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Enhanced Carotenoid Biosynthesis by Oxidative Stress in Acetate-Induced Cyst Cells of a Green Unicellular Alga, *Haematococcus pluvialis*

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In a green alga, *Haematococcus pluvialis*, a morphological change of vegetative cells into cyst cells was rapidly induced by the addition of acetate or acetate plus Fe^{2+} to the vegetative growth phase. Accompanied by cyst formation, algal astaxanthin formation was more enhanced by the addition of acetate plus Fe^{2+} than by the addition of acetate alone. Encystment and enhanced carotenoid biosynthesis were inhibited by either actinomycin D or cycloheximide. However, after cyst formation was induced by the addition of acetate alone, carotenoid formation could be enhanced with the subsequent addition of Fe^{2+} even in the presence of the inhibitors. The Fe^{2+}-enhanced carotenogenesis was inhibited by potassium iodide, a scavenger for hydroxyl radical, suggesting that hydroxyl radical formed by an iron-catalyzed Fenton reaction may be required for enhanced carotenoid biosynthesis. Moreover, it was demonstrated that four active oxygen species, singlet oxygen, superoxide anion radical, hydrogen peroxide, and peroxy radical, were capable of replacing Fe^{2+} in its role in the enhanced carotenoid formation in the acetate-induced cyst. From these results, it was concluded that oxidative stress is involved in the posttranslational activation of carotenoid biosynthesis in acetate-induced cyst cells.

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is accumulated in a potent producer, *Haematococcus pluvialis*, under unfavorable culture conditions such as nitrogen deficiency (23). Astaxanthin has been used not only as a pigmentation source for fish aquaculture (4) but also as a powerful antioxidative reagent (18). The massive accumulation of astaxanthin by the green alga during autotrophic growth under CO_2 has been investigated under conditions of nitrogen limitation (6) and illumination (15). In our previous study, an Fe^{2+}-rich medium with acetate as a carbon source was developed for improved astaxanthin production. In this heterotrophic growth medium, a rapid morphological change into an enlarged resting cell or a cyst was observed after only 6 days of cultivation (13, 14), whereas it took several weeks under autotrophic conditions (6). Moreover, astaxanthin formation was drastically stimulated in an Fe^{2+}-concentration-dependent manner when a high concentration of acetate was added to the vegetative growth phase (13). It was later demonstrated that the addition of acetate caused a high carbon/nitrogen ratio in the medium, leading to cyst formation possibly because of the relative deficiency of nitrogen (12). Thus, a question was raised: What is the role of Fe^{2+} in the enhanced carotenoid formation in the acetate-induced cyst cell? In the present article, from the regulation kinetics of astaxanthin biosynthesis in the green alga, it was shown that once the cyst formation was induced by the acetate addition, the carotenoid biosynthesis was enhanced by the Fe^{2+} addition even in the presence of transcriptional and translational inhibitors. Furthermore, four kinds of active oxygen species were able to replace Fe^{2+} in its role in the enhanced carotenoid formation, indicating that hydroxyl radical generated from an iron-involved Fenton reaction and other active oxygen species played an important role in the activation of carotenogenesis.

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MATERIALS AND METHODS

Algal strain. *H. pluvialis* Flotow NIES-144 was obtained from the National Institute for Environmental Studies, Tsukuba, Japan.

Basal culture. The basal medium (pH 6.8) consisted of 1.2 g of sodium acetate, 2.0 g of yeast extract, 0.4 g of L-asparagine, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 0.02 g of $CaCl_2 \cdot 2H_2O$ per liter of deionized water (13). For the basal culture, a 10-ml portion of a 4-day culture was inoculated into 100 ml of fresh basal medium in a 200-ml Erlenmyer flask. The flask was incubated at 20°C under a 12-h light–12-h dark illumination cycle at 1.5 klx (fluorescent lamp). The flask was shaken manually once a day. The 4-day culture (vegetative growth phase, ca. 5.5×10^5 cells per ml) was employed for the supplementation culture.

Supplementation culture. Sodium acetate solution (2.25 M, pH 7.0), ferrous sulfate solution (22.5 mM, pH 1.5), or both were added to the 4-day culture at 45 mM and 450 μM, respectively. After the addition, the light intensity was increased from 1.5 to 8.6 klx, and the illumination period was extended from 12-h light–12-h dark to continuous illumination. The supplementation culture was incubated at 20°C under gentle mixing with a magnetic stirrer. All of the cultures were done in duplicate for analysis.

Transcriptional and translational inhibitors. A transcriptional inhibitor (actinomycin D) and translational inhibitors (cycloheximide for cytoplasmic protein synthesis and chloramphenicol for mitochondrial and chloroplastic protein synthesis) were used to study the regulatory role of the acetate and/or Fe^{2+} addition in carotenoid formation. Actinomycin D was added 12 h before the addition of acetate plus Fe^{2+} to the 4-day culture. The translational inhibitors were also added 4 h before the addition of acetate plus Fe^{2+}. To examine the effect of the Fe^{2+} addition in acetate-induced cyst cells on carotenoid formation, only acetate was added to the 4-day culture. The inhibitors were added 36 h after the acetate addition. Fe^{2+} was added to the culture 48 h after the

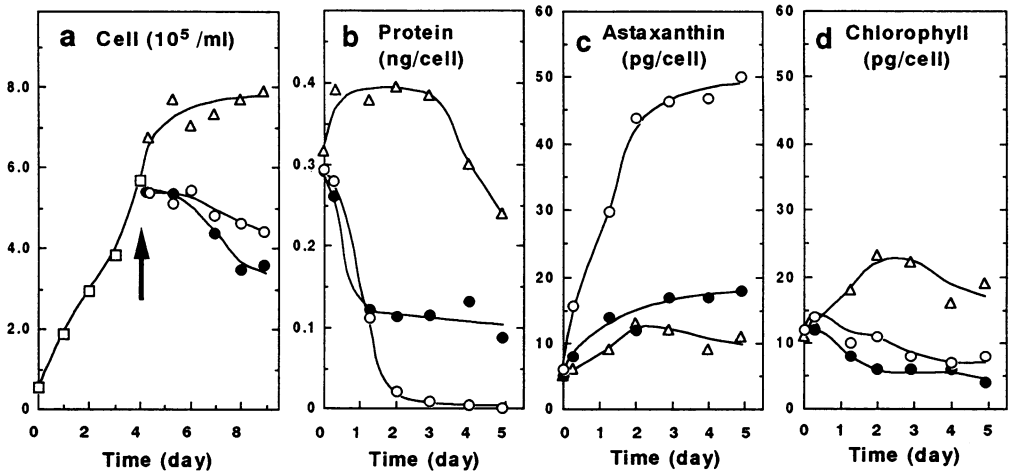

FIG. 1. Effects on cell growth (a), protein content (b), carotenoid formation (c), and chlorophyll content (d) of addition of acetate, Fe^{2+}, or both to the vegetative growth phase of *H. pluvialis*. The arrow indicates the time of the addition of acetate (45 mM), Fe^{2+} (450 μM), or both. The supplementation time was scaled as day zero in panels b, c, and d. Symbols: △, Fe^{2+}; ●, acetate; ○, acetate plus Fe^{2+}.

acetate addition, followed by the equivalent pretreatment with the inhibitors.

Active oxygen generating reagents. Four active oxygen reagents were used: methylene blue for singlet oxygen ($^1O_2$) (18), methyl viologen for superoxide anion radical (O_2^-) (10), H_2O_2, and 2,2′-azo-*bis*(2-amidinopropane)-dihydrochloride (AAPH) for peroxy radical (AO_2·) (10, 16, 22). The oxidative reagent solutions were filtered through a membrane filter (Dismic-25, 0.45-μm pore size; Advantec, Tokyo, Japan) and added to the 4-day culture. At the time of addition of the oxidative reagents, acetate was also added at 45 mM.

Quencher and scavengers. 1,4-Diazabicyclo[2.2.2]octane (DABCO) was employed as a specific quencher for $^1O_2$ (20). 1,2-Dihydroxy-benzene-3,5-disulfonic acid (Tiron) and potassium iodide were used as specific scavengers for O_2^- and hydroxyl radical (HO·), respectively (20). At the time of addition of the oxidative reagents and acetate, the quencher or the scavengers were also added to the 4-day culture.

Analyses. The cell number was counted with a hemacytometer. For the protein assay, the algal cells were suspended in 2 M NaOH for 1 h on ice as described by Whitelam and Codd (26), and the alkaline-solubilized protein was determined by the Bradford method (7), with bovine serum albumin as the standard. After the algal cells were ground with a pestle and a mortar, carotenoids and chlorophylls were extracted with 90% (vol/vol) acetone for 1 h. Astaxanthin was determined at 480 nm by using an absorption coefficient, $A_{1\%}$, of 2,500. In this study, it was ascertained that the sum of the diester and monoester forms of astaxanthin was invariably more than 90% of the total extracted carotenoids determined by thin-layer chromatography analysis as described previously (13, 14). Thus, astaxanthin was regarded as the major carotenoid in this green alga. The chlorophyll was calibrated against chlorophyll *a* as the major chlorophyll component by the method of Strickland and Parsons (24). All of the analyses were carried out for duplicate cultures, and their averages are reported.

Chemicals. Actinomycin D, cycloheximide, and chloramphenicol were obtained from Sigma Chemical Co. AAPH, Tiron, and DABCO were purchased from Wako Pure Chemical Industries, Osaka, Japan, and all other reagents were from Katayama Chemical, Osaka, Japan.

RESULTS

Addition of acetate and/or Fe^{2+} to the vegetative growth phase. Acetate, Fe^{2+}, or both were added to the 4-day culture to investigate their effect on carotenoid formation (Fig. 1). In the case of Fe^{2+} addition, the algal cell maintained vegetative growth until the stationary phase (Fig. 1a), swimming actively with the two flagella (Fig. 2a). The algal cells remained green because of the relatively high chlorophyll content and the low carotenoid content (Fig. 1c and d), indicating no significant metabolic changes after the Fe^{2+} addition. In contrast, when acetate was added at 45 mM, the algal cell rapidly underwent a morphological change from a biflagellated oval cell to an enlarged round cyst cell with a thick cell wall (Fig. 2b) and was slightly decreased in number. The cyst formation was accompanied by a decrease in both protein content and chlorophyll content (Fig. 1b and d). In our previous study, it was shown that the encystment took place under a high C/N ratio, while it was delayed under a low C/N ratio (12).

With the addition of acetate plus Fe^{2+}, the cyst formation proceeded markedly, with a disappearance of the cellular protein even more extensive than that when acetate alone was added (Fig. 1b). Furthermore, the carotenoid formation was drastically enhanced (Fig. 1c), resulting in the distinct appearance of the enlarged dark-red cyst cells (Fig. 2c). Therefore, how the carotenoid biosynthesis in the green alga would be regulated by the addition of acetate plus Fe^{2+} was of interest.

Effect of transcriptional and translational inhibitors on enhanced carotenoid formation. Actinomycin D or cycloheximide was added to the vegetative growth phase before the addition of acetate plus Fe^{2+} as described in Materials and Methods. In the presence of either actinomycin D or cycloheximide at the concentration tested, enhanced carotenoid formation was significantly inhibited, as shown in Fig. 3. When the algal cells were pretreated with actinomycin D or cycloheximide, the encystment was not observed. On the other hand, chloramphenicol was inhibitory neither for carotenoid formation nor encystment at 50 μg/ml. Since cyst formation could be induced by the sole addition of acetate (12), it was indicated that both encystment and enhanced carotenoid formation should be regulated at the transcrip-

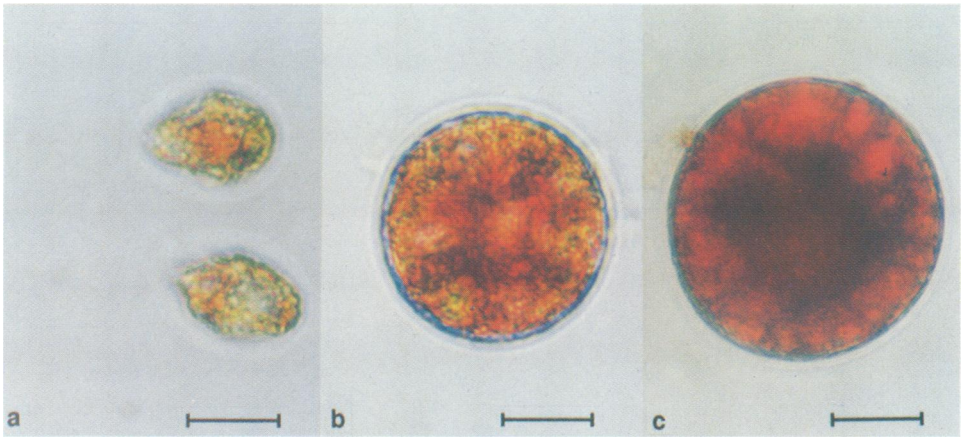

FIG. 2. Morphological changes of *H. pluvialis* after addition of acetate, Fe^{2+}, or both. Major cells observed in 4-day culture after the supplementation, namely, vegetative cells after Fe^{2+} addition (a), an enlarged cyst cell after acetate addition (b), and an enlarged red cyst cell after the addition of acetate plus Fe^{2+} (c), are shown. Bar, 20 μm.

tional level of the algal chromosomal genes, possibly not of organellic genes, and these metabolic changes could be induced by the addition of acetate plus Fe^{2+}. Since the sole addition of Fe^{2+}, however, did not bring any significant metabolic changes, it was necessary to examine the role of Fe^{2+} in the acetate-induced cyst cells.

To investigate the effect on carotenoid formation of the Fe^{2+} addition to the acetate-induced cyst cells, encystment was induced by adding only acetate to the 4-day culture. The flagella of most algal cells disappeared during the 36-h incubation period after the acetate addition. At 36 h, the algal cells were treated with the transcriptional or translational inhibitors. Fe^{2+} was then added to the culture 48 h after the acetate addition. As shown in Fig. 4, after encystment was induced by the acetate, the carotenoid formation enhanced by the addition of Fe^{2+} was no longer blocked by the presence of the inhibitors. However, when the inhibitors and Fe^{2+} were added 18 and 30 h after the acetate addition, respectively, the carotenoid formation was completely arrested (data not shown). Therefore, the induction of encystment, as well as the carotenogenic enzyme(s), should have been accomplished between 18 and 36 h after the acetate addition. The synthesis of several proteins was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by L-[^{35}S]methionine incorporation at 3 h after the addition of acetate or acetate plus Fe^{2+} (data not shown), while most proteins disappeared rapidly after the acetate addition, as shown in Fig. 1b. The incorporation of radioactivity was drastically reduced with a longer incubation time (over 6 h)

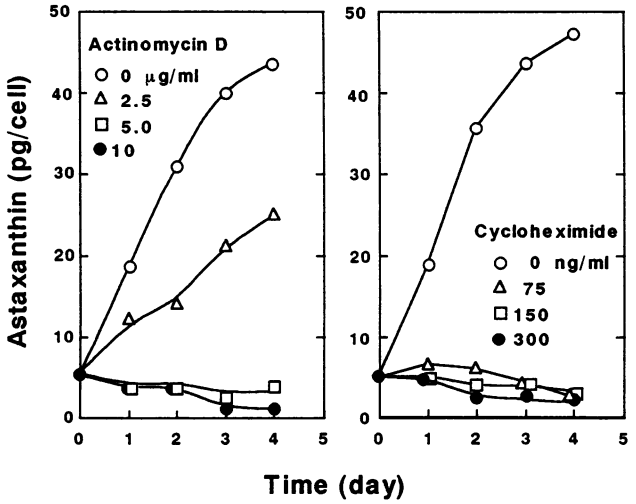

FIG. 3. Effect of transcriptional and translational inhibitors on carotenoid formation of *H. pluvialis*. Actinomycin D or cycloheximide was added to the culture 12 or 4 h before the addition of acetate plus Fe^{2+} to the 4-day culture. The supplementation time was scaled as day zero.

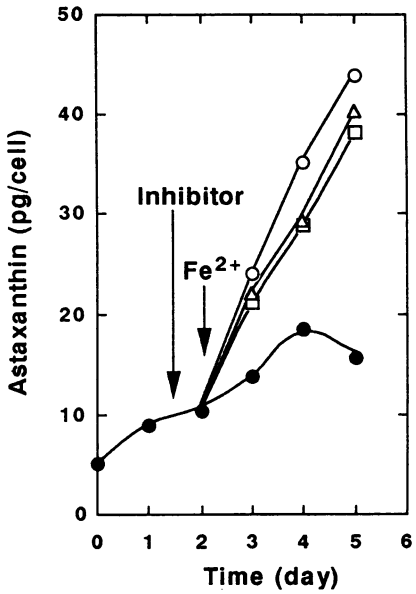

FIG. 4. Effect of inhibitors on carotenoid formation in acetate-induced cyst cells of *H. pluvialis*. Acetate was added as described in the legend to Fig. 1. The inhibitors and Fe^{2+} were added at the times indicated by the arrows. The concentrations were as follows: acetate, 45 mM; Fe^{2+}, 450 μM; actinomycin D, 10 μg/ml; cycloheximide, 300 ng/ml. Symbols: ●, acetate; ○, acetate plus Fe^{2+}, △, acetate, actinomycin D, and Fe^{2+}; □, acetate, cycloheximide, and Fe^{2+}.

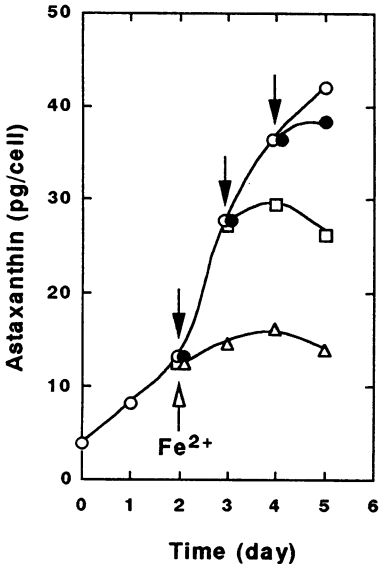

FIG. 5. Effect of potassium iodide, a specific scavenger for hydroxyl radical, on Fe^{2+}-enhanced carotenoid formation in acetate-induced cyst cells of *H. pluvialis*. Acetate and Fe^{2+} were added as described in the legend to Fig. 4. The scavenger was added at the times indicated by the arrows. The concentrations were as follows: acetate, 45 mM; Fe^{2+}, 450 μM; potassium iodide, 10^{-3} M. Potassium iodide was added at 2 (△), 3 (□), or 4 (●) days after the acetate addition or not at all (○).

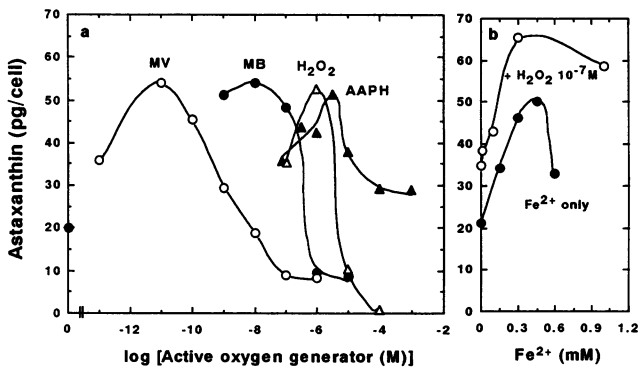

FIG. 6. Effect of active oxygen species on carotenoid formation of *H. pluvialis*. Active oxygen species were supplemented with acetate in the 4-day culture. Carotenoid formation was determined for the 4-day culture after the addition of acetate at 45 mM and each active oxygen generator at the indicated concentrations. Abbreviations: MV, methyl viologen; MV, methylene blue.

after the addition, which may be due to the dilution of the labeled methionine caused by the rapid protein degradation.

On the basis of these results, it was indicated that the enhanced carotenoid biosynthesis by the Fe^{2+} addition in the acetate-induced cyst cells does not require de novo protein synthesis. In other words, regulation of the Fe^{2+}-enhanced carotenogenesis may be at the posttranslational level, possibly at the activation of the carotenogenic enzyme(s) system by the Fe^{2+} addition.

Effect of active oxygen generators. Ferrous ion is known to form a highly reactive oxygen-free radical, hydroxyl radical (HO·), by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow OH^- + HO\cdot + Fe^{3+}$ (10). To examine the possible function of Fe^{2+} as the free radical generator, first, potassium iodide, a specific scavenger for HO·, was added to the different stages of the carotenogenesis enhanced by the Fe^{2+} addition (Fig. 5). From the result, it seems likely that HO· generated in the presence of Fe^{2+} was essential for the stimulated carotenogenesis at any stage tested. It has been ascertained that potassium iodide itself was not inhibitory for the carotenoid formation, as shown in Table 1. Second, available active oxygen-generating reagents other than hydroxyl radical were employed to study whether the other types of active oxygen species can replace Fe^{2+} in its role in enhanced carotenoid formation in the acetate-induced cyst. Four active oxygen generators were added simultaneously together with acetate to the 4-day culture. As shown in Fig. 6a, all of the active oxygen species were capable of replacing the Fe^{2+} in its role in enhanced carotenoid formation to almost the same extent. Methyl viologen, which generates superoxide anion radical, was effective at an extremely low concentration of 10 pM, while AAPH, which degrades to form two molecules of peroxy radical, and hydrogen peroxide were stimulative for

TABLE 1. Effect of scavengers and a quencher on enhanced carotenoid formation by oxidative stress

Scavenger or quencher added	Concn (M)	Astaxanthin content (pg/cell)[a]				
		Methylene blue (1.0×10^{-8} M)	Methyl viologen (1.0×10^{-11} M)	AAPH (3.0×10^{-6} M)	H_2O_2 (1.0×10^{-6} M)	Fe^{2+} (4.5×10^{-4} M)
No addition		53.9 (100)	54.1 (100)	51.0 (100)	52.3 (100)	50.0 (100)
Tiron	10^{-2}	49.6 (92)	19.5 (36)	46.4 (91)	55.4 (106)	ND[b]
	10^{-3}	56.6 (105)	44.4 (82)	43.9 (86)	59.6 (114)	ND
	10^{-4}	59.3 (110)	52.5 (97)	43.9 (86)	54.4 (104)	ND
KI	10^{-3}	44.7 (83)	23.3 (43)	52.5 (103)	54.4 (104)	17.0 (34)
	10^{-4}	44.7 (83)	44.4 (82)	55.1 (108)	53.3 (102)	30.0 (60)
	10^{-5}	54.4 (101)	47.1 (87)	51.0 (100)	50.7 (97)	42.5 (85)
DABCO	10^{-2}	3.2 (6)	15.2 (28)	9.7 (19)	13.1 (25)	3.0 (6)
	10^{-3}	18.3 (34)	33.5 (62)	17.9 (35)	14.6 (28)	30.0 (60)
	10^{-4}	32.3 (60)	26.5 (49)	41.3 (81)	57.0 (109)	34.0 (68)

[a] Relative values given the sole addition of each active oxygen species as the control (100%) are shown in parentheses. The active oxygen species were $^1O_2$ (from methylene blue), O_2^- (from methyl viologen), $AO_2\cdot$ (from AAPH), and HO· (from Fe^{2+}).

[b] ND, not determined because of precipitation of Tiron and Fe^{2+}.

carotenogenesis at concentrations on the order of 1 μM. As a singlet-oxygen-generating catalyst under illumination, methylene blue exhibited enhanced carotenoid formation at a concentration as low as nanomolar. With an excessive addition, however, carotenoid formation was drastically reduced in all of the active oxygen species. Moreover, the combined effect of the addition of hydrogen peroxide and Fe^{2+} was superior to the sole addition of either Fe^{2+} or hydrogen peroxide, as shown in Fig. 6b. Thus, it was verified that Fe^{2+} can work as an HO· generator through an iron-catalyzed Fenton reaction in the cyst cells to enhance carotenogenesis. The enhanced carotenoid formation by these oxidative stresses was also inhibited by pretreatment with actinomycin D or cycloheximide (data not shown) but was unaffected by the same inhibitors after the encystment was induced by the acetate addition. The result was consistent with the Fe^{2+}-enhanced carotenoid formation, as shown in Fig. 4. From these results, it was concluded that active oxygen species may be required for the posttranslational activation of carotenoid biosynthesis in cyst cells.

Effect of scavengers and quencher on enhanced carotenoid formation. Scavengers for O_2^- (Tiron) and for HO· (KI) or a quencher for $^1O_2$ (DABCO) was added together with acetate and each active oxygen species to the 4-day culture (Table 1). Since Tiron was, as expected, inhibitory for the carotenoid formation enhanced by methyl viologen only, it was verified that O_2^- generated by methyl viologen was involved in the activation of carotenogenesis. Potassium iodide, however, inhibited the enhanced carotenoid formation not only by HO· but also by O_2^-, suggesting that O_2^- may be converted to HO· through an iron-catalyzed Haber-Weiss reaction: $O_2^- + H_2O_2 \rightarrow HO\cdot + OH^- + O_2$ (10). For the specificity of the scavengers, it should be noted that Tiron and potassium iodide themselves were not inhibitory for the carotenogenesis because the carotenoid formation enhanced by AAPH or H_2O_2 was not at all affected by the addition of Tiron or potassium iodide (Table 1). In the case of a $^1O_2$ quencher, i.e., DABCO, distinct degrees of inhibition were observed at 10^{-4} M: 40 to 50% inhibition for $^1O_2$ and O_2^- but negligible inhibition for H_2O_2. In contrast, nearly complete inhibition by DABCO was obtained at 10^{-2} M in all of the active oxygen species. These results implied that there may be interconversion among the active oxygen species from one species to another, depending on the intracellular redox potential, as suggested by Chance et al. (9). Therefore, it was very likely that $^1O_2$ may be the most directly effective active oxygen species for enhanced carotenogenesis. In the carotenoid formation enhanced by oxidative stress, however, it cannot be excluded that DABCO might directly inactivate the carotenoid biosynthesis not as a $^1O_2$ quencher.

DISCUSSION

While a number of articles have been published on the photoinduction of carotenoid formation in microbial secondary metabolism (8), only a few studies have focused on the involvement of oxidative stress in carotenoid biosynthesis. An oxygen-tolerant mutant of *Azospirillum brasilense* could produce more carotenoids in response to a higher dissolved-oxygen concentration in culture (11). The addition of hydrogen peroxide to a *Fusarium aquaeductuum* culture in the dark induced carotenoid synthesis, which normally took place only under illumination. The result suggested that a photooxidative product(s) formed under illumination may be replaceable by hydrogen peroxide for induction of fungal carotenogenesis (25).

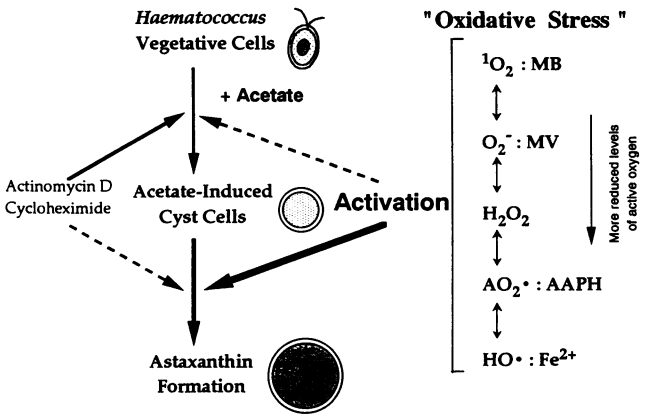

FIG. 7. Diagram of carotenogenesis enhanced by active oxygen species in an acetate-induced cyst cell of *H. pluvialis*. Abbreviations: MB, methylene blue; MV, methyl viologen. A broken line indicates that the addition was ineffective.

At the isoprenogenic enzyme level, it was recently reported that Fe^{2+} stimulated the synthesis of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) catalyzed by acetoacetyl-coenzyme A thiolase and HMG-CoA synthase, which were partially purified from radish seedlings, and that the stimulation was inhibited by the presence of a radical scavenger, hydroxyurea (2). Moreover, HMG-CoA reductase that is controlled by allosteric effectors and by phosphorylation-dephosphorylation was suggested to be also regulated by changes in the oxidation state of protein thiols (27).

Oxygen-radical-dependent activation of enzyme activity has been demonstrated for glutathione transferases from rat liver. The purified enzymes were activated either by hydrogen peroxide (1) or xanthine and xanthine oxidase, a superoxide-generating system (21), and the activation was reversed by the addition of dithiothreitol or superoxide dismutase, respectively. Furthermore, it was shown that the hydrogen peroxide-dependent activation was associated with the formation of the protein dimer. In some flavoproteins such as glutathione reductase, the formation of a stabilized cysteine-sulfenic acid bridge (-S-O-S-) instead of a normal disulfide bridge was demonstrated after H_2O_2 treatment (19). From these results, it was concluded that such enzymes may be regulated in vivo by a reactive oxygen metabolite(s) generated by cytochrome P-450 or by the environmental oxidative stress (10).

In our previous study, the astaxanthin formation of *H. pluvialis* was drastically enhanced in Fe^{2+}-rich modified medium, which led us to pursue the role of Fe^{2+} in algal carotenoid formation (13). From several lines of evidence in the present study, it was shown that Fe^{2+} would possibly function as an HO· generator via an iron-catalyzed Fenton reaction, and that HO· or other active oxygen species ($^1O_2$, O_2^-, H_2O_2, and $AO_2\cdot$) play an essential role(s) in the enhanced carotenoid formation in the algal cyst cells. It was also indicated that the carotenogenesis stimulated by oxidative stress could be regulated at the posttranslational level because only before cyst formation, not after the induction of encystment, the Fe^{2+}-enhanced carotenoid formation was inhibited by actinomycin D or cycloheximide. The results in the present study are illustrated in Fig. 7.

Of the active oxygen generators, singlet oxygen, an excited state of molecular oxygen with inverted spins, is involved in photooxidative and photodynamic reactions with

a variety of biological substances because of its electrophilic nature. These reactions include peroxidation of lipid and the specific oxidation of histidine, methionine, tryptophan and the nucleic acids, particularly guanine (10, 18). Methyl viologen, also known as the herbicide paraquat, catalyzes the formation of a superoxide anion radical by cyclic reduction of NADPH and reoxidation of oxygen (10). These two active oxygen generators were effective for algal carotenoid formation at strikingly low concentrations (Fig. 6a), which may be explained by the nature of these generators as catalysts and the high reactivities of $^1O_2$ and O_2^-. On the other hand, H_2O_2 is a weak oxidizing agent but it can cross the cell membrane easier than O_2^- and possesses a steady-state concentration as high as 10^{-9} to 10^{-7} M (9). The combined effect of H_2O_2 and Fe^{2+} on enhanced carotenoid formation (Fig. 6b) agreed well with the fact that the toxicity of H_2O_2 is derived from HO· formed by an iron-catalyzed Fenton reaction rather than H_2O_2 itself (10). For a unique active oxygen generator, AAPH, known as a water-soluble azo initiator (10, 16, 22), decomposes in a temperature-dependent manner to form carbon-centered radicals (A-N=N-A→N_2 + 2A·). The radicals can react rapidly with O_2 to yield peroxy radicals (A· + O_2 → AO_2·). It is generally accepted that peroxy radicals are capable of abstracting hydrogen from membrane lipids (10).

In the present article, it was indicated that all five kinds of active oxygen species are capable of activating astaxanthin biosynthesis, possibly without de novo protein synthesis. Therefore, it can be postulated that the active oxygen species may be involved in the structural modification of a carotenogenic enzyme(s), as indicated in glutathione transferases (1) and glutathione reductase (19). Alternatively, it would be conceivable that the active oxygen species can participate directly in the carotenogenic enzyme reactions as an oxidizer or an H acceptor (5). For example, it has been assumed that the biosynthesis of ketocarotenoids, or xanthophylls from β-carotene, should contain oxygenase-dependent reactions because molecular oxygen was introduced into some ketocarotenoids (8). Although carotenoid biosynthesis has not been characterized to date with purified enzymes, phytoene desaturase that catalyzes four-step dehydrogenation reactions from phytoene to lycopene was recently identified as flavin adenine dinucleotide- and NAD(P)-binding protein from the deduced amino acid sequence of the cloned photosynthetic bacterial gene (3). In addition, the subsequent lycopene cyclase reactions require two more dehydrogenation reactions as well. Thus, there must be effective cyclic regeneration of a flavin adenine dinucleotide and/or NAD(P) system for the carotenogenic pathway. Moreover, it can be speculated that the oxidative stress on algal cyst cells might be utilized as an oxidizer for oxygenation and hydroxylation of β-carotene or as an H acceptor for the NAD(P) regeneration. The latter hypothesis was in good agreement with a recent report that artificial quinone compounds can be substituted for molecular oxygen as a terminal electron acceptor in phytoene desaturation of chromoplasts of daffodil flowers (17). Therefore, how the green alga would respond to the environmental oxidative stress with carotenoid formation is of interest. To examine the role of the active oxygen species in the enhanced carotenogenesis in the algal cyst cells, the carotenogenic enzyme reactions should be characterized in terms of the enzyme kinetics under the oxidative stress.

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REFERENCES

1. **Aniya, Y., and M. W. Anders.** 1992. Activation of rat liver microsomal glutathione *S*-transferase by hydrogen peroxide: role for protein-dimer formation. Arch. Biochem. Biophys. **296**:611–616.
2. **Bach, T. J., A. Boronat, C. Caelles, A. Ferrer, T. Weber, and A. Wettstein.** 1991. Aspects related to mevalonate biosynthesis in plant. Lipids **26**:637–648.
3. **Bartley, G. E., T. J. Schmidhauser, C. Yanofsky, and P. A. Scolnik.** 1990. Carotenoid desaturases from *Rhodobacter capsulatus* and *Neurospora crassa* are structurally and functionally conserved and contain domains homologous to flavoprotein disulfide oxidoreductases. J. Biol. Chem. **265**:16020–16024.
4. **Bernhard, K.** 1990. Synthetic astaxanthin—the route of a carotenoid from research to commercialization, p. 337–363. *In* N. I. Krinsky, M. M. Mathews-Roth, and R. F. Taylor (ed.), Carotenoids—chemistry and biology. Plenum Press, New York.
5. **Beyer, P., and H. Kleinig.** 1990. On the desaturation and cyclization reactions of carotenes in chromoplast membranes, p. 195–206. *In* N. I. Krinsky, M. M. Mathews-Roth, and R. F. Taylor (ed.), Carotenoids—chemistry and biology. Plenum Press, New York.
6. **Borowitzka, M. A., J. M. Huisman, and A. Osborn.** 1991. Culture of the astaxanthin-producing alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type. J. Appl. Phycol. **3**:295–304.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
8. **Britton, G.** 1988. Biosynthesis of carotenoids, p. 133–182. *In* T. W. Goodwin (ed.), Plant pigments. Academic Press Ltd., London.
9. **Chance, B., H. Sies, and A. Boveris.** 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. **59**:527–605.
10. **Halliwell, B., and J. M. C. Gutteridge.** 1989. Free radicals in biology and medicine, 2nd ed. Oxford University Press, Oxford.
11. **Hartmann, A., and T. Hurek.** 1988. Effect of carotenoid overproduction on oxygen tolerance of nitrogen fixation in *Azospirillum brasilense* Sp7. J. Gen. Microbiol. **134**:2449–2455.
12. **Kakizono, T., M. Kobayashi, and S. Nagai.** 1992. Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, *Haematococcus pluvialis*. J. Ferment. Bioeng. **74**:403–405.
13. **Kobayashi, M., T. Kakizono, and S. Nagai.** 1991. Astaxanthin production by a green alga, *Haematococcus pluvialis*, accompanied with morphological changes in acetate media. J. Ferment. Bioeng. **71**:335–339.
14. **Kobayashi, M., T. Kakizono, N. Nishio, and S. Nagai.** 1992. Effects of light intensity, light quality, and illumination cycle on astaxanthin formation in a green alga, *Haematococcus pluvialis*. J. Ferment. Bioeng. **74**:61–63.
15. **Lee, Y. K., and C. W. Soh.** 1991. Accumulation of astaxanthin in *Haematococcus lacustris* (Chlorophyta). J. Phycol. **27**:575–577.
16. **Lim, B. P., A. Nagao, J. Terao, K. Tanaka, and T. Suzuki.** 1992. Antioxidant activity of xanthophylls on peroxy radical-mediated phospholipid peroxidation. Biochim. Biophys. Acta **1126**:178–184.
17. **Mayer, M. P., P. Beyer, and H. Kleinig.** 1990. Quinone compounds are able to replace molecular oxygen as terminal electron acceptor in phytoene desaturation in chromoplasts of *Narcissus pseudonarcissus* L. Eur. J. Biochem. **191**:359–363.
18. **Miki, W.** 1991. Biological functions and activities of animal carotenoids. Pure Appl. Chem. **63**:141–146.
19. **Miller, H., and A. Claiborne.** 1991. Peroxide modification of monoalkylated glutathione reductase-stabilization of an active-

site cysteine-sulfenic acid. J. Biol. Chem. **266:**19342–19350.
20. **Murata, A., H. Suenaga, S. Hideshima, Y. Tanaka, and F. Kato.** 1986. Hydroxyl radical as the reactive species in the inactivation of phages by ascorbic acid. Agric. Biol. Chem. **50:**1481–1487.
21. **Murata, T., I. Hatayama, K. Satoh, S. Tsuchida, and K. Sato.** 1990. Activation of rat glutathione transferase in class mu by active oxygen species. Biochem. Biophys. Res. Commun. **171:** 845–851.
22. **Niki, E.** 1990. Free radical initiators as source of water- or lipid-soluble peroxy radicals. Methods Enzymol. **186:**100–108.
23. **Renstrøm, B., G. Borch, O. M. Skulberg, and S. Liaaen-Jensen.** 1981. Optical purity of (3S, 3'S)-astaxanthin from *Haematococcus pluvialis*. Phytochemistry **20:**2561–2564.
24. **Strickland, J. D. H., and T. R. Parsons.** 1968. IV.3. Pigment analysis, p. 185–206. *In* A practical handbook of seawater analysis. Fisheries Research Board of Canada, Ottawa, Ontario, Canada.
25. **Theimer, R. R., and W. Rau.** 1970. Light dependent carotenoid synthesis. V. Extinction of the photoinduction by reducing substances and substitution of hydrogen peroxide for light. Planta **92:**129–137.
26. **Whitelam, G. C., and G. A. Codd.** 1982. A rapid whole-cell assay for superoxide dismutase. Anal. Biochem. **121:**207–212.
27. **Ziegler, D. M.** 1985. Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. Annu. Rev. Biochem. **54:**305–329.