**In Vitro Characterization of Astaxanthin Biosynthetic Enzymes***

(Published February 6, 1996, and revised December 19, 1996)

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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, that the *CrtZ* enzymes formed zeaxanthin from β-carotene via β-cryptoxanthin, as well as astaxanthin from canthaxanthin via phenoixanthin (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 CrtWBKT 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 Fe2+ 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′-dihydroxy and CrtWBKT carotenoid 4,4′-dione ring oxygenase.

A 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*, astaxanthan as well as substantial quantities of various intermediates are synthesized (15). Because of the limitations of *in vitro* 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 astaxanthan via phenoixanthin (adonirubin) or CrtW can convert zeaxanthin to astaxanthan via 4-ketozeaxanthin (adonixanthin).

At present no specific *in vitro* assay systems, cofactor requirements, or properties of the enzymes originating from an astaxanthan-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 astaxanthan biosynthesis are reported for the first time as well as a comparison with the *Erwinia CrtZ* and *Haematococcus BKT*.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids pCAR16ΔcrtX and pCAR25ΔcrtX, corresponding to pCAR16delB and pCAR25delB (16), contain the *Erwinia ure-
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**Doxor* genes for the synthesis of β-carotene and zeaxanthin, respectively. pACCAR16ΔcrtX has been described (18). pCRT-Z containing the E. uredovora crtZ gene was constructed by inserting the SphaI (5599 − EcoRl 6505) fragment of pCAR15 (16) into the EcoRl-Spha site of pUC18. Plasmids pAK916 and pAK96NK, which contain the A. aurantiacum crtW and crtZ genes, respectively, were previously described (15). pFC17-3 (18) and pFC15 (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 pHPS1 (19) as a template, where ATG at nucleotide position 264 (19) was placed next to the HindIII site of pUC19 to form a fusion protein with the amino terminus of β-glucosidase as follows:

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**Organisms and Culture Conditions**—The E. coli strains were grown in 2 X 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-galactopyranoside and the following antibiotics depending on the recombinant strain. Strains used and antibiotic requirements follow. E. coli JM109 containing pACCAR16ΔcrtX and pAK916, which synthesized canthaxanthin (18), required ampicillin (150 μg/ml) and chloramphenicol (30 μg/ml). All E. coli strains were grown at 30 °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 °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 g × 30 min. Pelleted cells were placed on ice and resuspended in 100 mM Tris-HCl, pH 8.0, containing 1 mM diithiothreitol, protease mixture (0.1 mM phenylmethylsulfonyl fluoride, 1 μM μg/ml leupeptin, 1 μM μg/ml pepstatin) as well as 5% glycerol w/v. The suspension was passed through a French press cell at a internal pressure of 500 p.s.i. DNase (50 μg) was added to the broken extract, and the mixture was incubated on ice for 30 min. The crude extract was centrifuged at 10,000 × 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 diithiothreitol.

**Incubation Conditions**—Incubations were carried out in a total volume of 900 μl. Typically 400 μl 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 μl was buffered with 0.4 M Tris-HCl, pH 8.0, containing 1 mM diithiothreitol, 0.1% w/v Tween 80, 3 mM ATP, 0.5 mM FeSO4, and 0.5 mM 2-oxoglutarate. A 1 μM μg/ml catalase was optional. The mixture was incubated by shaking in the dark for 4 h at 30 °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 °C (3 volumes). After mixing, a partition was formed by centrifugation at 3,000 × g for 5 min at 4 °C. The organic phase was removed and aqueous phase was extracted 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 HPLC<sup>1</sup> on a reversed phase C<sub>18</sub> column (Nova-pak HR C<sub>18</sub> 3.9 × 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 λ<sub>max</sub>, respectively, are as follows: astaxanthin, 5.31 min, 480 nm; 4-ketoastaxanthin, 5.96 min, 465 nm; phycocyanin, 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/μl of protein enzyme activity. Below this level novel peaks were detectable, however, 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 vitro 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.

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1 The abbreviation used is: HPLC, high performance liquid chromatography.
(30–35%) of its mono-keto intermediate echinone was also detected. The ratio of canthaxanthin to echinone 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 cochromatograph 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 e-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) 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 ± 0.3 pmol/h/mg of protein, respectively.
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 *E. coli* strain) | Gene product assayed | Specific enzyme activity | pmol/h/mg protein |
|---------------------------------------------|----------------------|-------------------------|------------------|
| JM109 (pAK96NK)                             | *A. aurantiacum* CrtZ | β-Carotene → zeaxanthin | 9 ± 1.0          |
| JM109 (pPC13)                                | *Alcaligenes* PC-1 CrtZ | Canthaxanthin → astaxanthin | 3.4 ± 0.9 |
| JM109 (pCRT-Z)                               | *Erwinia* CrtZ        | β-Carotene → canthaxanthin | 37 ± 6.0 |
| JM109                                       |                      | Zeaxanthin → 4-ketozeaxanthin | 0 |
| JM109 (pAK96NK/pPC13/pCRT-Z)                | Heat-denatured CrtZ  | 0                       | 0 |

| II. JM109 (pAK916)                           | *A. aurantiacum* CrtW | β-Carotene → canthaxanthin | 5 ± 0.7 |
| JM109 (pUCBKT)                               | *Hematoecoccus* BKT   | Zeaxanthin → 4-ketozeaxanthin | 12 ± 2.0 |
| JM109 (pPC17–3)                              | *Alcaligenes* PC-1 CrtW | 5 ± 1.0 |
| JM109                                        |                      | 10 ± 3.0 |
| JM109 (pAK96NK/pPC13/pCRT-Z)                | Heat-denatured CrtW/BKT | 0 |

### Table II

**Oxygen dependence of CrtZ- and CrtW-mediated reactions**

The *A. aurantiacum* 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                | Aerobic Activity pmol/h/mg protein | Anaerobic Activity pmol/h/mg protein | % Inhibition |
|---------------------------|--------------------------|-----------------------------------|--------------------------------------|-------------|
| CrtZ                      | β-Carotene               | 22 ± 0.9                           | 4 ± 2.0                              | 76          |
| CrtZ                      | Canthaxanthin            | 219 ± 23.0                         | 24 ± 7.0                             | 88          |
| CrtZ (heat-denatured)     | β-Carotene/canthaxanthin | 0                                  | 0                                    | 0           |
| CrtW                      | β-Carotene               | 22 ± 5.0                           | 1 ± 0.5                              | 96          |
| CrtW                      | Zeaxanthin               | 39 ± 0.2                           | 0                                    | 100         |
| CrtW (heat-denatured)     | β-Carotene/canthaxanthin | 0                                  | 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 astaxanthin 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...
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-
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Fig. 4. Preferences of CrtZ (panel A) and W/BKT (panel B) in the presence of both utilizable substrates. Incubations were identical to those described under “Experimental Procedures.” Crude E. coli extracts had a protein content of 375 μg. Substrates (1.5 μg) 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. uredovora 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 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 μg 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. uredovora 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 canthaxanthin 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.
substrate preference compared with the CrtZ enzymes. When CrtW/BKT enzymes are significantly less selective in their zymehavethepotentialtoperformidenticalreactions (15). The mentation studies have established that both varieties of en-
uredovora glucoside but no ketocarotenoid endogenously. thin (Fig. 5 of the enzyme for the conversion of canthaxanthin to astaxan-
times greater than canthaxanthin did not alter the preference of the enzyme for the conversion of canthaxanthin to astaxan-
thin, which will accumulate because of the enzyme’s apparent ineffective ness to convert 4-ketozeaxanthin to astaxanthin. In the recent publication by Yokoyama and Miki (33), the astax-
athin 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 deter-
minded in vitro. Other pathways to astaxanthin via hydroxyechinenone intermediates must await further assay develop-
ment 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 astax-
athin 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 prop-
erties 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. Acknowledgments—We are grateful to the members of the Kirin Metabolic Engineering group and Dr. S. Ferri for helpful discussion. We also thank Dr. David E. Cane of Brown University for advice regarding possible CrtZW enzymatic mechanisms.

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