REACTIVE OXYGEN SPECIES REGULATE IRON UPTAKE IN *SCENEDESMUS INCRASSATULUS*

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Summary. Iron-sufficient cells of the green alga *Scenedesmus incrassatulus* Bohl, R-83 released in the medium strong and weak Fe^{3+}-complexing agents in response to freshly added Fe^{3+}. Applied in the dark, photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibited ferric reduction from ferric-EDTA or ferricyanide more than the chelator release. Fe-deficient cells and DCMU-treated Fe-sufficient cells took up Fe nonpreferentially both from strong and from weak complexes at high rates. On the contrary, intact Fe-sufficient cells took up Fe only from strong chelates at a lower rate. In vitro, when added into fresh lipid extracts, strong Fe chelates catalyzed the generation of 5 and 8 times, respectively, less malonaldehyde compared to the content catalyzed by weak Fe complexes or inorganic Fe^{3+}. 100 мM DCMU prevented a Fe^{3+}-induced release of chelators and an inorganic Fe uptake, but upregulated the uptake of exogenously added organic Fe. The hydroxyl radical (·OH) scavengers dimethylsulfoxide (DMSO), mannitol, formate, adenine and thiourea (but not urea) counteracted the effect of DCMU on ferric reduction and chelator release. DMSO did not affect the DCMU-induced stimulation of uptake of added organic Fe. Low concentrations of catalase added upregulated the Fe uptake from intact Fe-sufficient cells. Results show that Fe-sufficient cells possess a recognition system, which allows a selective uptake of Fe from non-toxic complexes. This recognition system does not operate in iron-limited and in DCMU-stressed Fe-sufficient cells. The DCMU-induced inhibition of ferric reduction and chelator release is caused by reactive oxygen species such as ·OH generated in the outer cell membrane. ·OH is not involved in the Fe deficiency- and in DCMU-induced upregulation of Fe uptake. H_2O_2 is involved

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in the regulation of Fe uptake. Ferric reduction is not involved in the induction of chelator release. Complexing agents released by Fe-sufficient cells in response to added Fe^{3+}, mediate the uptake of the metal at an adequate Fe supply.

Keywords: DCMU, hydroxyl radical, hydrogen peroxide, iron uptake, reactive oxygen, regulation, *Scenedesmus*.

Abbreviations: BPDS - bathophenanthroline disulfonate; HA - hydroxylamine hydrochloride; DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMSO - dimethylsulfoxide; FeCN – ferricyanide; ROS - reactive oxygen species

INTRODUCTION

DCMU herbicide has been widely used to inhibit PS II in plants under light. The effects of this chemical in dark have been neglected so far. Here we present evidence that DCMU is active in the dark, affecting both the reduction and the uptake of Fe.

Iron-induced release of complexing agents by Fe-sufficient cells is contrary to the concept of siderophore, which is supposed to be released by Fe-deficient cells during iron lack stages. Fe^{3+}-induced release of complexing agents has been recorded in *Emiliania huxleyi* marine alga (Boye and Berg, 2000), some green and red algae, and in cyanobacteria (Benderliev, 1999). Hydroxyl radical (·OH) inhibited the release of chelators; single oxygen and H_2O_2 (but not superoxide) were involved in the induction of chelator release from *Sc. incrassatulus* cells (Benderliev et al., 2003). With the present we give further evidence about the effect of ·OH on ferric reduction, chelator release and Fe uptake. Results show that a link between Fe complexes capacity to act as Fenton catalysts and the Fe uptake mechanism of intact Fe-sufficient cells exists.

MATERIAL AND METHODS

Scenedesmus incrassatulus

Bohl, R-83 green alga was obtained from the Algal Culture Collection in Plovdiv University. Cells were mixed with 2% CO_2 in the air, under light (170 mol $m^{-2}s^{-1}$) in diluted 3.5 times inorganic nutrient medium, which contained 56 M $FeCl_3$ and no EDTA (Benderliev and Ivanova, 1994). In the experiments Fe-sufficient cells, which during the first 3 hours of incubation do not release siderophores, were placed in an iron salt free medium. To asses the effects of ·OH, washed cells were preincubated simultaneously with ·OH scavengers and DCMU for 10 min in the dark. $FeCl_3$ from freshly prepared ten fold concentrated solutions in water, was added in the dark. The

effect of hydrogen peroxide on Fe uptake was estimated after 10 min preincubation of ferric complexes with catalase from *Micrococcus lysodeikticus* (Fluka) and subsequent addition of cells. The pretreatment of ferric complexes was done to estimate the diminished uptake catalytic activity. Suspensions were gently stirred for three min. Cells were removed by centrifugation at 8000 g for 15 min, and the released chelators were spectrophotometrically measured (Fedina and Benderliev, 2000). The concentration of ferric iron bound in hydroxylamine hydrochloride-labile complexes is the fraction of ferric iron, bound in hydroxylamine-reducible complexes. The concentration of ferric iron, bound in weak complexes was determined as follows: to 5 cm^3 cell-free supernatant 0.2 cm^3 solution of 5.9 M CH_3COONH_4 was added in 3 M CH_3COOH (pH 4.5). Then 0.1 cm^3 2.87 M hydroxylamine hydrochloride and 0.2 cm^3 solution of 25.25 μM *o*-phenanthroline chloride were added. The absorbance of the ferrous-phenanthroline complex was read after 15 min at 492 nm on a *Specol* spectrophotometer (*Carl Zeiss*, Iena, Germany). The absence of inorganic iron in samples reported as "iron bound in weak complexes", was proved by the complete solubility of hydroxylamine-reducible iron at pH 9.5 (results not shown). Total iron content was determined following the same procedure, but instead of hydroxylamine hydrochloride, to the 5 cm^3 cell-free supernatant a 0.1 cm^3 fresh daily-prepared 10% solution of ascorbic acid was added. As a measure for the released strong chelators, hydroxylamine-stable ferric iron content was measured as the difference between the contents of total soluble and hydroxylamine labile iron. Fe^3+ complexes catalytic activity was measured in methanolic lipid extracts. Hot methanol (50 ml) was poured over 0.1 g washed algal cells, then 100 ml chloroform was added and the cells were removed by a filtration. Then 10 ml 0.2 N NaCl was added in water and the lipid-containing layer was decanted. 5 g Na_2SO_4 was added and the filtration was repeated. The solvent was evaporated at 45°C in a rotary vacuum evaporator. The samples were temperated at room temperature, weighed and solubilized in methanol to achieve 2 mg lipid per ml of extract. One ml from the lipid extract was solubilized in 4 ml methanol, then 5 ml of the nutrient medium containing 0.0184 μM $FeCl_3$ or Fe bound to algal chelators ml^{-1} were added. Mixtures were gently stirred for 1 min at temperature 20°C and the content of MDA was measured as thiobarbituric acid reacting substances (TBARS) (Esterbauer and Cheeseman, 1990). Lipid extract, placed in the same nutrient iron salt free medium was used as a blank.

To specify the involvement rate of hydroxyl radical (·OH), scavengers such as dimethylsulfoxide (DMSO), mannitol, formate, adenine and thiourea, were added. Results were compared to these after the addition of urea (the inactive analog of thiourea).

Ferric reduction from ferricyanide (FeCN) or ferric EDTA (sodium salt) was measured in the dark according to Lynnes et al. (1998) by gentle stirring of suspension at pH 6.9.

To determine Fe uptake rate, cells were thoroughly washed in the same medium, in which Fe was not added. Gently stirring them for 10 min in the dark, they were incubated in media, which contained Fe^{3+} bound either to weak complexing agents, or to strong chelators released by algae in response to added Fe^{3+}. These organic media were prepared according to Benderliev and Ivanova (1997). Supernatants were aerated with 2% CO_2 for 40 min in air to favor the redox-cycle necessary for the formation of strong ferric chelates. Transformation of weak complexes into strong chelates presumably involved structural changes, but preserved the basic chemical characteristics of the complexes, since their molecular weights remained the same. Fe^{3+} uptake was measured after the cells were washed with titanium reagent to remove the extracellular Fe. Cells were ashed and Fe was spectrophotometrically measured after the reduction of Fe with ascoric acid or with hydroxylamonium hydrochloride (Benderliev and Ivanova, 1996). Moderately Fe-deficient cells were produced by 24 h incubation in an iron free medium. DCMU, catalase and hydroxyl radical scavengers used in the present study, did not intervene in Fe determination.

RESULTS

When applied in the dark, DCMU inhibited ferric reduction stronger than the chelator release (Table 1). At 0.1 M DCMU completely abolished ferric reduction, while 100 M DCMU were needed to stop the release of complexing agents in response to Fe^{3+}. When applied simultaneously with DCMU, the ·OH scavenger DMSO efficiently removed the DCMU-induced inhibition of chelator release and ferric reduction. Same effect was induced from other ·OH scavengers like mannitol, formate, adenine, and thiourea (Table 2), while urea almost completely failed to remove the inhibitory effect of DCMU even at high concentrations. The concentration of chelators released by cells in response to Fe^{3+} added diminished under Fe limitation and the DCMU-induced inhibition of Fe^{3+}-induced release of chelators was alleviated from DMSO in Fe-deficient cells (results not shown).

Intact Fe-sufficient cells took up ferric iron only from a medium containing strong chelates and the rate of Fe uptake was low (Table 3). These cells did not take up Fe^{3+} from a medium containing weak complexes of Fe^{3+} as a single iron source. Both Fe-deficient and DCMU-treated Fe-sufficient cells took up Fe^{3+} not selectively from strong and weak complexes at high rates. DMSO did not affect the uptake of Fe by organic complexes in intact and in DCMU-treated cells. 100 M DCMU that completely inhibited the release of complexing agents (Table 1) prevented the uptake only of inorganic Fe^{3+}, but stimulated the uptake of exogenously added organic Fe^{3+}. Fe-sufficient cells failed to take up Fe from $FeCl_3$ suspension in non-aerated cultures in the dark - the chelators released in these conditions initially formed weak com-

Table 1. Effect of DCMU and 10 μM DMSO on the Fe^{3+}-induced release of strong chelators and on the ferric reduction. Fe was added at 60 μM; 60 μM EDTA and 500 μM BPDS were added in ferric reduction experiments; the presence of strong chelators was recorded after 80 min of aeration of cell-free supernatants. Mean values from three separate experiments ±SE.

Treatment DCMU (M)	100	10	1	0,1	0,01
	Released strong chelators (% of control)				
	0	5.5±3	11±3	16.5±4	22±5
	Released weak complexing agents (% of control)				
	0	7±1	12±2	18±3	25±4
	Ferric reduction (% of control)				
FeEDTA +BPDS	0	0	0	0	10±2
FeCN +BPDS	0	0	0	0	9±2
Treatment DCMU+DMSO					
	Released strong chelators (% of control)				
	10±3	20±4	37±4	70±3	100±2
	Released weak complexing agents (% of control)				
	12±2	24±3	40±4	75±5	100±4
	Ferric reduction (% of control)				
FeEDTA +BPDS	0	0	10±2	30±3	60±4
FeCN +BPDS	0	0	9±2	28±3	55±4

plexes with Fe^{3+} (Benderliev and Ivanova, 1996,1997). Contrariwise, Fe-deficient cells took up some Fe from $FeCl_3$ medium - these cells preserved some capacity to release chelators in response to added Fe^{3+} (results not shown). DCMU-treated Fe-

Table 2. Effect of ·OH scavengers on the removal of the inhibitory effect of 0.1 μM DCMU, on ferric reduction and on chelator release, as induced by 56 μM $FeCl_3$addition. Urea served as a negative control. Mean values from three experiments ±SE.

Inhibitor	Released strong chelators	Ferric reduction	
		FeEDTA (% of control)	FeCN
DCMU	16.5±4	0	0
+ mannitol (100 mM)	70±20	30±10	25±8
(50 mM)	40±6	10±2	8±3
+ Na-formate (20 mM)	80±10	33±8	30±8
(10 mM)	16±7	10±3	8±2
+ adenine (10 mM)	60±10	22±7	10±6
+ thiourea (50 mM)	70±18	30±6	22±6
+ urea (100 mM)	17±5	0	0

Table 3. Effect of 100 M DCMU and 10 µM DMSO on the iron uptake. Moderately Fe-deficient cells were prepared by 24 h incubation in a Fe-limited medium. Medium 1 is a cell-free supernatant which contains ferric iron bound by weak complexing agents, immediately released by cells in response to added Fe^{3+}. Medium 2 is the same as Medium 1, but after aeration. Medium 3 is an inorganic nutrient medium containing ferric chloride as a single iron source. Mean values from three experiments ±SE.

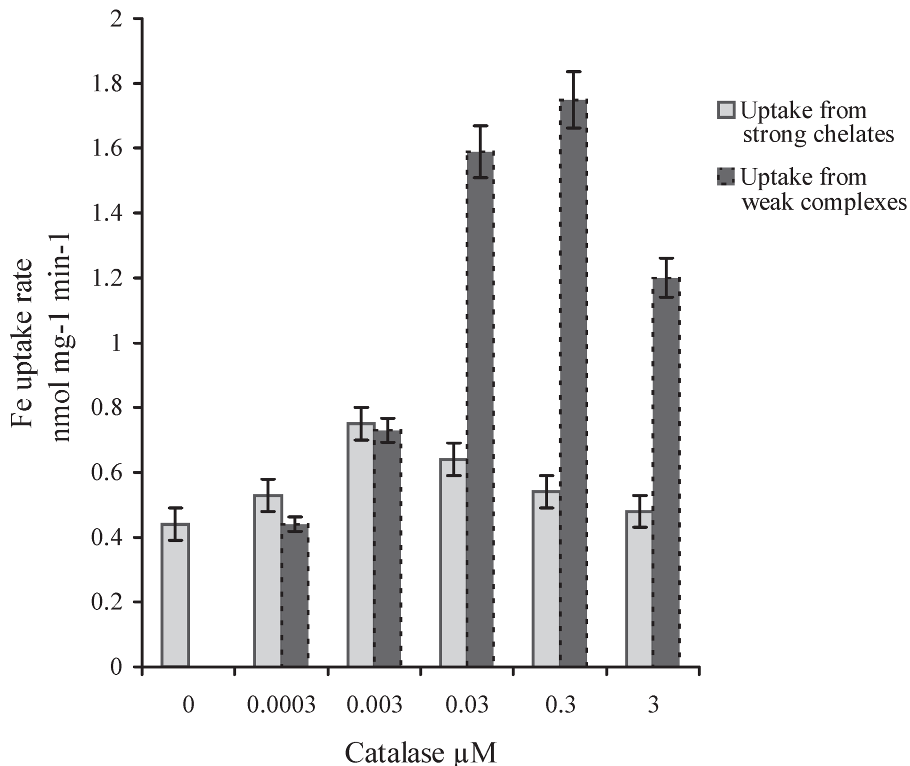
	Medium 1 Organic Fe Weak (nmol ml^{-1}) 108	Medium 2 Organic Fe Strong + Weak 32 76	Medium 3 $FeCl_3$ 108
		Fe uptake (nmol $mg^{-1}min^{-1}$)	
Fe-sufficient cells			
Control	0	0.44±0.03	0
+ DCMU	1.3±0.3	1.3±0.2	0
+ DCMU + DMSO	1.3±0.3	1.3±0.2	0.2
+ DMSO	0	0.44±0.03	0
Fe-deficient cells			
Control	1.75±0.4	1.75±0.4	0.8
+ DCMU	1.9±0.2	1.9±0.3	0
+ DCMU + DMSO	1.9±0.2	1.9±0.3	0.2
+ DMSO	1.75±0.4	1.75±0.2	0.8

deficient cells failed to take up Fe from a $FeCl_3$ medium, while DCMU-and-DMSO treated cells took up some Fe. Low concentrations of catalase up regulated Fe^{3+}uptake from strong chelates and made possible the uptake from weak complexes, while higher catalase concentrations were less effective (Fig.1). The uptake rate from catalase-pretreated weak complexes was higher than that from catalase-pretreated strong chelates.

Iron bound to strong chelators (released by cells in response to Fe^{3+}), when exogenously added into lipid extracts, catalyzed the generation of five and eight times less MDA, respectively, as compared to iron bound to weak complexing agents or inorganic Fe^{3+} (Table 4).

Table 4. Effect of catalytic activity of Fe^{3+} bound by complexing agents released from cells in response to freshly added $FeCl_3$. Capacity of Fe^{3+} to catalyze the generation of malonaldehyde in lipid extracts was a measure for the catalytic activity. Mean values from three experiments ±SE.

Catalyst	MDA (mol g $lipid^{-1}$ mol Fe^{-1})
$FeCl_3$	6.39±0.9
Weak Fe complexes	1.30±0.4
Strong Fe chelates	0.80±0.2



Mean values from three experiments ±SE.

Fig. 1. Uptake of iron by Fe-sufficient cells as affected by pretreatment of Fe complexes with catalase

DISCUSSION

The lower capacity of strong Fe^{3+} chelates to catalyze lipid peroxidation in lipid extracts as compared to weak Fe^{3+} complexes is related to their higher inertness to reduction and, probably, higher affinity to bind iron. Apart from these properties, the strong chelates may differ from weak complexes in their net charge and/or in their interactions with putative receptors at the plasma membrane, similarly to phytosiderophores of higher plants (Wiren *et al.,* 2000). The selective Fe uptake from strong chelates (but not from weak complexes) by intact Fe-sufficient *Scenedesmus* cells cannot be explained in terms of the different basic chemical properties or the different molecular weight of chelators, since aeration transforms weak complexes into strong chelates with the same molecular weights (Benderliev and Ivanova, 1996). Differences in the oxidation state of Fe are also not decisive, because small part of Fe^{3+} bound to weak complexes was reduced by cell membrane reductases and was

not taken up by cells. It should be noted that all complexes were 100% soluble in water at pH 6.9 and that all experiments were accomplished at this pH. Further research is needed to establish which property of the strong chelates as compared to weak complexes is actually sensed by the cell membrane receptors, and to find out the signal generated by Fe bound to weak complexes. We presume that the significant difference in the capacity of Fe complexes to act as Fenton catalysts is of importance in the Fe uptake process. It is probably safer for cells to adsorb at their surface and to take up less toxic Fe - behaviour which seems to be reasonably abandoned in periods of Fe limitation. The up-regulation of Fe uptake from strong chelates, and especially from weak complexes, in intact Fe-sufficient cells (after pretreatment of the complexes with low concentrations of catalase) shows that strong chelates, which have low capacity to generate extracellular hydrogen peroxide, may be preffered as substrates for Fe uptake. Diminishment of the stimulating effect of catalase at higher enzyme concentrations suggests that H_2O_2 may function as the signal involved in Fe uptake induction from the complexes, while lower or zero concentrations of H_2O_2 down-regulate Fe uptake. On the other hand, high H_2O_2 concentrations,generated by weak Fe complexes, may be responsible for the Fe unavailability for Fe sufficient cells.

Fe uptake rate from strong chelates equalled the rate of Fe uptake from weak complexes both in DCMU-stressed and in iron-limited cells, showing that stressed cells have completely abandoned the selective behaviour in Fe uptake and did not differentiate between strong chelates and weak complexes of Fe. Fe uptake upregulation in stressed cells is well known - it has been attributed to superoxide-induced release of “free” iron in the cytosol (Maringanti and Imlay, 1999), and/or to activation of IRP-1 by H_2O_2 and lipid hydroperoxides (Pantopoulos *et al.,* 1997;Tampo *et al*., 2003). Results suggest that the stress-induced neutralization of the selective mechanism for Fe uptake may be more important for the Fe uptake upregulation than the activation of iron transporters. The activation of iron transporters alone is not expected to induce equal rates of Fe uptake from different substrates, while the selectivity ceasing widens the spectrum of substrates suitable for uptake.

The suppressive effect of hydroxyl radical on chelator release suggests that the cells have developed a mechanism to restrict chelators release when generation of ROS, such as ·OH, in cells was stimulated. This mechanism could be used either to down-regulate the stress-stimulated uptake of iron or to preserve the capacity for intracellular Fe chelation. It has been found that H_2O_2-induced generation of ·OH affected the function of cell membrane channels responsible for the uptake of ions different from iron (Kourie, 1998). Here we demonstrate that ·OH did not affect the uptake of Fe^{3+} from added organic complexes, showing that ·OH had not affected the function of iron channels both in intact and in stressed cells, while H_2O_2 regulated Fe uptake independently from ·OH.

DCMU is widely used to inhibit the electron transport light-stimulation in chloroplasts. The present results show that this herbicide is active in the dark, as well. At 100 μM it generated ·OH at concentrations sufficient to completely inhibit cell membrane-bound reductases, to stop the release of iron-complexing agents from cells, and also to abolish the selective uptake of Fe from preexisting organic complexes, thus stimulating Fe uptake. The stimulated uptake of organic iron and the complete unavailability of inorganic ferric iron for DCMU-treated cells suggests that organic complexation of ferric iron is an obligatory step in the uptake of the metal.

DCMU at 100 μM induced only a 14% inhibition of FeEDTA reduction by iron-limited *Chlamydomonas* cells (*Eckhardt* and *Buchkhout*, 1998). This demonstrates a low susceptibility of Fe limitation-stimulated ferric chelate reductase in *Chlamydomonas* cells to DCMU treatment. On the contrary, reductases in Fe-sufficient *Scenedesmus* cells were extremely susceptible to DCMU treatment. The complete inhibition of ferric reduction did not result in a complete inhibition of chelator release, showing that the release of chelators from *Scenedesmus* cells was not mediated by ferric reduction.

DCMU does not react to iron, and does not change the pH of the nutrient medium, so the effects reported were due to the direct interaction between the cells and DCMU. It could be presumed that a site-specific iron-catalyzed generation of ·OH in the outer cell membrane may be responsible for the reported DCMU-induced inhibitory effects. The DCMU-induced generation of ·OH in plant cells is not quite unexpected, considering the long known action of DCMU as a skin irritant.

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