BIOLOGIA PLANTARUM 47 (4): 523-526, 2003/4

Singlet oxygen and other reactive oxygen species are involved in regulation of release of iron-binding chelators from *Scenedesmus* cells

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Abstract

Freshly-added iron only slightly affected the growth of iron-sufficient cells of the green alga *Scenedesmus incrassatulus* Bohl, strain R-83, but induced accumulation of malondialdehyde (MDA) in cells and excretion of MDA in the medium. These effects were stronger in response to Fe^{2+} as compared to Fe^{3+}, but Fe^{3+} induced the release of more iron-binding chelators from these cells than Fe^{2+}. Fe^{3+} added either in dark or in light induced release of equal concentrations of iron-complexing agents, part of which formed strong chelates with iron in the medium. Exogenously added hydrogen peroxide inhibited iron-induced release of chelators but the effect was removed by addition of the hydroxyl radical scavenger dimethylsulfoxide (DMSO). Malondialdehyde also inhibited the release of chelators. Release of chelators was induced in the absence of iron salts by photoexcited chlorophyll (Chl). The Chl-induced release was efficiently inhibited by singlet oxygen scavengers such as dimethylfuran, β-carotene, sodium azide and vitamin B_6, and stimulated in D_2O or DMSO. Exogenously added catalase inhibited the release more than added superoxide dismutase. The Fe^{3}-induced release of chelators was also inhibited by scavengers of singlet oxygen, but was not affected by sodium azide and by ethanol. Hence both H_2O_2 and singlet oxygen were involved in induction of chelator release in the absence of iron in light. The induction of chelator release by iron in dark involved H_2O_2, but not singlet oxygen.

Additional key words: catalase, chlorophyll, hydrogen peroxide, malondialdehyde, ROS, superoxide dismutase.

Introduction

Much attention has been given to the response of organisms to conditions of iron limitation, and not to how plants acquire iron at adequate Fe supply. *Scenedesmus incrassatulus* released organic iron-binding molecules in response to freshly added Fe^{3+} (Benderliev and Ivanova 1996). The released complexing agents enhanced Fe solubility at neutral pH. Upon aeration part of these agents formed strong chelates with Fe^{3+} in the medium. Fe-sufficient cells took up iron only from these chelates using a low-affinity uptake system (Benderliev and Ivanova 1997). Fe-deficient *Scenedesmus* cells released siderophores in the medium to alleviate Fe stress (Benderliev and Ivanova 1994). The marine alga *Emiliania huxleyi* released iron-solubilizing compounds in response to freshly added Fe^{3+} (Boye and Berg 2000). Fe^{3+}-induced release of strong chelators was reported in seven strains of green algae, red algae and cyanobacteria (Benderliev 1999). In contrast, the cyanobacterium *Synechococcus leopoliensis* did not release chelators in response to Fe^{3+} (unpublished results).

Iron-induced type of release is contrary to the concept of a siderophore, which is supposed to be released in periods of lack of iron. Here we report involvement of different reactive oxygen species (ROS) in induction and in regulation of chelator release from cells at adequate Fe supply.

Materials and methods

The green alga *Scenedesmus incrassatulus* Bohl, strain R-83 was cultivated in 3.5-fold diluted inorganic nutrient medium (Benderliev and Ivanova 1994). The experiments were done with Fe-sufficient cells that typically did not

Received 24 March 2003, *accepted* 22 August 2003.
Abbreviations: BHT - butylated hydroxytoluene; Chl - chlorophyll, DMSO - dimethylsulfoxide; MDA - malondialdehyde; ROS - reactive oxygen species; SOD - superoxide dismutase, TBARS - thiobarbituric acid reacting substances; TCA - trichloroacetic acid.
Acknowledgment: The authors are indebted to anonymous referee for the helpful suggestions and comments.
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release siderophores during the first 3 h of incubation in medium in which iron salts were not added. The cells were preincubated with chemicals or in D_2O for 10 min in the dark, then $FeCl_3$ or $FeCl_2$ from freshly prepared ten fold concentrated solutions in water was added in the dark. The suspensions were gently stirred for three min, the cells were removed by centrifugation at 8000 *g* for 15 min, and the released chelators were measured spectrophotometrically as described by Fedina and Benderliev (2000).

For extracellular generation of $^1O_2$ we used photo-excited chlorophyll (Chl). Three cm^3 from fresh ethanolic Chl extracts containing 130 μg Chl *a* cm^{-3} were added to 6.7 cm^3 cell suspension, in which Fe was not added, but which contained scavengers or quenchers (each dissolved in 0.3 cm^3 of water or in ethanol). To assess the effect of prolonged lifetime of singlet oxygen, D_2O was used instead of water in the same medium (pH 6.9) in which iron salts were not added. After pretreatment with scavengers, the suspension was irradiated (1300 μmol m^{-2} s^{-1}) for 2 min, to induce extracellular generation of reactive oxygen species (ROS) from the photoexcited Chl. Then the cells were removed and $FeCl_3$ was added to form complexes with the released chelators.

The cell density was measured by determination of dry mass. Malondialdehyde (MDA) for exogenous addition was prepared using malondialdehyde bismethyl-acetal. Intracellular MDA was determined in trichloro-acetic acid (TCA) extracts (Esterbauer and Cheeseman 1990). The released MDA was measured directly in cell-free supernatants as thiobarbituric acid reacting substances (TBARS). Chl *a* in ethanol extracts was measured spectrophotometrically (*Specol, Carl Zeiss*, Jena, Germany) after Lichtenthaller and Wellburn (1983).

Results

Fe^{2+} induced the release of less chelators than Fe^{3+} (Fig. 1), but induced a longer growth lag phase, higher accumulation of MDA in cells and release of more TBARS in the medium (Fig 2*A,B*). Irradiated cells (170 μmol m^{-2} s^{-1}) at the time of addition of Fe^{3+} released the same concentration of chelators as non-irradiated cells (results not shown).

Exogenously added ·OH scavenger dimethylsulfoxide (DMSO) stimulated chelator release much more in the Fe^{2+} than in the Fe^{3+}-induced system, and removed the inhibiting effect of H_2O_2 (Table 1). Exogenously added H_2O_2 (0.001 - 50 μmol) failed to induce chelator release in absence of iron salts (results not shown). Exogenously added MDA also did not induce chelator release in absence of iron salts (results not shown), but abolished the Fe^{3+}-induced release. Butylated hydroxytoluene (BHT) slightly alleviated the inhibitory effect of H_2O_2.

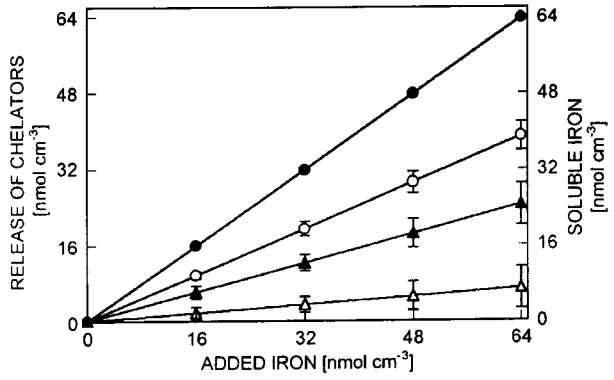

Fig. 1. Effect of oxidation state of freshly added inorganic iron on the release of strong iron-binding chelators in the medium from iron-sufficient cells of *Scenedesmus incrassatulus*. The release of weak iron-complexing agents that increased the concentration of total soluble iron is also shown. Fe(III)-induced responses: released strong chelators (*open circles*), and total soluble Fe (*closed circles*). Fe (II)-induced responses: released strong chelators (*open triangles*), and total soluble Fe (*closed triangles*). Means of 4 experiments ± SE.

Table 1. Effect of exogenously added 10 μM DMSO, 25 μM BHT, 25 μM H_2O_2, and 9 μM MDA on iron-induced release of chelators as influenced by oxidation state of Fe. Fe was added at concentration 54 μM. Means from three experiments ± SE.

	Released strong chelators [% of control]					
	DMSO	BHT	H_2O_2	DMSO+ H_2O_2	BHT+ H_2O_2	MDA
Fe(II)	130 ± 4	100	0	91 ± 3	10 ± 1	0
Fe(III)	103 ± 3	100	33 ± 2	98 ± 3	50 ± 2	0

Dimethylfuran, vitamin B_6, and β-carotene completely suppressed the release of chelators in both experimental systems (Table 2) while D_2O and DMSO

Table 2. Effect of ROS scavengers and D_2O on induction of chelator release by Fe^{3+} in dark and by photoexcited chlorophyll. Means of 3 experiments ± SE.

Treatment	Released chelators [μM]	
	Fe(III)-induced	Chl-induced
Control (untreated cells)	36.0 ± 1.8	46.0 ± 1.4
SOD (518 U cm^{-3})	32.4 ± 1.1	41.0 ± 1.4
Cat (282 U cm^{-3})	16.2 ± 1.4	19.3 ± 1.8
Ethanol (6.95 M)	36.0 ± 1.8	0 (without Chl)
DMSO (10 μM)	37.0 ± 1.1	49.5 ± 1.4
Dimethylfuran (1 mM)	0	0
Vit B_6 (30 μM)	0	0
β-carotene (0.93 μM)	0	0
D_2O 67 % (v/v)	38.5 ± 1.1	53.4 ± 1.4

stimulated the release of chelators. At equimolar enzyme concentration and lower enzyme activity, catalase inhibited the release of chelators more than superoxide dismutase (SOD).

Sodium azide abolished the Chl-induced release of chelators at very low concentration, but failed to affect the Fe^{3+}-induced release. Ethanol did not affect the release as induced by iron and did not induce release in the absence of Chl.

Discussion

The removal of the inhibitory effect of added H_2O_2 on iron-induced release of chelators by the ·OH scavenger DMSO suggests that the added H_2O_2 stimulated the generation of ·OH and that ·OH inhibited the release of chelators. The lower release of chelators in response to Fe^{2+} as compared with Fe^{3+} was due to higher generation of ROS in the presence of Fe^{2+}, though partial oxidation of Fe^{2+} may also affect the result. Though the growth lag phase was very short in both cases thus indicating that iron was not damaging at this Fe/cell ratio (54 μmol Fe/27.7 × 10^6 cells cm^{-3}), the results show that Fe^{2+} was more toxic than Fe^{3+}. It is comprehensible that iron surplus may increase the generation of ·OH, and assuming their strong damaging effect it is desirable to stop uptake of iron. Similarly, oxidative breakdown products such as MDA may have inhibitory effect on Fe uptake. The equal release of chelators as induced by ferric iron in dark or in light shows that the induction was light independent.

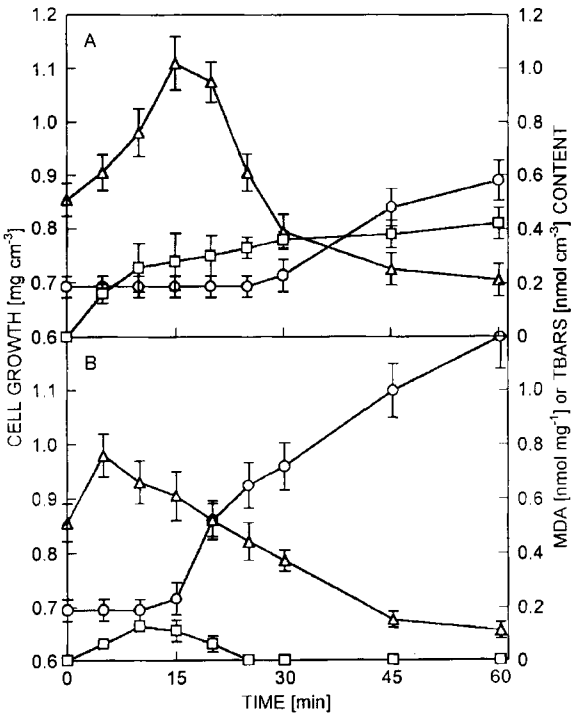

Fig. 2. Effect of Fe^{2+} (*A*) or Fe^{3+} (*B*) on cell growth (*open circles*), intracellular MDA (*open triangles*), and extracellular TBARS (*open squares*). The cells were treated with 54 μM iron in light (170 μmol m^{-2} s^{-1}) and bubbled with 2 % CO_2 in air. Zero time values were taken prior to treatment. Means from 3 experiments ± SE.

Dimethylfuran, vitamin B_6, sodium azide and β-carotene react quickly with singlet oxygen. Their suppresive effect on chelator release suggests involvement of $^1O_2$ in induction of chelator release by photoexcited Chl in light. This is supported also by the stimulation of the process in D_2O, known to enhance the lifetime of $^1O_2$. The DMSO-dependent stimulation of Chl-induced release shows that ·OH was not involved in induction of chelator release in this system but, on the contrary, suppressed the release. Dimethylfuran, β-carotene, vitamin B_6 and sodium azide react not only with $^1O_2$, but also with other strong oxidants such as superoxide and ·OH. If scavening of ·OH was involved, the scavengers of singlet oxygen would not inhibit but would stimulate the release. Apart from $^1O_2$, D_2O is known to prolong also the lifetime of superoxide. The low efficiency of SOD in inhibition of chelator release argues against involvement of superoxide in induction of the release and against suppression of release due to reaction of dimethylfuran, vitamin B_6 or β-carotene with superoxide. The inhibitory effect of SOD may be due to generation of H_2O_2, but not to scavenging of superoxide. Taken together these results suggest that the release of chelators in response to photoexcited Chl was mediated by singlet oxygen.

Catalase inhibited the release of chelators in both experimental systems, showing that H_2O_2 was involved in the induction of the release. We suggest that H_2O_2, which had been either released by cells in response to iron, or had been generated by photoexcited Chl with participation of $^1O_2$, reacted with other extracellularly generated intermediate(s) to produce the species responsible for initiation of the release. In the iron-induced system the ·OH scavenger sodium azide failed to inhibit the release of chelators, and ethanol known to prolong the lifetime of $^1O_2$ did not affect the process, suggesting that $^1O_2$ may not be involved in Fe^{3+}-induced release of chelators. This is supported by the inability of light to stimulate the Fe^{3+}-induced release and with the inability of exogenously added H_2O_2 to induce release of chelators in absence of iron salts in dark. The inhibitory effect of exogenously added SOD or H_2O_2 on iron-induced release of chelators was probably due to shift in the equilibrium of the proposed H_2O_2-dependent reactions towards Fenton chemistry.

Adaptation of cells to utilize H_2O_2 and $^1O_2$ in induction of chelator release may be tentatively explained with generation of both H_2O_2 and $^1O_2$ under Fe-deficient

conditions due to problems in photosynthetic electron flow (*e.g.* overreduction of photosystem 2) because of lack of components containing iron. Similarly to H_2O_2 and superoxide radical (Dat *et al.* 2000, Droge 2002), singlet oxygen is involved in selective activation of genes (Ryter and Tyrrell 1998) and in induction of signaling events at concentrations below these required for cytotoxicity (Briviba *et al.* 1997). In contrast to 'OH, both H_2O_2 and singlet oxygen have longer lifetime and can cross biomembranes.

The release of chelators in presence of iron seems to be an adaptive strategy, both because free iron is practically insoluble at neutral and alkali pH and because of its potential toxicity. The Fe^{3+}-induced release of chelators enhances manifold the capacity of cells to scavenge and later to take up iron in comparison with cell surface area-restricted (Morel *et al.* 1991) Fe uptake. Further research is needed for better understanding of the regulation of the release of chelators by algal cells.

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