3,000 $\times$ g for 5 min at 4 $^{\circ}$ C. The organic phase was removed and aqueous phase reextracted with the same solvent. The remaining aqueous phase was further reextracted with chloroform (2 volumes). The organic extracts were pooled and brought to dryness under a stream of nitrogen.

Carotenoids formed *in vitro* were separated by HPLC¹ on a reversed phase C₁₈ column (Nova-pak HR 6 μ C₁₈, 3.9 $\times$ 300 mm, Waters) using an isocratic mobile phase of acetonitrile/methanol/isopropyl alcohol (90:6:40, v/v/v). On-line photodiode array detection enabled peak identification by comparison of spectral data with authentic standards (24). Retention times and $\lambda_{\max}$, respectively, are as follows: astaxanthin, 5.31 min, 480 nm; 4-ketozeaxanthin, 5.96 min, 465 nm; phenoxixanthin, 6.61 min, 475 nm; zeaxanthin, 7.09 min, 456 nm; canthaxanthin, 9.05 min, 475 nm; β -cryptoxanthin, 20.3 min, 452 nm; echinenone, 22 min, 460 nm; β -carotene 60.6 min, 456 nm. Quantification of carotenoids was achieved by injecting known amounts of carotenoid standards to generate a standard curve. In addition, further confirmation of the amount eluting and detected on-line was performed using the method devised by Sandmann (35). The detection limit of HPLC-photodiode array detection system was 0.0003 absorbance units corresponding to 0.5 pmol/h/mg of protein enzyme activity. Below this level novel peaks were detectable having the correct retention time, but definitive spectral data were difficult to derive and therefore are referred to as trace levels.

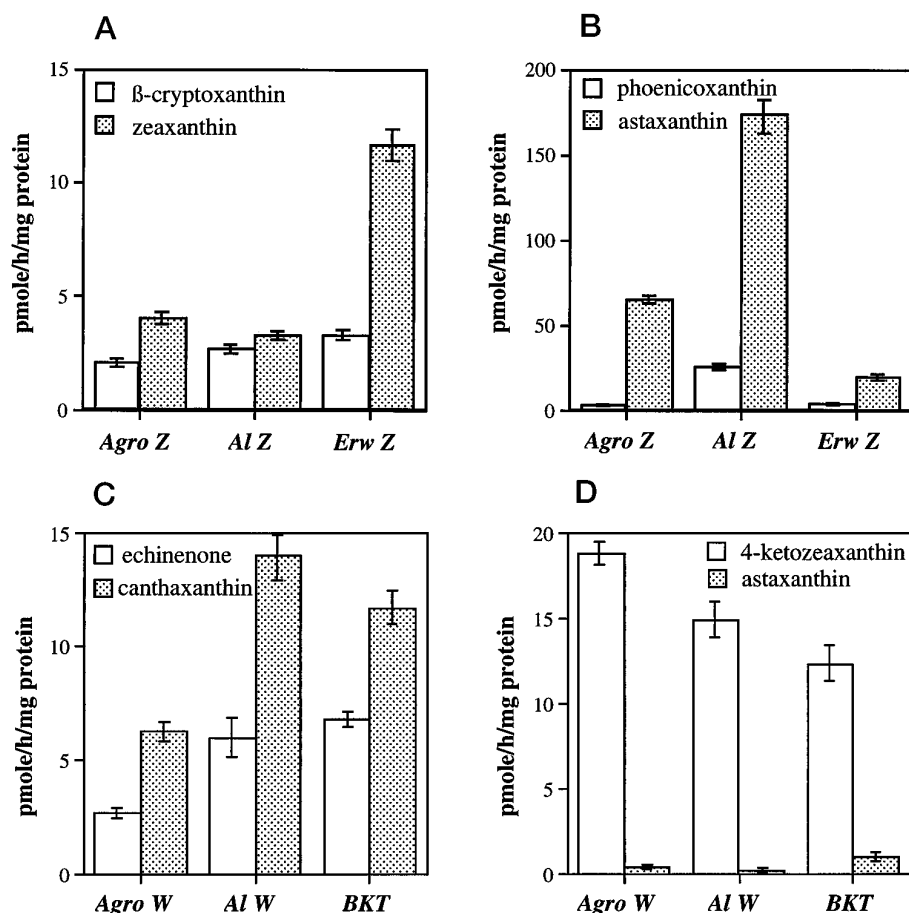
Protein and Other Determinations—Protein present in *E. coli* extracts was estimated using the Bio-Rad protein assay dye reagent protocol following the manufacturer's instructions. Enzyme activity was expressed as pmol formed per h/mg of protein in all cases. When determining the *in vivo* rate of carotenoid formation carotenoid-synthesizing *E. coli* cultures were induced and time points taken at 1-h intervals. *In vivo* and *in vitro* comparisons were made between cells induced for the same time period in the case of recombinant strains.

RESULTS AND DISCUSSION

Specific Determination of CrtZ and W/BKT Enzyme Activities—Substrates were supplied in the form of extracts prepared from recombinant *E. coli* strains accumulating β -carotene, zeaxanthin, and canthaxanthin in the all-*trans* configuration. This procedure overcame difficulties experienced when introducing carotenoids into aqueous incubation mixtures. All varieties of the CrtZ enzymes formed zeaxanthin from β -carotene via the monohydroxylated compound β -cryptoxanthin. The intermediate β -cryptoxanthin was detectable at significant levels (20–40%) as shown in Fig. 2A. When canthaxanthin was supplied to the CrtZ enzymes astaxanthin was formed via phenoxixanthin in all cases. Astaxanthin was the predominant product constituting (>70%) of the total products (Fig. 2B). Canthaxanthin was the predominant (63–68%) product synthesized by CrtW/BKT from β -carotene; significant amounts

¹ The abbreviation used is: HPLC, high performance liquid chromatography.

FIG. 2. Products formed by CrtZ enzymes from *A. aurantiacum*, *Alcaligenes* sp. strain PC-1, and *E. ureidovorae* with the substrates β -carotene (panel A) and canthaxanthin (panel B). Products formed by CrtW from *A. aurantiacum*, *Alcaligenes* sp. strain PC-1 and BKT of *H. pluvialis* with the substrates β -carotene and zeaxanthin are shown in panels C and D, respectively. Determinations were performed with crude extracts of *E. coli* (protein content 500 μ g). Cofactors and incubation conditions are as given under "Experimental Procedures." Substrates were supplied at 2 μ g. Agro, Al, and Erw refer to *A. aurantiacum*, *Alcaligenes* PC-1, and *E. ureidovorae*, respectively. CrtZ reactions using β -carotene as the substrate are shown in panel A, mean $\pm$ S.E. ($n = 3$) and with canthaxanthin as substrate in panel B, mean $\pm$ S.E. ($n = 7$). CrtW/BKT conversions using β -carotene are shown in panel C, mean $\pm$ S.E. ($n = 5$), and zeaxanthin as substrate in panel D, mean $\pm$ S.E. ($n = 3$).



(30–35%) of its mono-keto intermediate echinenone were also detected. The ratio of canthaxanthin to echinenone was independent of species (Fig. 2C). When supplied with zeaxanthin, CrtW and BKT preparations only formed trace levels (0.5 pmol/h/mg of protein) of astaxanthin, but 4-ketozeaxanthin was formed predominantly by the enzyme from all varieties (Fig. 2D). Why the CrtW and BKT enzymes were unable to catalyze the conversion of 4-ketozeaxanthin to astaxanthin from the initial substrate zeaxanthin efficiently is difficult to explain. It is possible that the experimental conditions were not optimal. However, *in vivo* 4-ketozeaxanthin can accumulate (up to 50% of the total carotenoid content) in recombinant *E. coli* strains forming ketocarotenoids (15), and in the marine bacteria it is often more predominant than astaxanthin (14). Such findings correlate with the *in vitro* data reported in this study and could suggest that the formation of the 4' keto group on 3,3'-hydroxylated β -ionone ring-containing carotenoids is prevented if prior keto group formation at position 4 has occurred.

Reaction products were separated by HPLC and identified with an on-line photodiode array detector, from which spectral data could be derived (described under "Experimental Procedures"). Comparison with authentic standards enabled conclusive identification by cochromatography and comparative spectral data. In all cases the CrtZ/W and BKT reaction products were of the all-*trans* configuration and constituted novel compounds not present in control samples. Controls were performed with both extracts of *E. coli* strains not harboring astaxanthin-forming genes and heat-denatured extracts. Some compounds present at low levels did occur in the controls and were also detectable in the experimental incubations, but they did not cochromatogram with the CrtZ/W and BKT reaction products and were spectrally unrelated. Presumably these compounds were derived from the substrates due to nonspecific

oxidation. Thus it can be confirmed that the enzymes CrtZ/W of marine bacteria, CrtZ of *Erwinia*, and BKT of *Haematococcus* are bifunctional in their activity. Hydroxylation of carotenoid β -ionone rings by CrtZ can be performed specifically at the 3,3' positions regardless of the presence of previous keto group formation at positions 4,4'. Although the formation of keto groups by CrtW/BKT is specific at positions 4,4' on the β -ionone rings retrospective of previous 3,3' hydroxylation, it must be stated that the mono-keto reaction product accumulates if prior 3,3' hydroxylation has occurred. In this study, we have only used carotenoids containing β -ionone rings as they are the precursors involved in astaxanthin formation. It would be interesting to ascertain whether the substrate specificity and product diversity would extend to ϵ -ring cyclic carotenoids or even acyclic carotenoids.

In Table I the maximum catalytic activities are presented. These data indicate that compared with the *Erwinia* CrtZ the marine bacteria enzymes possess greater (e.g. *A. aurantiacum*, 10-fold) ability to convert canthaxanthin to astaxanthin. In contrast, the *Erwinia* enzyme shows greater (e.g. 10-fold compared with *Alcaligenes*) activity for the conversion of β -carotene to zeaxanthin than canthaxanthin to astaxanthin. Of the CrtZ enzymes the marine bacterium *A. aurantiacum* possessed the greatest activity. Comparison of CrtW/BKT activities using β -carotene as substrate indicated that the *Alcaligenes* PC-1 CrtW was about 2-fold greater than the other varieties which were similar in activity (Table I). 4-Ketozeaxanthin was formed from zeaxanthin at a similar rate by all of the enzymes examined.

The *in vivo* rate of carotenoid formation in recombinant strains synthesizing zeaxanthin (CrtZ), canthaxanthin (CrtW), and astaxanthin (CrtZ + W/BKT) as well as the marine bacteria were determined as 5.3 ± 1.9 , 1.8 ± 0.2 , 2.95 ± 0.95 , and $3.1 \pm$

TABLE I
In vitro activities of CrtZ (I) and CrtW/BKT (II) enzymes

Incubations were performed at 30 °C, in dark, aerobic conditions, for 3 h. Substrate levels (1.5 µg) and protein content (500 µg) were standardized. Incubations contained the following cofactors ATP (3 mM), FeSO₄ (0.5 mM), 2-oxoglutarate (0.5 mM), and ascorbic acid (5 mM) as described under "Experimental Procedures." Data represent the means ± S.E. (n = 5).

Enzyme source (recombinant <i>E. coli</i> strain)	Gene product assayed	Specific enzyme activity	
		β -Carotene $\rightarrow$ zeaxanthin	Canthaxanthin $\rightarrow$ astaxanthin
<i>pmol/h/mg protein</i>			
I. JM109 (pAK96NK)	<i>A. auratiacum</i> CrtZ	9 $\pm$ 1.0	60 $\pm$ 8.0
JM109 (pPC13)	<i>Alcaligenes</i> PC-1 CrtZ	3.4 $\pm$ 0.9	28 $\pm$ 4.0
JM109 (pCrt-Z)	<i>Erwinia</i> CrtZ	37 $\pm$ 6.0	6 $\pm$ 1.3
JM109		0	0
JM109 (pAK96NK/pPC13/pCrt-Z)	Heat-denatured CrtZ	0	0
		β -Carotene $\rightarrow$ canthaxanthin	Zeaxanthin $\rightarrow$ 4-ketozeaxanthin
<i>pmol/h/mg protein</i>			
II. JM109 (pAK916)	<i>A. auratiacum</i> CrtW	5 $\pm$ 0.7	12 $\pm$ 2.0
JM109 (pUCBKT)	<i>Hematococcus</i> BKT	5 $\pm$ 1.0	10 $\pm$ 3.0
JM109 (pPC17-3)	<i>Alcaligenes</i> PC-1 CrtW	12 $\pm$ 3.0	9 $\pm$ 3.0
JM109		0	0
JM109 (pAK96NK/pPC13/pCrt-Z)	Heat-denatured CrtW/BKT	0	0

TABLE II
Oxygen dependence of CrtZ- and CrtW-mediated reactions

The *A. auratiacum* CrtZ/W are shown as representative examples of the determination of oxygen dependence. Incubations contained ATP (3 mM), FeSO₄ (0.5 mM), 2-oxoglutarate (0.5 mM), and ascorbic acid (5 mM) as described under "Experimental Procedures." Crude extracts contained 450 µg of protein. Data represent means ± S.E. (n = 8) of a typical experiment. Activities represent total products formed, e.g. in the case of CrtZ with the substrate β-carotene the products are zeaxanthin + β-cryptoxanthin. Control experiments were carried out under identical conditions except that *E. coli* extracts not harboring CrtZ/W and boiled extracts were used.

Gene product assayed	Substrate	Activity		% Inhibition
		Aerobic	Anaerobic	
<i>pmol / h / mg protein</i>				
CrtZ	β -Carotene	22 $\pm$ 0.9	4 $\pm$ 2.0	76
CrtZ	Canthaxanthin	219 $\pm$ 23.0	24 $\pm$ 7.0	88
CrtZ (heat-denatured)	β -Carotene/canthaxanthin	0	0	
CrtW	β -Carotene	22 $\pm$ 5.0	1 $\pm$ 0.5	96
CrtW	Zeaxanthin	39 $\pm$ 0.2	0	100
CrtW (heat-denatured)	β -Carotene/canthaxanthin	0	0	

0.7 µg/g dry weight/h, respectively. Corresponding to typical *in vitro* rates for CrtZ enzymes of 2.5 µg/g dry weight/h (mean from typical experiment) and CrtW/BKT 0.83 µg/g dry weight/h (mean from typical experiment). The *in vitro* rates for CrtZ and W/BKT, respectively, are 47 and 46% of the *in vivo* rate. To our knowledge no previous data have been reported comparing *in vivo* and *in vitro* rates of carotenoid formation. Other enzymes involved in terpenoid biosynthesis have been compared with their *in vivo* biosynthetic capacity. In these instances it was found that the enzymes camphor hydroxylase, abietadiene and abietadienol hydroxylase only constituted 10–20% (25) or 1% (26) of the *in vivo* capacity. Considering that carotenoids like other terpenoids are hydrophobic (preventing effective use in aqueous solution), easily degraded, oxidized, and prone to isomerization, it is not surprising that *in vitro* rates are typically a poor reflection of their *in vivo* counterparts. Taking these facts into account along with the strong analogy between qualitative product formation *in vitro* and *in vivo*, we believe it is justified that at present the *in vitro* system described represents a creditable representation of the *in vivo* situation.

Oxygen Dependence of CrtZ- and W/BKT-catalyzed Reactions—The absence of oxygen on carotenoid hydroxylation and oxygenation *in vitro* was assessed. Table II shows experimental data obtained with the *A. auratiacum* enzymes using the substrates β-carotene, canthaxanthin, and zeaxanthin independently. It was evident that all catalyzed reactions studied were oxygen-dependent, each having a similar degree of sensitivity to the absence of oxygen. Phoenicoxanthin was formed at a rate of 17.0 pmol/h/mg of protein, quantitatively similar to the aerobic rate of 22.0 pmol/h/mg of protein. However, when

the levels of phoenicoxanthin formed aerobically and aerobic are compared in a qualitative manner with respect to their proportion of total CrtZ products it is significant that phoenicoxanthin the monohydroxylated product is the predominant product compared with astaxanthin (dihydroxylated) under aerobic conditions. When the aerobic CrtZ activity was reduced to a total rate quantitatively similar to the anaerobic level, qualitatively astaxanthin remained the principal product. Thus, predominance of phoenicoxanthin in the anaerobic incubation was a finding specific to those conditions. Similar findings of mono-ketolated product accumulation were observed with CrtW and β-carotene as substrate. It was impossible to observe any difference in the product profile formed from zeaxanthin by CrtW/BKT as the activity was not detectable, and as stated previously 4-ketozeaxanthin is the principal product of this reaction. From the experimental data it can be concluded that oxygen is involved in all of the reactions performed by the CrtZ and W enzymes from marine bacteria; this also reflects the situation found with BKT and *Erwinia* CrtZ (data not shown). At present we cannot conclusively ascertain whether the oxygen moieties present in the carotenoid molecule resulting from enzymatic synthesis are derived directly from molecular oxygen or if oxygen is indirectly utilized in the catalysis. These findings are the first *in vitro* evidence that the enzymes involved in astaxanthin biosynthesis are oxygen-dependent, and they complement previous *in vivo* studies described for zeaxanthin formation by *Flavobacterium* species (27), xanthophyll synthesis in leaf tissue (28), astaxanthin accumulation in *P. rhodozyma* (29), and xanthophyll formation in marine bacteria (34). The changing pattern of products formed

TABLE III
Influence of Fe^{2+} , ascorbic acid, 2-oxoglutarate, and catalase on activity

The amount of substrate used and protein content of the extracts were standardized at 2 μg and 300 μg , respectively. The basal activity without the presence of additional cofactors (20% of the maximum) has been subtracted from all determinations for clarity. The greatest rate determined for each reaction is shown as 100%; all other determinations are relative to this value (see footnotes to table for precise values). Basic mixture is described under "Experimental Procedures" without the stated additions. JM109 extract is a control extract not expressing CrtZ/W. All data are the mean of duplicate or triplicate incubations from a typical experiment.

Cofactor additions	CrtZ		CrtW	
	β -Carotene	Canthaxanthin	β -Carotene	Zeaxanthin
	% relative activity		% relative activity	
Basic mixture	0	0	0	0
+ Fe^{2+} , ascorbic acid, 2-oxoglutarate, catalase	100 ^a	43	70	100 ^b
+ Fe^{2+} , ascorbic acid, 2-oxoglutarate	16	100 ^c	100 ^d	59
+ Fe^{2+} , ascorbic acid	4	63	0	74
+ Fe^{2+}	7	63	0	74
+ Fe^{2+} , 2-oxoglutarate	14	7	10	26
+ Fe^{2+} , catalase	12	7	0	19
+ Fe^{2+} , 2-oxoglutarate, catalase	9	63	0	15
+Ascorbic acid, 2-oxoglutarate, catalase	2	0	0	0
+Ascorbic acid, 2-oxoglutarate	0	0	0	0
+Ascorbic acid, catalase	0	0	0	0
+Ascorbic acid	0	0	0	0
+2-Oxoglutarate, catalase	0	0	0	0
+2-Oxoglutarate	0	0	0	0
+Catalase	0	5	0	0
+ Fe^{2+} , ascorbic acid, 2-oxoglutarate, catalase, heat-denatured CrtZ/W	0	0	0	0
+ Fe^{2+} , ascorbic acid, 2-oxoglutarate, catalase, JM109 extract	0	0	0	0

^a 33.3 pmol/h/mg of protein.

^b 150 pmol/h/mg of protein.

^c 417 pmol/h/mg of protein.

^d 92 pmol/h/mg of protein.

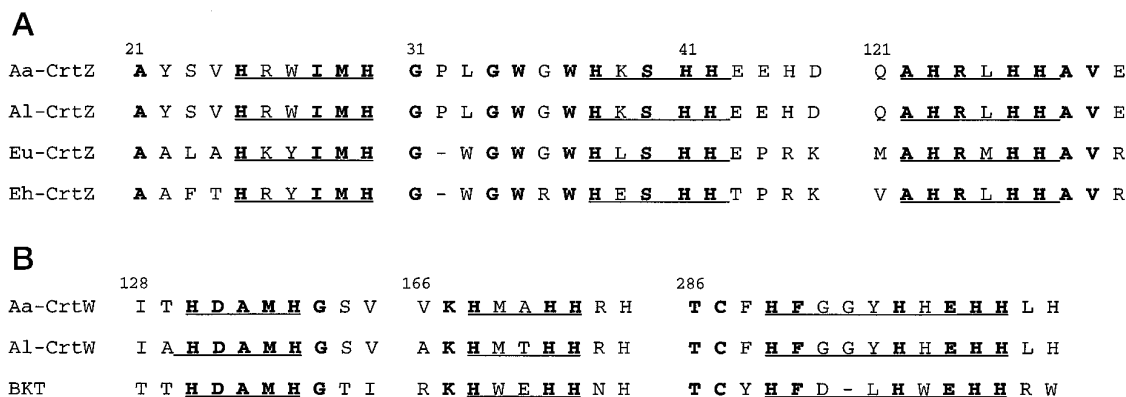


FIG. 3. Alignment of *A. aurantiacum* (Aa), *Alcaligenes* PC-1 (Al) CrtZ/W, *E. uredovora* (Eu) and *E. herbicola* (Eh), and *H. pluvialis* BKT protein sequences. Conserved iron binding motifs are underlined. Bold type indicates conserved residues among all sequences.

with reduced oxygen levels suggests that its presence or absence could also play a crucial regulatory role over product formation and could possibly be influenced by environmental conditions.

Effect of Cofactors on CrtZ- and W/BKT-catalyzed Reactions—In the absence of any exogenously added cofactors significant activity (20%) was detectable. Therefore there was no absolute requirement for cofactors using these enzyme preparations. The addition of the dinucleotides NAD^+ , NADP^+ and flavins FAD, FMN (at a concentration of 1 mM) as well as divalent ions Mg^{2+} and Mn^{2+} to the incubations individually and in combination had no stimulatory effect. In fact they were often inhibitory. The presence of NADPH^+ (1 mM) did yield some elevation (2.5-fold) in activity. These findings were consistent among the marine bacteria CrtZ/W, *Erwinia* CrtZ and BKT enzyme.

Combinations of cofactors used for typical monooxygenase (30) or dioxygenase (31) reactions were tested subsequently. The monooxygenase cofactors consisted of FAD, NADPH^+ , and

ATP. This cofactor combination increased the activity of CrtW and Z about 2.5- and 1.5-fold greater than the control, respectively. When the dioxygenase mixture (Fe^{2+} , 2-oxoglutarate, ascorbic acid, and catalase) was used, far greater stimulation in activity was found, particularly in the case of CrtZ, where about a 6-fold elevation was experienced. CrtW activity was also increased significantly (4-fold). Based on the observed stimulation in activities, a detailed analysis of the components was made. A similar profile in activities was observed between the CrtZ/W-catalyzed reactions as shown in Table III. The presence of Fe^{2+} , 2-oxoglutarate, and ascorbic acid in combination was responsible for the greatest enzymatic activity; however, all incubations containing Fe^{2+} possessed an activity higher than the control value, indicating that Fe^{2+} was the most influential effector. A series of control experiments confirmed that this cofactor mixture could not perform these conversions without the presence of the enzymes. Their presence enhanced specific 3,3' hydroxylation by CrtZ' or 4,4' oxygenation by CrtW. Comparison between the gene sequences encod-

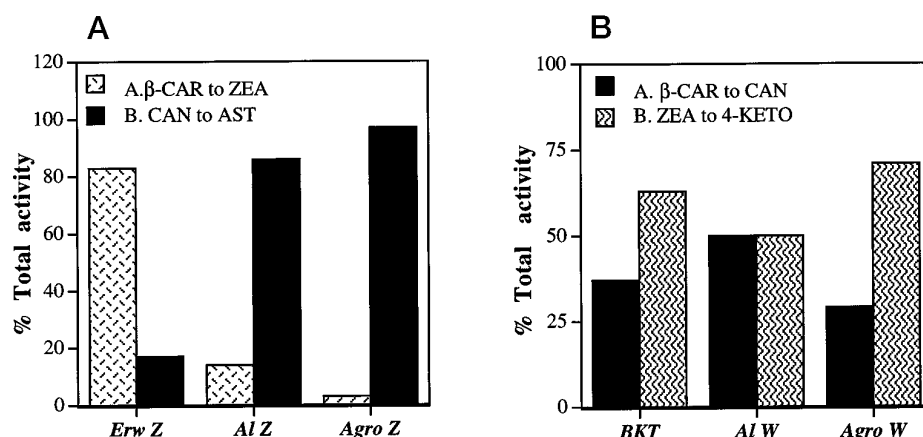


FIG. 4. Preferences of CrtZ (panel A) and W/BKT (panel B) in the presence of both utilizable substrates. Incubation conditions were identical to those described under "Experimental Procedures." Crude *E. coli* extracts had a protein content of 375 μ g. Substrates (1.5 μ g) were supplied in identical volumes. β -CAR indicates β -carotene; CAN, canthaxanthin; ZEA, zeaxanthin; AST, astaxanthin; and 4-KETO, 4-ketozeaxanthin. Data are presented as percent total activity correlating to rates of 401 pmol/h/mg of protein *A. aurantiacum* CrtZ (Agro), 210 pmol/h/mg of protein *Alcaligenes* PC-1 CrtZ (Al), and 510 pmol/h/mg of protein *E. uredoovora* CrtZ (Erw). In panel B *A. aurantiacum* CrtW is equivalent to 210 pmol/h/mg protein *Alcaligenes* PC-1 CrtW 140 pmol/h/mg of protein, whereas *H. pluvialis* (BKT) is 210 pmol/h/mg of protein. These enzyme activities are the mean of duplicate or triplicate determinations from a typical experiment. Control incubations performed under identical conditions, but with heat-denatured extracts as well as extracts from *E. coli* strains not expressing *crt* genes, showed no activity.

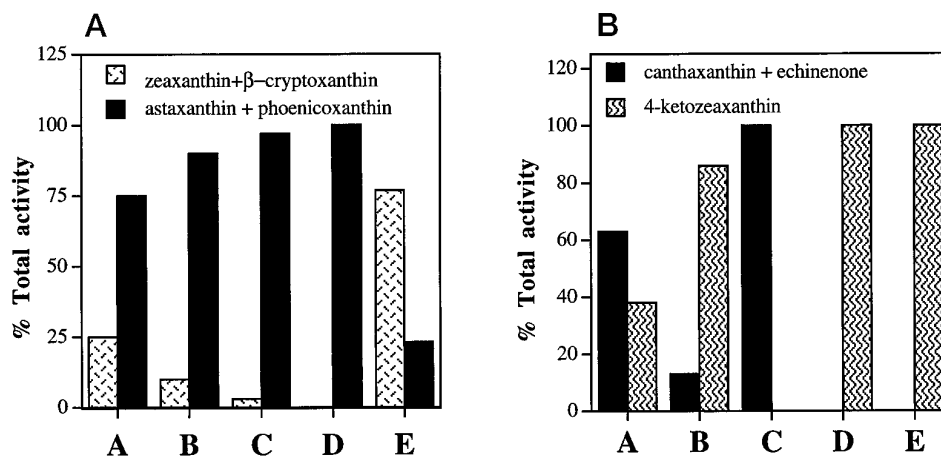


FIG. 5. Preferences of CrtZ (panel A) and W (panel B) with varying levels of both utilizable substrates. Incubations were performed in a manner identical to those described in Fig. 4, with the exception that the substrate ratios were varied; this was done by altering the volume of substrate extract added. The basal substrate level (*i.e.* ratio value 1) was 1 μ g of carotenoid. Where no histogram bar is given, no activity was detected. In panel A, A and B indicate *Alcaligenes* PC-1 CrtZ with the substrates β -carotene and canthaxanthin present in 1:2 and 3:1 ratios, respectively; total activity corresponds to 113 and 93 pmol/h/mg of protein, respectively. C and D refer to *A. aurantiacum* CrtZ β -carotene and canthaxanthin 6:1 and 1:2 ratios, respectively; total activities are 438 and 450 pmol/h/mg of protein. E represents *E. uredoovora* CrtZ; the β -carotene and canthaxanthin ratio was 1:8; total activity was 500 pmol/h/mg of protein. Panel B letters represent *Alcaligenes* PC-1 CrtW. Substrate ratios for β -carotene and zeaxanthin were 6:1 (A) and 1:3 (B); total activities were 60 and 21 pmol/h/mg of protein, respectively. C and D are *A. aurantiacum* CrtW at β -carotene and zeaxanthin ratios of 6:1 and 1:2, respectively; total activity was 281 and 500 pmol/h/mg of protein, respectively. E shows the *H. pluvialis* BKT at a β -carotene:zeaxanthin ratio of 1:2. The total activity was 213 pmol/h/mg of protein. Data represent typical experiments; determinations are the means of duplicate or triplicate incubations. Controls with denatured extracts as well as *E. coli* extracts not expressing the genes of interest were performed under identical conditions and showed no activity.

ing the marine bacteria CrtZ and W, *E. uredoovora* and *Erwinia herbicola* as well as *bkt* revealed a number of conserved histidine residues in a formation consistent with reported Fe^{2+} binding motifs (HX_3H , HX_2HH , or HX_2HH). Such motifs are characteristic of enzymes containing non-heme iron such as membrane-bound fatty acid desaturases (32). Fig. 3 shows the alignment of the postulated Fe^{2+} binding motifs present in the CrtZ genes from marine bacteria and *Erwinia* species. Three conserved Fe^{2+} motif regions exist occurring at $\text{H}^{25}\text{X}_4\text{H}$, $\text{H}^{38}\text{X}_2\text{HH}$, and $\text{H}^{123}\text{X}_2\text{HH}$. In the case of BKT and the CrtW enzymes of marine bacteria such motifs occur at $\text{H}^{130}\text{X}_3\text{H}$, $\text{H}^{168}\text{X}_2\text{HH}$ as well as an overlapping conserved sequence between $\text{H}^{289}\text{X}_3\text{H}$ and $\text{H}^{294}\text{X}_2\text{HH}$. Thus both biochemical and molecular data suggest that Fe^{2+} is a cofactor involved in the catalysis performed by these enzymes. Of the other cofactors (2-oxoglutarate and ascorbic acid) present in the optimal com-

bination no independent stimulatory role was witnessed. Perhaps their function may be as oxidizable cosubstrates, which generate a reactive ferryl ion in the process. Such a mechanism would be similar to the gibberellin oxidases and hydroxylases (31). However, in the experimental system described in this article it is likely that ascorbic acid is substituting for a genuine cosubstrate as ascorbic acid is not present endogenously in *E. coli*. Purification of these enzymes will hopefully enable determination of the precise role performed by these postulated cofactors as well as *in vitro* mutagenesis of the postulated Fe^{2+} binding sites.

Selectivity of Substrate Utilization by CRTW/Z—To investigate the preference of one substrate above the other by these bifunctional enzymes experiments were designed whereby both utilizable substrates were added to the enzyme preparation simultaneously, initially in an equal ratio. The resulting activ-

ities were calculated for the individual reactions rates then expressed as the percent of the total activity. The marine bacteria CrtZ enzymes showed that canthaxanthin was the favored substrate for conversion, 36-fold in the case of *A. aurantiacum* and 6-fold in the case of *Alcaligenes* PC-1. The *E. uredovora* CrtZ in contrast showed a 5-fold preference for β -carotene as the substrate to zeaxanthin (Fig. 4A). The CrtW enzymes and BKT gene product were less conclusive in their selectivity with both BKT and *A. aurantiacum* possessing about a 2-fold preference for zeaxanthin as the substrate for 4-ketozeaxanthin formation. *Alcaligenes* CrtW, however, showed no significant favorability to zeaxanthin as the principal substrate for conversion (Fig. 4B). To determine if this preference for substrates was consistent regardless of varying substrate levels, different ratios of substrates were incubated with the respective enzymatic preparations; the findings are presented in Fig. 5, A and B. In the case of CrtZ from *Alcaligenes* PC-1, increasing the level of β -carotene to a level three times greater than canthaxanthin did not alter the preference of the enzyme for the conversion of canthaxanthin to astaxanthin (Fig. 5A). In fact it appeared to some extent that such elevated levels of β -carotene could inhibit the conversion of β -carotene to zeaxanthin presumably by substrate inhibition. The *A. aurantiacum* CrtZ also showed no alteration in its preference for canthaxanthin conversion even when β -carotene levels were increased six times greater than that of the canthaxanthin substrate (Fig. 5A). In contrast, only relatively small increases (1.5–3.0-fold) in canthaxanthin levels were required to eliminate completely the presence of zeaxanthin formation (Fig. 5A) by the CrtZ enzymes from either marine bacteria. The *E. uredovora* CrtZ was not studied extensively, but it was evident that increases in the proportion of canthaxanthin did alter comparatively the preference of the enzyme for the conversion of canthaxanthin to astaxanthin to some extent (Fig. 5A).

The *A. aurantiacum* CrtW gene product showed a significant response to changes in substrate concentration, as when β -carotene was in excess (6-fold) canthaxanthin was the product formed. When zeaxanthin was supplied to excess (2-fold) 4-ketozeaxanthin was formed preferentially (Fig. 5B). Comparison of the two situations suggested that the zeaxanthin to 4-ketozeaxanthin is the favored reaction as smaller incremental increases in substrate were required to result in a greater commitment to this reaction. The *Alcaligenes* PC-1 CrtW also showed an ability to respond to changes in precursor levels, as when β -carotene was in excess canthaxanthin was the sole product formed, and when zeaxanthin levels were elevated 4-ketozeaxanthin resulted and canthaxanthin formation eliminated. Although not investigated to the same extent, BKT clearly responded to changes in precursor levels in a manner similar to that of the marine bacteria enzymes.

Collectively, data shown in Table I along with results determined in the presence of both utilizable substrate in equal (Fig. 4) and varying concentrations (Fig. 5), indicate that the marine bacteria CrtZ enzymes significantly favor performing the conversion of canthaxanthin to astaxanthin, whereas the *Erwinia* CrtZ has a recognizable preference for converting β -carotene to zeaxanthin. In some respects this is not too surprising considering that the *Erwinia* species accumulate zeaxanthin or its glucoside but no ketocarotenoids endogenously. *In vivo* complementation studies have established that both varieties of enzyme have the potential to perform identical reactions (15). The CrtW/BKT enzymes are significantly less selective in their substrate preference compared with the CrtZ enzymes. When equal ratios of substrates are used the enzymes utilization is similar; but compared with the CrtZ enzymes only small ele-

vations of zeaxanthin or β -carotene substrates are required to alter the product profile to 4-ketozeaxanthin or canthaxanthin, respectively.

Based on the enzymes' substrate utilization we predict that the astaxanthin pathway in marine bacteria occurs ideally through the initial formation of canthaxanthin via echinenone, which is then acted upon by CrtZ enzymes to form astaxanthin. This proposal is based on the facts that β -carotene is the initial precursor to oxygenated carotenoids, and the marine bacteria CrtZ favors the conversion of canthaxanthin to astaxanthin *in vitro*. However, the bifunctional nature of the enzymes must be taken into account, and therefore if zeaxanthin is formed initially either due to environmental influences or in the case of recombinant studies altered expression levels, the CrtW/BKT enzymes will utilize this as a substrate-forming 4-ketozeaxanthin, which will accumulate because of the enzyme's apparent ineffectiveness to convert 4-ketozeaxanthin to astaxanthin. In the recent publication by Yokoyama and Miki (33), the astaxanthin pathway was proposed from the accumulation of intermediates under different culture conditions. The study indicated that when zeaxanthin was formed, substantial 4-ketozeaxanthin also accumulated, a finding supporting the pathway deduced from the capabilities of the enzymes determined *in vitro*. Other pathways to astaxanthin via hydroxyechinenone intermediates must await further assay development before they can be confirmed. The similarities of BKT to the CrtW enzymes would suggest that the pathway in *Haematococcus* is like the marine bacteria, as suggested in previous *in vivo* studies (34), but the yet to be isolated hydroxylase gene from this organism prevents confirmation.

Concluding Remarks—The *in vitro* characterization of astaxanthin biosynthetic enzymes described in this article provides the first enzymological data on their substrate utilization and product diversity as well as possible cofactor requirements. The data presented indicate that the CrtZ-type enzymes show properties of enzymes frequently categorized as hydroxylases, and CrtW-type enzymes, oxygenases. Both are also bifunctional with regard to substrates containing β -ionone rings. It is for these reasons that we propose that the enzymes be referred to as 3,(3')- β -ionone ring hydroxylase and 4,(4')- β -ionone ring oxygenase as standard enzyme nomenclature.

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