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Evidence of β-carotene 7,8 (7′,8′) oxygenase (β-cyclocitral, crocetindial generating) in *Microcystis**

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Abstract. A β-carotene oxygenase is described which occurs in the Cyanobacterium *Microcystis*. It cleaves β-carotene and zeaxanthin specifically at the positions 7,8 and 7′,8′, while echinenone and myxoxanthophyll are not affected. The oxidative cleavage of β-carotene leads to the formation of β-cyclocitral and crocetindial and that of zeaxanthin to hydroxy-β-cyclocitral and crocetindial in nearly stoichiometric amounts. Oxidant is dioxygen as has been demonstrated by high incorporation (86%) of $^{18}O_2$ into β-cyclocitral. β-Carotene oxygenase is membrane bound, sensitive to sulfhydryl reagents, antioxidants and chelating agents. Iron seems to be an essential part of the enzyme activity. Cofactors necessary for the reaction could not be detected.

Key words: β-Carotene oxygenase – β-Cyclocitral – Crocetindial – $^{18}O_2$ Labelling – *Microcystis* – *Cyanobacteria*

In *Cyanobacteria*, *Mucorales*, *Ascomycetes* and unicellular algae numerous excretion products have been found whose structures point to their genesis from carotenes. Methyl ketones and the corresponding alcohols with irregular terpenoid structures represent such components and have been termed nor-carotenoids (Enzell et al. 1977). One group of them probably derives from acyclic carotenes by oxidative cleavage of a double bond. Widely distributed components of this type are 6-methylhept-5-en-2-one and 6-methylhept-5-en-2-ol which were detected as early as 1950 (Birkinshaw and Morgan) in *Ceratocystis (Endoconidiophora) coerulescens* and were later confirmed (Sprecher 1962; Sprecher et al. 1975) in several strains of this species and in *C. virescens* (Collins 1976). The same components have also been found to belong to the major compounds excreted by *Cyanidium caldarium* (Jüttner 1979), *Synechococcus* (Henatsch and Jüttner 1983) and *Anabaena cylindrica* (Jüttner et al. 1983). Another typical substance of this series is *trans*-geranyl acetone which has up to now among microorganisms exclusively been found in the medium of *Cyanidium caldarium* (Jüttner 1979). A second group of nor-carotenoids possesses a β-ionone ring. Typical representatives are β-ionone and β-cyclocitral isolated from *C. caldarium* and *Anabaena cylindrica* and *Microcystis aeruginosa*, respectively (Jüttner 1976). In addition, the trisporins, trisporols and trisporic acids belong to this group. The latter have, however, a more complex structure by introduction of additional substituents and act in a complex way as sex hormones and prohormones in several genera of the *Mucorales* (Bu'Lock et al. 1976). Physiological activity has also been observed for *trans*-geranyl acetone that specifically inhibits carotene synthesis in *Cyanobacteria* (Jüttner 1979) and β-ionone that causes an up-regulation of β-carotene synthesis in *Phycomyces* (Eslava et al. 1974).

In spite of the close structural relationship of the nor-carotenoids to the carotenes, no enzyme has yet been described in microorganisms that can catalyze the generation of these substances. In this contribution we describe the occurrence of an enzymic reaction in the genus *Microcystis* that catalyzes the oxidative cleavage of β-carotene and generates specifically β-cyclocitral and crocetindial.

Materials and methods

Origin and culture of Microcystis. Microcystis PCC 7806 was obtained from R. Rippka, Institut Pasteur, Paris. The conditions for axenic growth in 300 ml culturing tubes, the medium used and control for sterility were essentially the same as previously published (Jüttner et al. 1983).

Quantitative determination of pigments. The cell pellet obtained after centrifugation of 25 ml of a suspension of *Microcystis* (1.1×10^8 cells/ml) was extracted either directly or after freezing with 5 ml of a mixture of acetone/methanol (7/3; v/v) after a 5 s sonification. The extraction was repeated once with the same solvent mixture and twice with 3 ml of diethyl ether. The combined solutions were brought to dryness in a rotary evaporator. The residue was redissolved in a small volume of chloroform/methanol (2/1; v/v) for pigment separation by reversed phase thin layer chromatography according to Egger (1962). However, before impregnation with fat, the plates layered with Kieselgur G (Merck, Darmstadt, FRG) were dipped in a solution of 2,6-di-*tert*.-butyl-4-methyl phenol (10 ml of a 1% ethanolic solution in 100 ml of petrol ether). Developing solvent was a mixture of methanol/acetone/water (20/8/3; v/v/v). The separated pigments were eluted with ethanol and recorded on a spectrophotometer (model Cary 14). Crocetindial was measured in petrol ether (40–60° C). The specific absorption coefficients as stated by Seely and Jensen

* Dedicated to Professor G. Drews on occasion of his 60th birthday

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Abbreviations. TLC, thin layer-chromatography; PIPES, piperazine-N,N′-*bis*-(2-ethanesulfonate) Na; TES, 2{[*tris*-(hydroxymethyl)-methyl]-amino} ethanesulfonic acid

(1965) and listed up by Davies (1976) were used to calculate the amounts of chlorophyll *a* and the carotenoids, respectively.

Quantitative determination of β-cyclocitral. The cell pellet of a 50 ml *Microcystis* suspension (1.1×10^8 cells/ml) which had been frozen at $-16°$C for at least 3 h was taken up in 50 ml of water and transferred into a 100 ml round bottom flask with ground neck. β-Cyclocitral was stripped in a closed loop system, adsorbed on 150 mg of Tenax TA (60–80 mesh) and subsequently determined by gas liquid-chromatography by separation on a 25 m glass capillary column (coated with UCON 50 HB 5100) as previously described (Jüttner 1984). However, isothermal conditions (100° C) were chosen. Decanone-2 was added as an internal standard (1 ml of a 1% methanolic solution). Defined amounts of authentic β-cyclocitral were added to an equal volume of water in such quantities to give the same peak height as the peak obtained from the cyanobacterial suspension.

Incorporation of $^{18}O_2$ into nor-carotenoids. 1.7×10^{11} cells of *Microcystis* were harvested and resuspended in 20 ml of water. To remove any oxygen, the suspension was gassed for 5 min with Ar in a sealed 60 ml serum bottle. Subsequently 10 ml of $^{18}O_2$ (98.7%, ORIS, Gif sur Yvette, France) was added, the bottle vigorously shaken and frozen at $-16°$C for 12 h. Prior to thawing a further amount of $^{18}O_2$ was injected to maintain the overpressure. After thawing, the suspension was shaken by hand for 30 min to equilibrate with the oxygen/argon atmosphere and centrifuged (10 min, $3{,}000 \times g$). Crocetindial was isolated from the pellet and β-cyclocitral from the supernatant.

Determination of $^{18}O_2$ labelling in β-cyclocitral. β-Cyclocitral was stripped from the supernatant in a closed loop system for 2 h and absorbed on a Tenax TA odour trap as described above. The loaded odour trap was removed from the assembly and eluted with a minimum volume of diethyl ether. The gas chromatographic separation of β-cyclocitral was achieved on a 12 m quartz capillary column coated with OV-1 and He as the carrier gas. The mass ratios of the fragment ions were obtained with a coupled mass selective detector (Hewlett Packard, model 5970 A). As a reference standard unlabelled β-cyclocitral was used that has been worked up identically.

Determination of $^{18}O_2$ labelling in hydroxy-β-cyclocitral. Hydroxy-β-cyclocitral was isolated by liquid-solid sorption from the supernatant. For this purpose 250 µl of the supernatant were applied to the Tenax TA of an odour trap and the water removed in a hydrogen gas stream at 40° C. The further analytical procedures to obtain mass spectra were the same as for β-cyclocitral.

Determination of $^{18}O_2$ labelling in crocetindial. The ^{18}O-labelled cell pellet was extracted with 60 ml of a mixture of acetone/methanol (7/3; v/v). The extract was brought to dryness in a rotary evaporator. The residue redissolved in 0.5 ml of a acetone/methanol mixture was applied for reversed phase separation on a column filled with 1.5 g of C_8 Bond Elut (Analytichem Intern., Harbor City, USA). The first fraction eluted with methanol/acetone/water (20/4/3; v/v/v) contained crocetindial together with myxoxanthophyll. In a next step crocetindial was separated from myxoxanthophyll using a 9×40 mm Al_2O_3-column (activity I, neutral, Merck No 1077). The pigments were added in a 250 µl solution of acetone/methanol to a column which had previously been inactivated by methanol and subsequently reconditioned with pentane. Crocetindial was eluted with pentane and the fraction brought to dryness in a rotary evaporator. The residue was redissolved in a small volume of dichloromethane, transferred into vessels for mass spectrometry and vacuum-dried for 2.5 d. Mass spectra at 70 eV were recorded at 200° C using the direct inlet system of a MAT mass spectrometer.

Incorporation of $H_2{}^{18}O$ into nor-carotenoids. 0.5 ml of $H_2{}^{18}O$ (98.1 atom % ^{18}O) was added to 0.5 g wet weight of *Microcystis* obtained after centrifugation for 15 min at $20{,}000 \times g$. The suspension was frozen and subsequently worked-up for β-cyclocitral and crocetindial as described above.

Assay of carotene oxygenase. Usually the assay of the carotene oxygenase was performed after activation of 3 ml of *Microcystis* suspension (1.1×10^8 cells/ml) by freezing at $-16°$C for at least 3 h. When oxygen free conditions were employed, the *Microcystis* suspension was firstly gassed with Ar in sealed test-tubes. Then to remove traces of oxygen 250 µl of glucose (1 M), 30 µl of katalase (130,000 U/ml) and 30 µl of glucose oxidase (2,000 U/ml) were added to the suspension and incubated for about 8 min. After addition of the agents to be tested the assay was frozen for activation. To maintain strictly anaerobic conditions, after removal from the freezer it was necessary to again flush the gas space of the test tube with Ar during thawing. Oxygen was added for 0.5 h during the subsequent stripping and β-cyclocitral determined by quantitative gas liquid-chromatography.

Derivation of carotenoids. The reduction of the carotene aldehyde groups to the corresponding alcohols was performed with $NaBH_4$ in 70% ethanol. Ready formation of the O-methyloximes was achieved applying the method given by Mawhinney et al. 1980.

Chemicals. β-Cyclocitral and hydroxy-β-cyclocitral were obtained from BASF, Ludwigshafen, FRG. The salts in p.a. grade were purchased from Merck/Darmstadt, the antioxidants and β-apo-8′-carotenal from Fluka, Neu-Ulm, FRG, the inhibitors, fatty acids and carotene from Sigma, München, FRG, and the enzymes from Boehringer, Mannheim, FRG.

Results

Formation of nor-carotenoids. When a suspension or cell paste of the cyanobacterium *Microcystis* is frozen, large amounts of β-cyclocitral are liberated the identity of which has been reported previously (Jüttner 1976). The generation of this compound occurs also upon addition of salt to a high concentration (20% NaCl, w/v), addition of ethanol (20%), french press treatment or sonification of the cell.

The occurrence of β-cyclocitral is associated with a marked change of the pigment composition of the such treated cells. As listed in Table 1, β-carotene and zeaxanthin are markedly degraded in cells activated by freezing as compared to untreated native cells. Myxoxanthophyll and

Table 1. Pigment composition of native and activated cells of *Microcystis* PCC 7806. The concentration of pigments has been determined on the basis of the amount found in 50 ml of cell suspension. Activated cells were obtained after freezing of the cyanobacterial pellet for 12 h at − 16°C. The R_f-values of the pigments apply to reversed phase TLC

Pigments	R_f values	Native cells (μg/l)	Activated cells (μg/l)	Decrease/ increase of pigments (μM)
Myxoxanthophyll	0.95	200	199	0
Crocetindial	0.75	0	228	+ 0.77
Zeaxanthin	0.55	424	137	− 0.51
Echinenone	0.03	121	123	0
β-Carotene	0.01	602	405	− 0.37
Chlorophyll *a*	0.11	3,982	3,784	− 0.22

echinenone which also represent major carotenoids (Stransky and Hager 1970) are not affected. The decrease of β-carotene and zeaxanthin is accompanied by the generation of a new pigment not found in native cells. The electronic spectra of this pigment with A_{max} 444, 473 in benzene; 427, 455 in petrol ether and 443 in ethanol are consistent with those reported by Eugster et al. (1969) (A_{max} 445, 472.5 in benzene) and Vetter et al. (1971) (428 and 453 in petrol ether) for crocetindial (8,8′-diapocarotene-8,8′-dial). The identity of the pigment was further supported by its chromatographic properties, reaction with $NaBH_4$ to crocetindiol [A_{max} 375, 396 and 420.5 in petrol ether and 376, 397 and 421 in ethanol; according to Vetter et al. (1971) 375, 397 and 422 in petrol ether] and O-methyloxime formation. Further confirmation was obtained from the mass spectrum exhibiting ions of m/z 296, 57, 55, 91, 69, 71, 95, 83, 145, 105, 97, 81, 145 and characteristic fragment ions of m/z 213, 239, 257, 263 and 267 which is consistent with those reported by Enzell et al. (1969) and Eugster et al. (1969).

In activated cells beside crocetindial several other coloured substances appeared in trace amounts. One component of pink colour was identified as β-apo-8′-carotenal by its electronic spectra [A_{max} 452, 476 in petrol ether; 462 in ethanol; according to Yokohama and White (1966) 451, 478 and 463, resp.] formation of β-apo-8′-carotenol with $NaBH_4$ [A_{max} 423 and 449 in petrol ether and 427 and 452 in ethanol; according to Vetter et al. (1971) 425 and 452 in petrol ether], reaction to the O-methyloxims and chromatographic data which were consistent with those of an authentic sample of β-apo-8′-carotenal. However, the amounts available were not sufficient to enable mass spectrometry.

Assuming a cleavage reaction for zeaxanthin similar to that observed for β-carotene, hydroxy-β-cyclocitral should be formed in appreciable amounts. Since this substance exhibits low volatility the recovery values obtained by the stripping technique were very low. However, applying selective ion monitoring tracing the molecular ion of m/z 168 and the fragment ions of m/z 153, 150, 135 and 107, however, enabled the detection of this compound. High recovery values were achieved when a liquid-solid sorption technique was administered. The mass spectrum with prominent ions of m/z 29(100), 39(91), 41(96), 43(83), 55(65), 67(27), 79(67), 91(62), 107(89), 121(47), 135(79), 150(19), 153(15), 168(25) and the retention time obtained were in full accordance to the data of an authentic sample of 2,6,6-trimethyl-4-hydroxy-cyclohex-1-en-1-carboxaldehyde.

Table 2. Stoichiometric relationship between β-cyclocitral and β-carotene in native and cells of *Microcystis* (20 ml of 8.7×10^7 cells/ml) activated by freezing for 3 h

Native cells (μM)	Activated cells (μM)	Compounds
0.6	0.1	β-Carotene
0	1.4	β-Cyclocitral

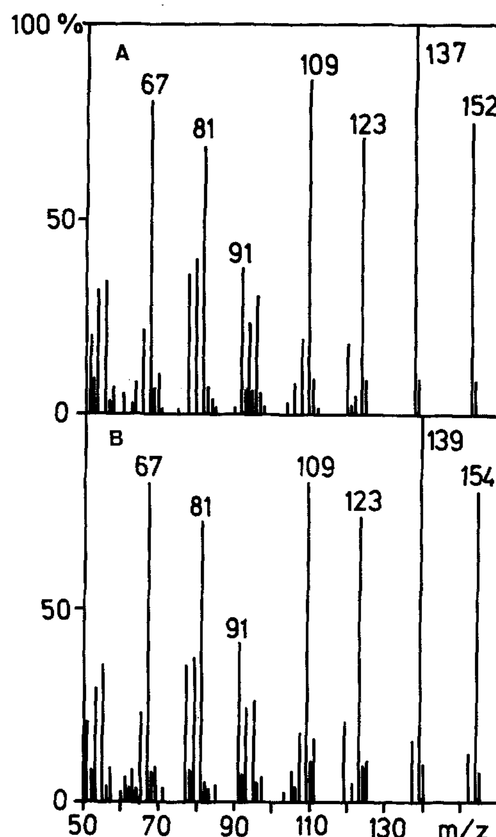

Fig. 1 A, B. Electron impact mass spectra of natural **A** and ^{18}O-labelled **B** β-cyclocitral both isolated from *Microcystis*

In further experiments the quantitative relationship between the β-carotene degradation and the formation of β-cyclocitral was studied. While the data on β-cyclocitral presented in Table 2 are corrected by spiking with authentic β-cyclocitral the actual concentrations of β-carotene must be assumed higher because a loss of about 20% during TLC can be taken as a basis.

$^{18}O_2$- and $H^{18}O$-labelling of the nor-carotenoids. To study the incorporation of dioxygen into both β-carotene cleavage products, β-cyclocitral and crocetindial, a *Microcystis* suspension was freed of oxygen by gassing with argon, subsequently equilibrated with $^{18}O_2$, frozen to active the β-carotene oxygenase and worked-up after thawing. Preceeding experiments in which the degradation of carotene had been stopped by addition of perchloric acid after different time intervals had indicated that the β-carotene degradation starts with the thawing process. The mass spectrometric analysis of β-cyclocitral performed after gas liquid-chromatography indicated a strong labelling with 18-oxygen (Fig. 1). Using either the molecular ions (M; m/z 152 and 154) or the fragment ions ($M-CH_3$; m/z 137 and

139) as the basis for the calculation, identical values of 86% labelling with 18-oxygen were obtained. The other oxygen containing fragment-ions ($M - C_2H_5$ and $M - C_3H_7$) which contribute a minor part together with hydrocarbon ions ($M - CHO$ and $M - CH_2-CHO$) to the masses *m/z* 109 and 123 were also labelled and could easily be diagnosed as newly formed fragment ions of *m/z* 111 and 125.

To analyse the incorporation of $^{18}O_2$ into hydroxy-β-cyclocitral, the molecular ion *m/z* 168 gave a signal of sufficient intensity to be used beside several fragment ions. The fragment ion *m/z* 150 derives from elimination of H_2O resulting from the loss of the hydroxy-group of the molecule. Cleavage of the tertiary methyl group accounts for the fragment ion of *m/z* 135. Removal of CHO and CH_2CHO results in fragment ions of *m/z* 121 and 107 giving intense signals, but which are free of aldehyde oxygen and therefore without diagnostic value. If the ion couples *m/z* 168/170, 151/153, 150/152 and 135/137 were used as a basis for calculation a percentage labelling of 20, 19, 25 and 18% with a mean average of 20.5% was observed.

Beside β-cyclocitral and hydroxy-β-cyclocitral 67 μg of crocetindial was isolated in the course of the same experiment and its mass spectrum recorded on a mass spectrometer with direkt inlet. No significant labelling (less than 2%) was observed in this molecule. While work-up of and isotope determination in β-cyclocitral was performed shortly after $^{18}O_2$-incorporation, isolation and purification of crocetindial needed more time during which a contact with water was given resulting in a complete exchange of the labelled aldehyde oxygen. The rapid exchange of the aldehyde oxygen could be demonstrated using unlabelled β-cyclocitral that had been incubated in $H_2{}^{18}O$. An exchange as high as 33% was observed after a 20 h incubation time.

To follow the incorporation of $H_2{}^{18}O$ into β-cyclocitral and crocetindial, a *Microcystis* cell paste was suspended in 0.5 ml of $H_2{}^{18}O$ and worked up as described above. β-Cyclocitral exhibited only a weak labelling of 19 and 17% corresponding to the fragment ions (*m/z* 137 and 139) and molecular ions (*m/z* 152 and 154), respectively. The labelling of crocetindial was in the range of the background.

Substrates of the β-carotene oxygenase. The β-carotene cleavage enzyme, further termed β-carotene oxygenase, is membrane bound and can be partly solubilized by the application of detergents combined with sonification. To retain enzyme activity, however, the handling has to be performed under strict exclusion of oxygen. This was achieved in an argon-atmosphere in combination with the use of a glucose oxidase based reaction in which the oxidase bounds oxygen with high affinity resulting in the consumption of even trace amounts thereof. Cells frozen under strict anaerobiosis did not yield any β-cyclocitral when treated with perchloric acid after thawing. Cells which had been activated by freezing or solubilized β-carotene oxygenase did not retain activity for long periods when oxygen had free access. Usually the occurrence of β-carotene oxygenase activity was accompanied by the presence of thylakoid fragments. To obtain some initial insight into the reaction mechanism of the oxygenase, all further experiments were performed using the cell-bound enzyme obtained by freezing and thawing whole cells. As a measure of the enzyme activity the formation of β-cyclocitral was used and determined quantitatively by gas liquid-chromatography.

Table 3. Effect of different substrates on β-cyclocitral formation in activated *Microcystis* cells. β-Carotene (20 μl of 1.07 mg/2 ml ethanol) and fatty acids (300 μl of a 10 mM solution containing 25 μg Tween 20/10 ml) were added to 3 ml of a *Microcystis* suspension (1.3×10^9 cells in TES-buffer) under aerobic conditions prior to freeze activation of β-carotene oxygenase

Concentration	Substrate	Activity (%)
	Nil (reference)	100
10 μM	β-Carotene	159
1 mM	Linoleic acid	90
1 mM	α-Linolenic acid	26
1 mM	γ-Linolenic acid	33
10 μM 1 mM	β-Carotene + Linoleic acid	33
10 μM 1 mM	β-Carotene + α-Linolenic acid	29
10 μM 1 mM	β-Carotene + γ-Linolenic acid	20

Table 4. Effect of sulfhydryl reagents and sulfhydryl compounds on the activity of β-carotene oxygenase. Prior to freezing the *Microcystis* cells for activation they were resuspended in TES-buffer containing one of the listed sulfhydryl substances (0.1 mM each)

Substance added	Activity (%)
Nil (reference)	100
L-Glutathione, red.	102
L-Cysteine	86
2-Mercaptoethanol	98
Dithioerythritol	108
4-Hydroxymercuribenzoate-Na	56
N-Ethylmaleimide	5
Jodoacetate-Na	94

Since a co-oxidation reaction could not initially be ruled out, several different unsaturated fatty acids common to *Cyanobacteria* were offered as substrates in addition to β-carotene. While β-carotene significantly increased the formation of β-cyclocitral the unsaturated fatty acids were all inhibitory (Table 3). The addition of lipase also slightly reduced the formation of β-cyclocitral. The application of an ethanolic lipid extract obtained from native cells was without effect.

Effects of sulfhydryl reagents and sulfhydryl compounds. Both the sulfhydryl reagents, 4-hydroxymercuribenzoate and, more strongly, N-ethylmaleimide inhibited the action of β-carotene oxygenase. Jodoacetate was not effective (Table 4). Addition of sulfhydryl compounds resulted in minor effects on the activity of the enzyme: dithioerythritol gave rise to a small increase in β-cyclocitral formation, cysteine was slightly inhibitory and the effects of glutathione and 2-mercaptoethanol were within the experimental error.

Effect of inhibitors on the β-carotene oxygenase reaction. Several inhibitors were found which effectively inhibited the formation of β-cyclocitral (Table 5). Among them antioxidants proved to be the most effective ones. *tert.*-Butylhydroxyquinone, methyl gallate and nordihydroguaiarectic acid resulted in a complete inhibition while *tert.*-

Table 5. Effect of inhibitors on the activity of β-carotene oxygenase. Inhibitors were added prior to activation of the enzyme under an atmosphere of air. In the case of Phen (argon) activation was carried out under strictly anaerobic conditions (Ar-atmosphere and glucose oxidase reagent) followed by incubation under air. (TBHQ = *tert.*-butylhydroquinone, TBHA = *tert.*-butyl-4-hydroxyanisol, MG = methyl gallate, NDGA = nordihydroguaiaretic acid, AET = 2-aminoethylisothiouronium bromide hydrobromide, Phen = *o*-phenanthroline)

Concentration (mM)	Inhibitor	Activity (%)
	Nil (reference)	100
0.5	TBHQ	0
0.5	TBHA	100
1.0	TBHA	39
2.0	TBHA	9
0.5	MG	0
0.5	NDGA	0
0.1	AET	58
1.0	AET	37
0.01	Phen	63
0.1	Phen	60
0.1	Phen (argon)	0

butylhydroxyanisol was only partially effective. A similar decrease in the amount of β-cyclocitral formation was also obtained with the radical scavenger 2-aminoethylisothiouronium bromide hydrobromide. *o*-phenanthroline, a powerful chelating agent, completely inhibited the reaction when the activation was carried out under oxygen free conditions and only for the subsequent reaction oxygen had free access. Under aerobic conditions a decrease of only 40% was observed. Gassing the suspension with a 50% CO/air mixture, a strong inhibitor for bacterial monooxygenases (Wagner 1983), was without any effect on the reaction.

Effect of metal ions upon the β-carotene oxygenase activity. To study the effect of different metal ions on the activity of β-carotene oxygenase, two series of experiments were conducted. In one series activation of the oxygenase was performed under exclusion, in the other under free access of oxygen. In both series of experiments performed at pH 7.6 (Table 6) exclusively iron and calcium ions proved to be stimulatory for the reaction. Applying a lower pH value of 6.5 (Table 7) calcium ions were inhibitory, however, iron ions remained stimulatory. The other ions assayed exhibited either no significant effect or inhibited the formation of β-cyclocitral.

Table 6. Effect of metal kations on the activity of the carotene oxygenase at pH 7.6. The carotene oxygenase of 6.6×10^7 cells was determined in TES-buffer of pH 7.6. In one set of experiments metal ions were added before freezing under an atmosphere of air. In the other series 0.1 mM *o*-phenanthroline was added prior to freezing under strictly anaerobic conditions. After thawing the supernatant was replaced by buffers with various metal ions and incubated in the air

Concentration (mM)	Kation	Activity (%) (argon)	Activity (%) (air)
Nil	Reference	100	100
0.1	Fe^{2+}	113	101
1.0	Fe^{2+}	107	95
0.1	Fe^{3+}	119	103
1.0	Fe^{3+}	124	116
0.1	Mn^{2+}	54	20
1.0	Mn^{2+}	54	87
0.1	Zn^{2+}	15	84
1.0	Zn^{2+}	–	41
0.1	Cu^{2+}	58	86
1.0	Cu^{2+}	25	43
0.1	Ni^{2+}	104	94
1.0	Ni^{2+}	78	90
0.1	Co^{2+}	80	101
1.0	Co^{2+}	70	105
0.1	Mg^{2+}	53	102
1.0	Mg^{2+}	65	79
0.1	Ca^{2+}	69	132
1.0	Ca^{2+}	114	118

Table 7. Effect of metal kations on the activity of the carotene oxygenase at pH 6.5. For the performance of the experiments see Table 6, however, TES-buffer was replaced by PIPES-buffer of pH 6.5

Environment	Concentration (mM)	Kation	Activity (%)
Argon	1.0	Ca^{2+}	54
	1.0	Mn^{2+}	75
	0.05	Fe^{3+}	99
	0.1	Fe^{3+}	99
	0.5	Fe^{3+}	120
	0.1	Fe^{2+}	106
	0.5	Fe^{2+}	117
Air	1.0	Ca^{2+}	96
	0.1	Mn^{2+}	65

Discussion

Carotene degrading enzymes have not been reported in microorganisms although the occurrence of several specific carotene degradation products pointed to their existence. Among the *Cyanobacteria* assayed for nor-carotenoids, the genus *Microcystis* is unique in its ability to liberate large amounts of β-cyclocitral (Jüttner 1976). The enzymatic reaction responsible for the formation of this compound can be activated by treatments that lead to the disintegration of the cells. The most convenient and reproducibly applicable method is freezing the cells which causes the enzyme to become fully active upon subsequent thawing.

The primary product of β-cyclocitral is β-carotene, the β-ionone rings of which are oxidatively split off. Taking a 20% loss of β-carotene during TLC separation into account a stoichiometry of about 2 mols β-cyclocitral formed per 1 mole β-carotene degraded was observed. Beside β-carotene, zeaxanthin is involved in an analogeous reaction that exhibited the same splitting location in the carotenoid molecule at the positions 7,8 and 7′,8′. In this case the β-ionone ring should be liberated as hydroxy-β-cyclocitral. In fact, the formation of this compound could be well established by mass spectrometry and gas liquid-chromatography. The identity of this compound with 2,6,6-

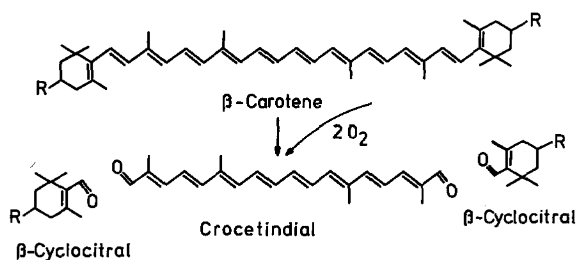

Fig. 2. Cleavage reaction of the β-carotene 7,8 (7′,8′)oxygenase. $R = \mathrm{H}$: β-carotene and β-cyclocitral; $R = \mathrm{OH}$: zeaxanthin and hydroxy-β-cyclocitral

trimethyl-4-hydroxy-cyclohex-1-en-1-carboxaldehyde was confirmed using an authentic sample.

The central molecular part of the oxidized β-carotene which is retained after splitting off the β-ionone rings can readily be recognized as a newly formed yellow pigment in *Microcystis* cells activated by freezing. It has the behaviour of a dialdehyde and its identity with crocetindial was established with several methods. Upon quantitative analysis of the pigments there appeared to be a stoichiometric relationship between the combined decrease in the amounts of β-carotene and zeaxanthin and the increase of crocetindial when these were expressed in molar terms. This is consistent with a cleavage reaction as outlined in Fig. 2. The slightly diminished molar amount of crocetindial observed in all experiments points to the formation of several minor pigment products which are detectable by TLC. However, these minor pigments do not surpass a few percent of the total amount of carotenoid pigments. One of these components was tentatively identified as β-apo-8′-carotenal and may possibly be regarded as an intermediate of the cleavage reaction. Echinenone and myxoxanthophyll were not degraded. This may be due to their different structures or their spatial separation in the cell which does not enable them to come in contact with the β-carotene oxygenase.

Oxidant for the carotene degradation reaction was dioxygen since no reaction could be observed under strictly anaerobic conditions. The direct incorporation of dioxygen into the aldehyde function was demonstrated using 18-dioxygen. Incorporation of label in β-cyclocitral was as high as 86% in two independent experiments. The small amount of unlabelled β-cyclocitral molecules is very likely the result of the ready exchange of oxygen generally observed between water and aldehyde groups (Jefford and Cadby 1981). Treatment of unlabelled synthetic β-cyclocitral with $H_2{}^{18}O$ for 20 h resulted in a labelling of 33% intensity indicating the rapid exchange reaction. The exchange of ^{18}O-labelled aldehyde with unlabelled water seems to be even more fascilitated in the case of hydroxy-β-cyclocitral in which molecule only 20.5% labelling was retained after work-up. In crocetindial no significant labelling was observed neither after application of 18-dioxygen nor with $H_2{}^{18}O$. This may be due to the rather lengthy work-up and cleaning procedures necessary for mass spectrometry resulting in a total exchange with unlabelled water.

β-Carotene can unequivocally be assumed to be a precursor of β-cyclocitral, but although addition of this compound stimulated the total reaction it did not support the reaction for long time intervals. This may be due to the sensitivity of the β-carotene oxygenase to dioxygen which had free access after starting the reaction. The need of a further co-factor that is used up in the reaction is not very likely since washing of activated cells did not result in a loss of activity and the addition of cell extracts failed to support or stimulate the reaction. A co-oxidation of β-carotene mediated by unsaturated fatty acids which has been observed in higher plants does not seem to be involved in the reaction (Grosch and Laskawy 1979). All unsaturated fatty acids added to the assay were inhibitory. From most dioxygenases that have been studied so far (Jefford and Cadby 1981), iron seems to be an essential part of the enzyme. This can be demonstrated by the application of *o*-phenanthroline, an effective chelating agent for divalent iron. When the activation of the oxygenase takes place in the presence of *o*-phenanthroline under strictly anaerobic conditions where iron is preferentially in the reduced state, subsequent addition of air failed to support the reaction. Under aerobic conditions of the activation when iron is oxidized to the trivalent state *o*-phenanthroline only partially inhibits β-cyclocitral formation. The involvment of iron in the reaction is supported by the fact that the addition of iron ions activates the β-carotene oxygenase after treatment with *o*-phenanthroline. This was not observed after identical treatment with a series of other divalent metal cations. Studies with several inhibitors, of which antioxidants proved to be the most powerful, indicated the occurrence of radicals as intermediates in the oxygenation reaction. TBHQ exhibited much higher effectivities than BHA and AET. It has been postulated that these substances attack different radicals. TBHQ has been reported to effectively scavange radicals of enzyme substrate complexes (Klein et al. 1984) while the latter preferentially attacks alkyl radicals (Bakker et al. 1983). Since physical data are not yet available considerations of the reaction mechanism must remain, however, speculative.

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