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Ferric and cupric reductase activities in the green alga *Chlamydomonas reinhardtii*: experiments using iron-limited chemostats

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Abstract. Cells of the green alga *Chlamydomonas reinhardtii* Dangeard were grown in Fe-limited chemostat culture over a range of growth rates (0.15–1.5 d^{-1}). Greater cell densities and culture chlorophyll levels were achieved using an excess of chelator [ethylenediamine di-(*o*-hydroxyphenylacetic acid)] relative to $FeCl_3$ (80:1), compared to growth using a 1:1 chelator:$FeCl_3$ ratio. The *C. reinhardtii* cells reduced extracellular ferric chelates, and ferric chelate reductase activity increased with increasing Fe-limited growth rates. However Fe-sufficient cells exhibited a low rate of ferric chelate reductase activity, similar to severely Fe-limited cells. Iron-limited cells were capable of reducing a wide variety of ferric chelates, representing a wide range of stability constants, at similar rates, suggesting that the stability constants of ferric complexes are not important determinants of ferric reducing activity. Cupric reductase activity also increased with increasing Fe-limited growth rates, and Cu(II) was preferentially reduced compared to Fe(III). These results suggest that both reductase activities may represent the same plasma-membrane enzyme. The rate of cupric reduction was a function of the free [Cu^{2+}], not the total [Cu(II)], suggesting that free Cu^{2+} is the actual substrate for cupric reductase activity.

Key words: *Chlamydomonas* (Fe-limitation) – Cupric reductase – Ferric chelate reductase – Iron-limited chemostat

Introduction

Iron is an essential element for photosynthetic organisms, but in aerobic environments it is typically found in the form of insoluble Fe(III) oxides (Byrne and Kester 1976; Lindsay 1979) and/or Fe(III)-organic complexes (Hermann and Gerke 1992; Rue and Bruland 1997). There is good evidence that iron availability limits primary productivity in parts of the ocean (e.g. Kolber et al. 1994; Coale et al. 1996), and perhaps as well in some non-marine aquatic systems (Chang et al. 1992; Evans and Prepas 1997).

In higher plants, iron limitation results in the induction of a suite of physiological and biochemical mechanisms for Fe(III) solubilization and uptake, which have been classified into two distinct "strategies". Some species use a strategy that involves extracellular acidification, reduction of Fe(III) to Fe(II), followed by uptake of Fe(II) by a specific transporter; this mechanism involves the obligatory reduction of extracellular ferric chelates at the plasma membrane by a ferric chelate reductase (FC-R), leading to chelate splitting and subsequent uptake of the released Fe(II) (Chaney et al. 1972; Römheld and Marschner 1983); this mechanism has been labeled "Strategy I". Conversely, "Strategy II" species export Fe(III)-specific siderophores, followed by uptake of the Fe(III)-siderophore complex across the plasma membrane (both strategies are reviewed in Marschner and Römheld 1994).

Among the algae, cyanobacteria excrete siderophores in response to iron limitation (e.g. Wilhlem and Trick 1994), perhaps in a mechanism analogous to higher plant "Strategy II" (Marschner and Römheld 1994; Moog and Brüggemann 1994). The mechanism(s) by which eukaryotic algae access extracellular Fe(III) is less clear. Anderson and Morel (1980) demonstrated that Fe-limitation leads to an increase in FC-R activity in the marine diatom *Thalassiosira weisflogii*, while Soria-Dengg and Horstmann (1995) provided similar evidence for the marine diatom *Phaeodactylum tricornutum*.

Among the green algae (Chlorophyta), *Scenedesmus incrassatulus* releases Fe(III)-specific siderophores in response to both Fe-limitation and Fe-toxicity (Benderliev and Ivanova 1994, 1996), and Fe-limitation does not induce detectable FC-R activity (Benderliev and Ivanova 1994). Similarly, Fe-limitation does not result in an

Abbreviations: BCDS = bathocuproine disulphonate; BPDS = bathophenanthroline disulphonate EDDHA = ethylenediamine di(*o*-hydroxyphenylacetic acid); FC-R = ferric chelate reductase

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increased level of FC-R activity in *Selenastrum minutum* (J.A. Lynnes and H.G. Weger, unpubl. data). However, Fe-limitation results in a large increase in FC-R capacity in two species of *Chlorella* (Allnutt and Bonner 1987; Moog and Brüggemann 1994).

More recently, it was demonstrated that Fe-limitation results in a large increase in FC-R activity in the green alga *Chlamydomonas reinhardtii* (Eckhard and Buckhout 1998; Lynnes et al. 1998); FC-R activity increased with duration of Fe-limitation. In both of these studies, Fe-limitation was achieved by growing the cells in iron-free or low-iron medium, similar to the manner in which most higher-plant Fe-limitation studies are performed. However, an algal culture system that may more closely approximate Fe-limited algal growth in the natural environment is an Fe-limited chemostat, in which the rate of Fe-limited growth is a function of the rate of iron supply. Using a chemostat system, it is possible to grow algal cells over a range of Fe-limited growth rates, and thus measure the response of FC-R activity to differing degrees of Fe-limitation. In this paper, a chemostat system for the growth of Fe-limited green algae is described, and the responses of both FC-R and cupric reductase activities to Fe-limitation are examined. The response of FC-R activity to Fe-limitation was clearly different from that described by Lynnes et al. (1998) for *C. reinhardtii* switched to iron-free medium.

Materials and methods

Cell culture. *Chlamydomonas reinhardtii* Dangeard UTEX 89 was obtained from the University of Texas Culture Collection. The cell wall-less mutant *C. reinhardtii* CW-15 was obtained from Dr. R.G. Smith, University College of the Cariboo (Kamloops, BC, Canada). Cells were grown in water-jacketed glass chemostat vessels, at a temperature of 20 °C and a photon fluence rate of 225 μmol m^{-2} s^{-1} (Weger et al. 1996). Cultures were continuously stirred and aerated with 2% CO_2 in air. Continuous light was supplied by a bank of high-output cool-white fluorescent tubes (F48T12/CW/HO; Philips Electronics, Scarborough, Ontario, Canada). The medium was a modification of that formulated by Hughes (Hughes et al. 1958), and contained 300 μM K_2HPO_4, 300 μM $MgSO_4$ and 245 μM $CaCl_2$. Both Na_2CO_3 and $NaSiO_3$ were omitted, and minor elements were added as in Allen (1968). The medium was buffered at pH 7.5 with 15 mM Hepes-KOH; acidification by the cells resulted in a final pH of approximately 6.5. Ammonium nitrate (3 mM) was used as the nitrogen source for UTEX 89 cultures, while 6 mM NH_4Cl was used for the CW-15 cells, which are unable to assimilate nitrate.

Ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDHA) was used as the iron chelator in the medium for all cultures; EDDHA stocks were dissolved in a 4-fold molar excess of KOH (Chaney and Bell 1987) prior to addition to the medium or addition of an iron source. For most experiments, EDDHA was added to the medium (from a stock of 10 mM EDDHA in 40 mM KOH) to a concentration of 20 μM prior to the addition of an iron source. Iron was added from a separate stock containing 1 mM EDDHA in 4 mM KOH, with either 1 mM $FeCl_3$ or 1 mM $FeSO_4$. The iron stock was added after autoclaving of the medium, and was sterilized by passage through a 0.2-μm filter. For experiments examining the effects of growing a chemostat culture using a 1:1 Fe(III):chelator ratio (i.e. "stoichiometric" cultures) the only source of EDDHA was the Fe(III)-EDDHA stock, which was added post-autoclaving at a concentration of 0.25 μM. Iron-limitation was confirmed for each growth rate (in the range 0.15–1.5 d^{-1}) by observed increases in chlorophyll and cell density upon addition of 25 μM Fe(III)-EDDHA to the chemostat vessel (added by sterile filtration). The medium reservoirs for the chemostat cultures were acid-washed 19-L Pyrex carboys. Chemostat vessels were acid-washed prior to sterilization.

Iron-sufficient cells were generated by the addition of either 25 μM Fe(II)- or Fe(III)-EDDHA to the medium reservoir. The dilution rate was set to 1.9 d^{-1}, which is slightly faster than the maximum growth rate of *C. reinhardtii* at 20 °C.

Chemostat cultures are a type of continuous culture, and operate on the principle of a limiting nutrient that determines the culture growth rate. This system was first devised for the continuous culture of bacteria (e.g. Novick and Sziland 1950), and was later adapted for algal cultivation (see Fogg 1976, for an overview). The culture medium is formulated such that one nutrient (e.g. iron) is present in low concentration, while all other nutrients are present in excess. Fresh medium is continuously pumped into the chemostat vessel, and displaces an equal volume of cell suspension. The growth rate of the culture is determined by the rate of pumping of fresh medium containing the limiting nutrient (i.e. by the culture dilution rate), and at steady-state the biomass in the chemostat vessel is constant, and the culture growth rate equals the dilution rate. Conversely, growth-rate-specific steady-state biomass is determined by the concentration of the limiting nutrient in the medium.

Reduction of Fe(III) and Cu(II). Ferric reduction was determined using a method modified from Lynnes et al. (1998). Cells were harvested by centrifugation (2000 *g* for 4 min), and twice washed in assay buffer (15 mM Hepes-KOH, pH 6.5, with 300 μM $MgSO_4$ and 245 μM $CaCl_2$). Resuspended cells were placed in water-jacketed reaction vessels (20 °C) in the dark, with gentle stirring. Ferric chelate reductase (FC-R) activity was determined using 250 μM Fe(III)-EDTA (added from a 100 mM stock solution) and 500 μM bathophenanthroline disulphonate (BPDS) to trap and quantify the Fe(II) produced from Fe(III) reduction. Previous studies have indicated that use of BPDS, which is membrane impermeant, effectively traps all extracellular Fe^{2+} (Lynnes et al. 1998), i.e. there is no detectable iron uptake by *C. reinhardtii* in the presence of BPDS. Concentrations of Fe(II)-$BPDS_3$ were determined spectrophotometrically (A536–A750) after the cells were removed by centrifugation, using a molar absorptivity of 22,140 (Blair and Diehl 1961). The activity of FC-R was calculated by linear regression over a 16-min course.

Cupric reductase activity was assayed using twice-washed cells resuspended in assay buffer, to which $CuSO_4$ chelated with either citric acid or EDTA was added at a concentration of 250 μM. To trap and quantify the Cu(I) produced from Cu(II) reduction, membrane impermeant bathocuproine disulphonate (BCDS) was added at a concentration of 500 μM. Concentrations of Cu(I)-$BCDS_2$ were determined spectrophotometrically (A484–A750), using a molar absorptivity of 12,250 (Blair and Diehl 1961). All determinations of FC-R and cupric reductase activities were performed in quadruplicate.

Competitive inhibition between ferric and cupric reductase activities was investigated using Fe-limited cells (growth rate = 0.3 d^{-1}) resuspended in assay buffer, as described above, to which Fe(III)-EDTA (1:1 Fe[III]:EDTA) and Cu(II)-citrate (1:1.5 Cu[II]:citrate) were each added at 250 μM, and BPDS and BCDS were each added at 500 μM. Concentrations of Fe(II)-$BPDS_3$ and Cu(I)-$BCDS_2$ were determined spectrophotometrically using empirically derived simultaneous equations to correct for the absorbance of the Fe(II)-$BPDS_3$ complex at 484 nm and the absorbance of the Cu(I)-$BCDS_2$ complex at 536 nm. The simultaneous equations were tested by adding mixtures of Fe(III) and Cu(II) to assay buffer, followed by reduction with dithionite to Fe(II) and Cu(I), respectively; recoveries for both Fe(II) and Cu(I) were quantitative (not shown). As well, preliminary experiments, designed to test the possible interactions between Fe(II) and Cu(I), were also performed (described in *Results and discussion*).

Other methods. Cell density was determined using a hemacytometer. Chlorophyll was quantified spectrophotometrically after extraction in 100% methanol (Porra et al. 1989). Free Cu^{2+} concentrations were calculated using Mineql+ (Environmental Research Software, Hallowell, Me., USA).

Results and discussion

Iron-limited chemostats. Initial experiments comparing the steady-state biomass of Fe-limited chemostat cultures (growth rate = 0.15 or 0.3 volumes per day [d^{-1}], 0.25 μM Fe[III]) grown using "stoichiometric" (0.25 μM) or "excess" (20.25 μM) EDDHA concentrations indicated that stoichiometric cultures produced only approximately 60% of the cell density of the cultures grown with excess EDDHA, while the per-cell FC-R activities were the same (data not shown). A similar decrease in biomass has been observed in hydroponically grown blueberry plants when stoichiometric chelator concentrations were used, compared to excess (Korcak 1989). In the latter study, the decreased growth in stoichiometric hydroponic solution was attributed to toxicity arising from non-chelated microelements. However, another possible explanation might be that stoichiometric solutions allowed some of the Fe(III) or microelements to precipitate and thus be unavailable for growth.

To examine the reason for decreased *C. reinhardtii* cell density in stoichiometric chemostat cultures, 25-mL aliquots of stoichiometrically grown Fe-limited cells (growth rate = 0.15 d^{-1}, [Fe(III)] = 0.25 μM) were aseptically removed from the chemostat vessels and transferred to 50-mL glass tubes equipped with bubblers. The cells were maintained in continuous light and aerated with 2% CO_2 in air. Tubes were amended with either additional microelements, chelated microelements, EDDHA or Fe(III)-EDDHA. Only the addition of Fe(III)-EDDHA resulted in an increase in cell density compared to the control (Fig. 1). Addition of EDDHA alone had no discernible effect, suggesting that microelement toxicity was not likely a factor. Addition of chelated microelements also had no discernible effect (Fig. 1), nor did addition of microelements in the absence of chelator (not shown). These results suggest that the decreased cell density apparent in the stoichiometric chemostat cultures was due to decreased Fe(III) availability compared to the excess chelator cultures, possibly due to precipitation of iron or adsorption to the reservoir walls. All subsequent experiments were performed using chemostat cultures with excess chelator (20.1–20.4 μM EDDHA and 0.10–0.40 μM $FeCl_3$ or $FeSO_4$).

At an iron concentration of 0.25 μM, changes in dilution rate had a major effect on the characteristics of the chemostat cultures. Both cell density and total chlorophyll content decreased with increasing dilution rate (Fig. 2, top and middle panels), while not surprisingly the amount of chlorophyll per cell increased with increasing dilution rate (Fig. 2, bottom panel). The reduction state of the iron source in medium (Fe[II] vs. Fe[III]) had no discernible effect on culture characteristics (Fig. 2). The cell wall-less mutant *C. reinhardtii*

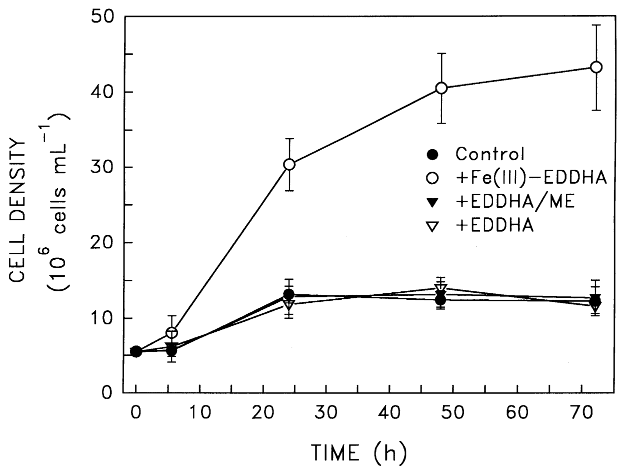

Fig. 1. Changes in cell density over a 3-d time course. Aliquots (25 mL) of a stoichiometric Fe-limited chemostat culture (*C. reinhardtii* UTEX 89, growth rate = 0.15 d^{-1}, [Fe(III)] = 0.25 μM) were placed in continuous light and bubbled with 2% CO_2 in air. Bars represent SE (n = 3). The EDDHA and Fe(III)-EDDHA were added at 5 μM, and 25 μL of minor elements (*ME*) (Allen 1968) was added

CW-15 was included in the study to test for possible effects due to binding of precipitated iron onto cell walls; however, culture characteristics were apparently identical to those of the wild type (Fig. 2).

Ferric chelate reductase. Somewhat unexpectedly, per-cell FC-R activity increased with increasing Fe-limited dilution rate (Fig. 3), i.e. FC-R activity was greater in cells that were less severely Fe-limited. In contrast, Fe-sufficient cells exhibited much lower FC-R activity (Fig. 3). Previous work from this laboratory (Lynnes et al. 1998), utilizing *C. reinhardtii* grown in semi-continuous culture, showed that switching to iron-free medium resulted in a progressive increase in per-cell FC-R activity with time. The results of Lynnes et al. (1998) are consistent with experiments utilizing "Strategy I" higher plants, in which root FC-R activity increases with time after imposition of Fe-deficiency (Moog and Brüggemann 1994). In contrast, the results shown in Fig. 3 clearly indicate that per-cell FC-R activity increases with increasing Fe-limited growth rates (i.e. with decreasing severity of Fe-limitation), suggesting that an Fe-limited chemostat yields a response that is physiologically distinct from that caused by sudden imposition of Fe-limitation by removal of iron. It could be speculated that during steady-state Fe-limitation achieved by using a chemostat system, the FC-R reductase capacity is adjusted to match the Fe-limited growth rate (i.e. the metabolic iron demands). In contrast, steady-state Fe-limited growth clearly would not be achieved in algal or plant experiments in which Fe-limitation is induced by suddenly removing iron from the system. This raises the possibility that FC-R activity, which is well-known to increase as a response to Fe-limitation (compared to Fe-sufficient conditions), might not be a good indicator of the degree of Fe-limitation, as less severely Fe-limited cells may exhibit

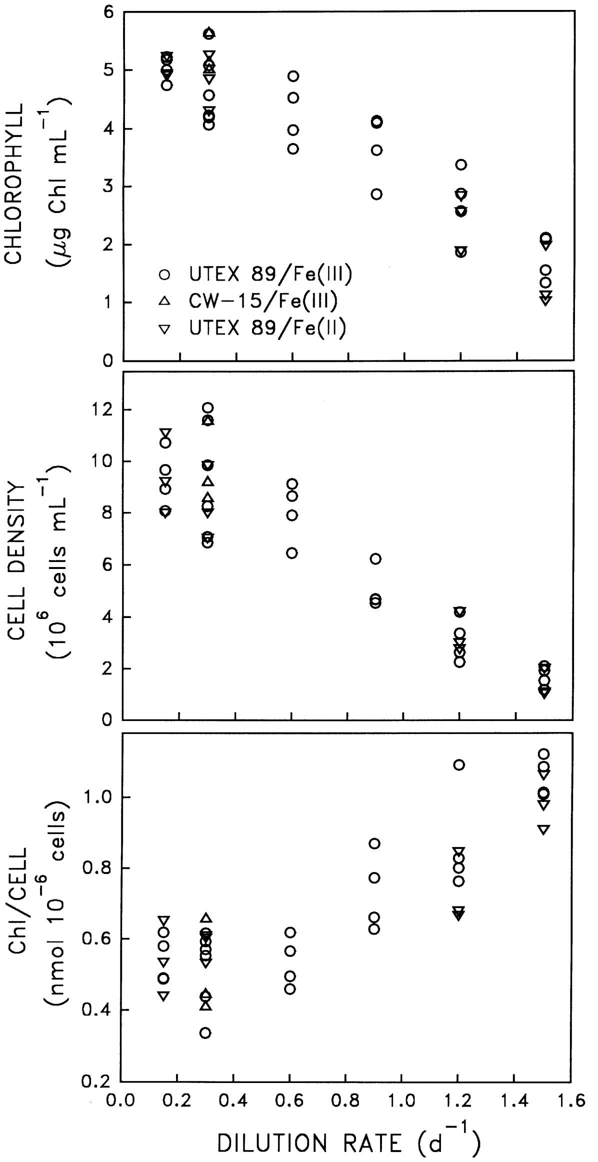

Fig. 2. Characteristics of Fe-limited culture as a function of dilution rate. Cultures were grown to steady-state, at which point dilution rate = growth rate. Iron was added at a final concentration of 0.25 μM, and EDDHA was added at 20.25 μM. Data points show *C. reinhardtii* UTEX 89 growing with Fe(III) (○), or Fe(II) (▽), and *C. reinhardtii* CW-15 growing with Fe(III) (△)

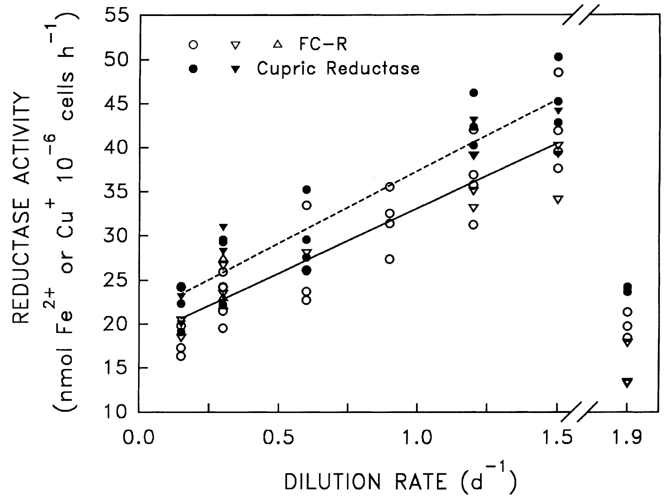

Fig. 3. Ferric chelate reductase (FC-R) and cupric reductase activities as a function of the dilution rate. A dilution rate of 1.9 d^{-1} exceeds the maximum growth rate, yielding an Fe-sufficient cell culture that is not in a steady state. At dilution rates of 1.5 d^{-1} and below, cultures were in a steady state and the dilution rate was equivalent to the Fe-limited growth rate. Iron was added to chemostat cultures at a final concentration of 0.25 μM, and EDDHA was added at 20.25 μM. *Hollow symbols*, FC-R; *solid symbols*, cupric reductase. Data show *C. reinhardtii* UTEX 89 growing with Fe(III) (○, ●), or Fe(II) (▽, ▼), and *C. reinhardtii* CW-15 growing with Fe(III) (△). Each symbol represents the mean of 4 replicate assays; SE is smaller than the symbol for all points. *Solid line*, ferric chelate reductase activity; *dashed line*, cupric reductase activity; lines were fitted by least-squares linear regression

greater FC-R activity than more severely Fe-limited cells.

The effects of Fe(III) concentration in the medium were also investigated (Fig. 4). Total culture chlorophyll levels responded approximately proportionally to [Fe(III)] in the medium (Fig. 4A), which also provided further evidence for iron limitation in the chemostat system. In contrast, while cell density also increased with increasing [Fe(III)], the relationship deviated slightly from linearity (Fig. 4B); cell density at [Fe(III)] = 0.05 μM averaged 4.47×10^{6} cells mL^{-1} while at [Fe(III)] = 0.40 μM it averaged 14.93×10^{6} cells mL^{-1}. Thus, the amount of chlorophyll per cell was higher at higher [Fe(III)], perhaps suggesting that self-shading of the culture was a factor at increased cell density. In contrast, per-cell FC-R activity was strictly a function of dilution rate (Fig. 3), and was not affected by the concentration of Fe(III) in the medium (Fig. 5).

Increases in cell density and total chlorophyll with increasing [Fe(III)] in the medium (Fig. 4) are consistent with chemostat theory. The only other algal Fe-limited-chemostat study to date, using the cyanobacterium *Synechococcus* PCC 7002, failed to show a clear correlation between medium [Fe(III)] and cell density (Wilhelm and Trick 1995). A semi-continuous-culture study utilizing three clones of marine *Synechococcus* also failed to show a linear relationship between chlorophyll or cell density and [Fe(III)] (Rueter and Unsworth 1991). One possible explanation for the apparent discrepancy between the *Synechococcus* studies and the present study might relate to the mechanism of iron uptake by *C. reinhardtii* compared to cyanobacteria. The latter cells are well-known to secrete Fe(III)-specific siderophores in response to Fe-limitation (e.g. Wilhelm and Trick 1994), and to date there is no evidence for a reductive mechanism for accessing extracellular Fe(III).

Iron-limited cells were able to reduce many different ferric chelates, with varying stability constants, at comparable rates (Table 1), suggesting that a wide range of chelated Fe(III) could be accessed in nature via FC-R activity. The lowest rates of FC-R activity were measured using non-chelated $FeCl_3$ as the iron source (Table 1). Furthermore, the rates shown in Table 1 for

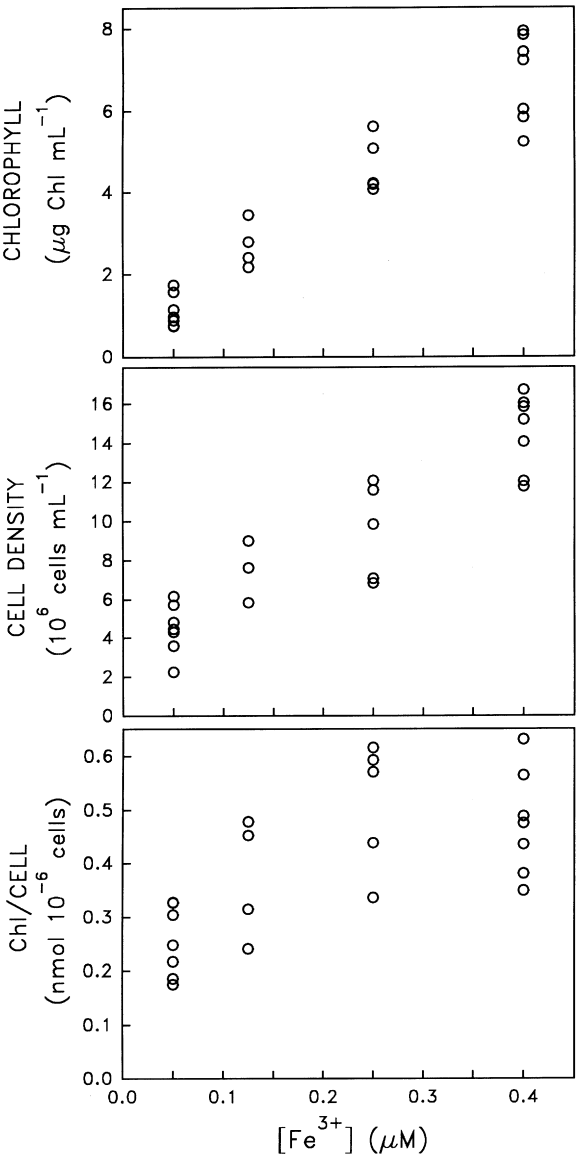

Fig. 4. Characteristics of Fe-limited culture as a function of [Fe(III)] in the medium. *Chlamydomonas reinhardtii* UTEX 89 was grown with Fe(III)-EDDHA as the iron source, with excess chelator (20 μM EDDHA), at growth rate = 0.3 d^{-1}

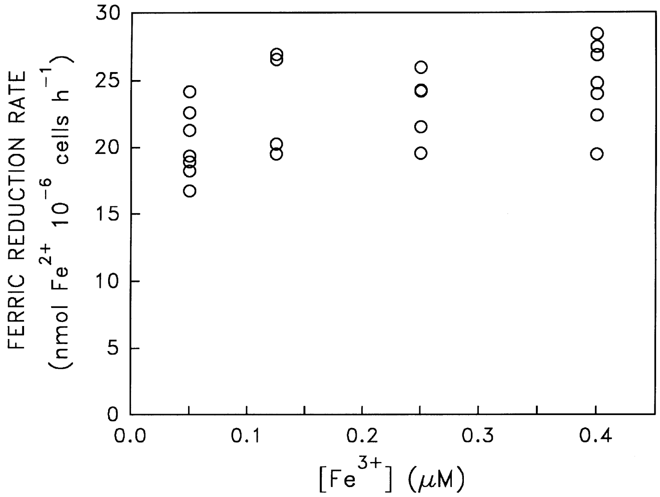

Fig. 5. Ferric reduction rate as a function of [Fe(III)] in the medium. *Chlamydomonas reinhardtii* UTEX 89 was grown in Fe-limited chemostats with Fe(III)-EDDHA as the iron source, with excess chelator (20 μM EDDHA), at growth rate = 0.3 d^{-1}

Table 1. Reduction of ferric chelate complexes. Ferric-chelate (1:1) was added at a concentration of 250 μM, and BPDS at 500 μM. Rates were determined in assay buffer using Fe-limited cells grown at growth rate = 0.3 d^{-1}. Results are the means of 6 replicates (± SE). The control rate of Fe(III)-EDTA reduction was 20.8 nmol Fe^{2+} 10^{-6} cells h^{-1} (±0.5, n = 6). Rates were calculated by linear regression over a 16-min time course. Stability constants (log $K^c_{0.1}$) are from Martell and Smith (1974a,b, 1981)

Chelator	Rate (% of EDTA control)	log $K^c_{0.1}$
EDTA	100	25.0
Citrate	105.0 ± 3.1	11.50
EDDHA	83.4 ± 1.3	33.9
EGTA	103.6 ± 2.2	20.5
HEDTA[a]	105.6 ± 2.0	19.8
Ferrioxamine mesylate	76.2 ± 3.8	30.60[b]
None (250 μM $FeCl_3$)	64.0 ± 5.6[c]	–

[a]Hydroxyethylethylenediaminetriacetic acid
[b]Stability constant for ferrioxamine B
[c]Rates for first 4 min of reduction only

$FeCl_3$ reduction were calculated only using the first 4 min of a 16-min assay, as the rate of reduction greatly decreased after that point (Fig. 6).

Concern has been expressed that the use of BPDS may lead to artifactual ferric reduction, in that the presence of BPDS may affect the equilibrium constant for ferric reduction (Thorstensen and Aisen 1990):

$$Fe^{3+} + e^- \Rightarrow Fe^{2+} \quad (1)$$

$$Fe^{2+} + 3\,BPDS \Rightarrow Fe(II)\text{-}BPDS_3 \quad (2)$$

Reaction (2) would serve to shift the equilibrium of reaction (1) towards Fe^{2+}, leading to artifactually high estimates of FC-R activity (Thorstensen and Aisen 1990). Hassett and Kosman (1995) used similar reasoning to argue that measurement of cupric reductase activities (which use BCDS to trap the cuprous ion) should be performed at low Cu(II):chelator ratios (i.e. excess Cu[II] chelator) to counteract the effect of the trapping of Cu^+.

In order to assess the potential of BPDS to influence apparent FC-R activity, effects of Fe(III):chelator ratios and effects of [BPDS] were measured (Table 2, Fig. 7). Excess EDTA had only a minor effect on FC-R activity, with a 20-fold excess of EDTA resulting in a 10% decrease in the rate of reduction (Table 2). Similarly, initial rates of Fe^{3+} reduction (i.e. measured as formation of Fe[II]-$BPDS_3$) were unaffected by BPDS concentrations ranging from 100 to 1000 μM (Fig. 7). These results suggest that the FC-R rates reported in this study reflect actual ferric reducing capacity, rather than an artifact of the assay system.

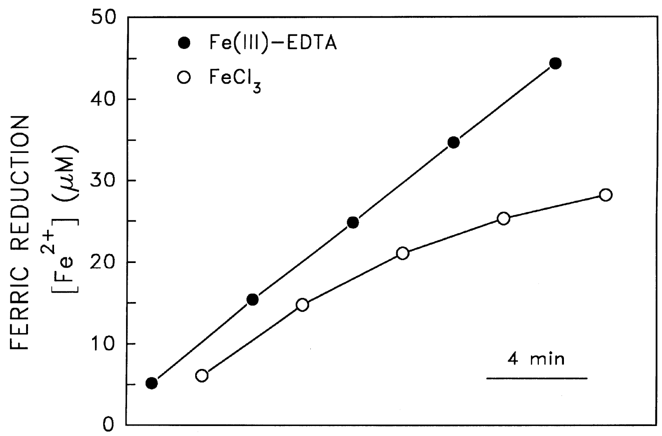

Fig. 6. Reduction of chelated (Fe[III]-EDTA) and non-chelated ($FeCl_3$) Fe(III) by Fe-limited cells (*C. reinhardtii* UTEX 89, growth rate = 0.3 d^{-1}). Ferric iron was added at 250 μM, and BPDS was added at 500 μM. Data are the means of 3 assays; SE is smaller than the symbol for all points

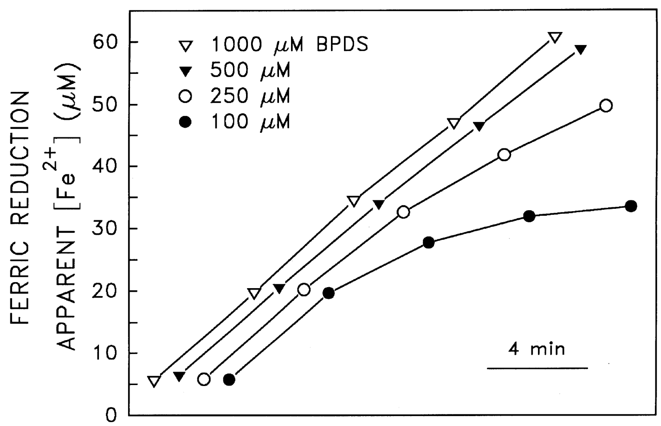

Fig. 7. Apparent reduction of Fe(III)-EDTA by Fe-limited *C. reinhardtii* UTEX 89 (growth rate = 0.3 d^{-1}) as a function of [BPDS]. The Fe(III)-EDTA was added at 250 μM. Data are the means of 4 experiments. SE was less than 5% of the mean for all points

Cupric reductase. Iron-limited cells also exhibited cupric reductase activity, which increased in parallel with FC-R activity as the Fe-limited growth rate increased (Fig. 3). Experiments using both higher plants (e.g. Norvell et al. 1993; Welch et al. 1993, Holden et al. 1995) and the yeast *Saccharomyces cerevisiae* (Hassett and Kosman 1995) have also provided evidence that Fe-limitation results in enhanced FC-R and cupric reductase activities, leading to the suggestions that the same reductase is responsible for both activities. Furthermore, both Fe-limitation and Cu-limitation result in increased FC-R activity by pea roots (Cohen et al. 1997).

The hypothesis that the same enzyme is responsible for both activities was assessed by assessing the potential for mutual competitive inhibition. As evident from Fig. 3, cupric reductase activity is usually slightly greater than is FC-R activity, for any given growth rate. This is also shown in Fig. 8A, for cells grown at 0.3 d^{-1}.

Addition of 250 μM Cu(II)-citrate to cells reducing Fe(III)-EDTA initiated cupric reduction, and completely inhibited the reduction of Fe(III)-EDTA (Fig. 8B). Furthermore, simultaneous addition of 250 μM each of Fe(III)-EDTA and Cu(II)-citrate resulted in only cupric reduction; FC-R activity was not detectable (not shown). These results suggest that Cu(II) and Fe(III)

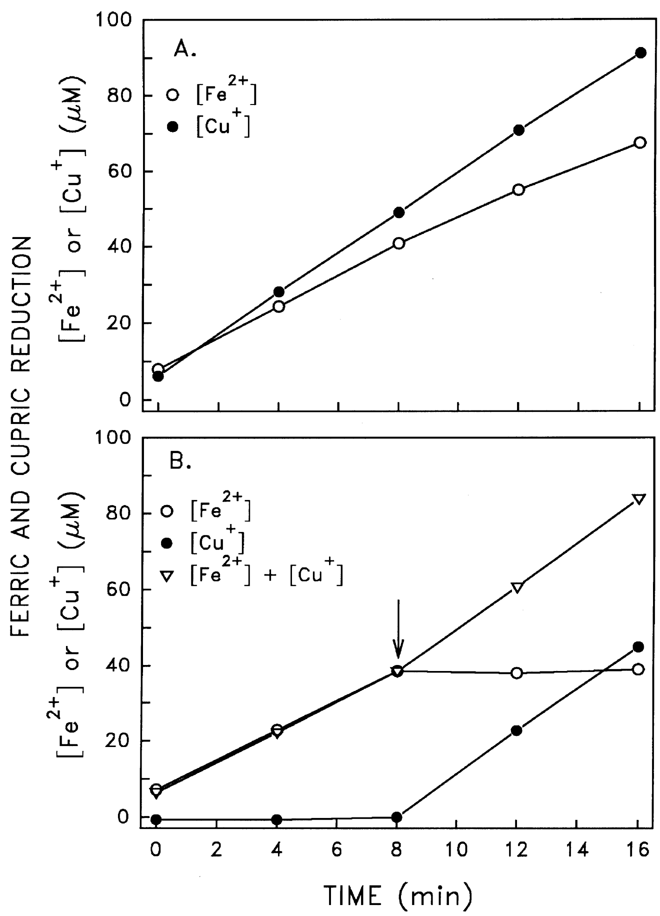

Fig. 8A,B. Competitive inhibition of Fe(III) reduction by Cu(II) in Fe-limited *C. reinhardtii* UTEX 89 (growth rate = 0.3 d^{-1}). **A** Separate assays of Fe(III) and Cu(II) reduction. **B** Addition of 250 μM Cu(II) (1:1.5 Cu[II]:citrate) to cells reducing Fe(III)-EDTA; *arrow* indicates time of addition of Cu(II). All experiments contained 500 μM each of BPDS and BCDS. The Fe(III) and Cu(II) were added at 250 μM. Both [Fe(II)] and [Cu(I)] were determined spectrophotometrically from simultaneous equations. Data are the means of 3 assays; SE is less than 5% of the mean for all points

Table 2. Effect of Fe(III):EDTA ratio on FC-R activity. Rates were determined in assay buffer using Fe-limited cells grown at growth rate = 0.3 d^{-1} with 0.25 μM Fe(III)-EDDHA. Bathophenanthroline disulphonate was added at 500 μM. Rates are expressed as percent of 1:1 Fe(III)-EDTA (± SE, n = 5)

Fe(III):EDTA	FC-R activity (% of 1:1 Fe[III]-EDTA ± SE)
1:2	98.5 ± 2.4
1:5	97.1 ± 3.1
1:10	95.8 ± 2.9
1:20	92.1 ± 2.9

Table 3. Reduction of Cu(II) by Fe(III). Experiments were run in assay buffer. The BPDS and BCDS were each added at 500 µM. When present, Cu(II) was added from a 1:1.5 $CuSO_4$:citrate stock. The reaction was initiated by the addition of 50 µM Fe(II) as either $FeSO_4$ (freshly prepared) or Fe(II)-EDTA. Both [Fe^{2+}] and [Cu^{+}] were determined from simultaneous equations (described in *Materials and methods*). Results are the means of 4 experiments (±SE)

	[Fe^{2+}] (µM)	[Cu^{+}] (µM)	[Fe^{2+}] + [Cu^{+}] (µM)
50 µM $FeSO_4$/0 µM Cu(II)-citrate	51.3 ± 1.1	0	51.3
50 µM $FeSO_4$/250 µM Cu(II)-citrate	46.5 ± 0.9	5.1 ± 0.6	51.6
50 µM Fe(II)-EDTA/ 0 µM Cu(II)-citrate	50.7 ± 1.0	0	50.7
50 µM Fe(II)-EDTA/250 µM Cu(II)-citrate	49.7 ± 0.5	0.3 ± 0.2	49.9

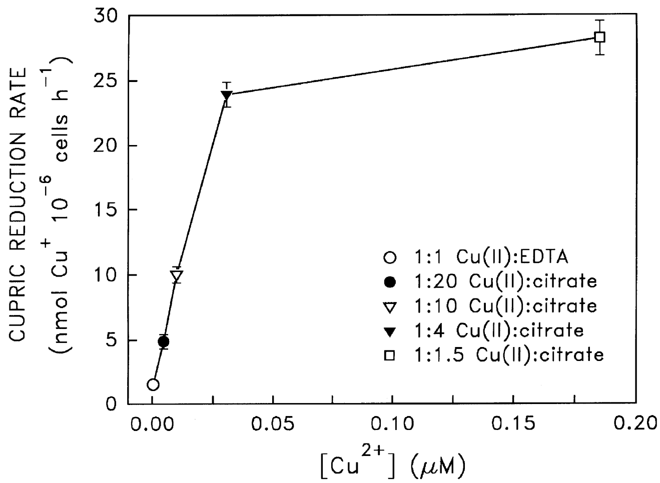

Fig. 9. Cupric reduction rate of Fe-limited *C. reinhardtii* UTEX 89 (µ = 0.3 $^{-1}$) as a function of free [Cu^{2+}]. Cupric-chelate was added at 250 µM, and BCDS was added at 500 µM. Data are the means of 4 experiments ± SE

are reduced by the same reductase, or, at the very least, access the same intracellular pool of reducing power.

The possibility that the apparent rapid reduction of Cu(II) versus Fe(III) is due to cellular reduction of Fe(III) to Fe(II), followed by subsequent reduction of Cu(II) to Cu(I) by Fe(II), was investigated in a series of cell-free experiments (Table 3). Addition of 50 µM non-chelated $FeSO_4$ to assay buffer containing both BPDS and BCDS, and 250 µM Cu(II)-citrate, resulted in production of only a small amount of Cu^{+} (Table 3). Furthermore, addition of Fe^{2+} in the form of Fe(II)-EDTA resulted in only a barely detectable level of Cu^{+} (Table 1). In other words, it is unlikely that the rapid reduction of Cu(II) versus Fe(III) is due to the reduction of Cu(II) by Fe(II).

Among phytoplankton and higher plants, there is some question about whether free Cu^{2+} or a Cu(II)-chelate is the species that is actually reduced. Evidence in favor of reduction of cupric complexes has been presented for *Thalassiosira weisflogii* (Jones et al. 1987), and reduction of cupric complexes has often been tacitly assumed in higher-plant experiments (by analogy with the reduction of ferric complexes). Conversely, Holden et al. (1995) provided evidence that cupric reduction by root plasma membrane vesicles isolated from iron-limited tomato plants was a function of the free [Cu^{2+}], rather than the concentration of Cu(II)-chelate. Using a similar approach with Fe-limited *C. reinhardtii* cells, it may be shown that the rate of reduction of 250 µM Cu(II)-chelate is also a function of the [Cu^{2+}] (Fig. 9), suggesting that free Cu^{2+} is the form of copper that is reduced by the reductase.

Conclusions. In Fe-limited *C. reinhardtii* cells, FC-R activity was a function of the Fe-limited growth rate, such that increasing Fe-limited growth rate (and decreasing degree of Fe-limitation) led to increasing FC-R activity. Iron-limited cells also exhibited cupric reductase activity, which also increased with increasing Fe-limited growth rate. The inhibition of FC-R activity by cupric reduction suggests that both reductases might represent the same plasma-membrane enzyme. The activity of FC-R is not necessarily a good indicator of the Fe status of algal cells, as severely Fe-limited cells and Fe-sufficient both exhibited low FC-R activities.

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