Running title:
Iron binding and internalization in *Dunaliella*

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**Research area:**
Environmental stress and adaptation to stress
Effects of Iron Deficiency on Iron Binding and Internalization into Acidic Vacuoles in *Dunaliella salina*¹

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FOOTNOTES

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Uptake of iron in the halotolerant alga *Dunaliella salina* is mediated by a transferrin-like protein, TTf, which binds and internalizes Fe$^{3+}$ ions (Fisher M, Zamir A, Pick U [1998] J Biol Chem 273:17553-17558). Recently we found that iron deficiency induces a large enhancement of iron binding which is associated with accumulation of three other plasma membrane proteins that associate with TTf (Paz J, Katz A, Pick U [2007] J Biol Chem 282:8658-8666). In the present study we characterized the kinetic properties of iron binding and internalization and identified the site of iron internalization. Iron deficiency induces a 4-fold increase in Fe binding but only 50% enhancement in the rate of iron uptake, and also increases the affinity for iron and for bicarbonate, a co-ligand for iron binding. These results indicate that iron deprivation leads to accumulation and to modification of iron binding sites. Iron uptake in iron-sufficient cells is preceded by an apparent time lag, resulting from pre-bound iron, which can be eliminated by unloading iron binding sites. Iron is tightly bound to surface-exposed sites and hardly exchanges with medium iron. All bound iron is subsequently internalized. Accumulation of iron inhibits further iron binding and internalization. The vacuolar inhibitor bafilomycin inhibits iron uptake and internalization. Internalized Fe was localized by electron microscopy within vacuolar structures that were identified as acidic vacuoles. Fe internalization is accompanied by endocytosis of surface proteins into these acidic vacuoles.

A novel kinetic mechanism for iron uptake is proposed, that includes two pools of bound/compartmentalized iron separated by a rate-limiting internalization stage. The major parameter that is modulated by iron deficiency is the iron binding capacity. We propose that the excessive iron binding in iron-deficient cells serves as a temporary reservoir for iron that is subsequently internalized. This mechanism is particularly suitable for organisms that are exposed to large fluctuations in iron availability.
INTRODUCTION

Iron is an essential element for survival for all living organisms, including photosynthetic organisms that have a special requirement for iron as a cofactor of multiple elements in their electron transport system. Because of its low solubility in aerobic solutions, iron is recognized as a major limiting factor for proliferation of plants and algae. To counterbalance Fe limitation, photosynthetic organisms evolved efficient high affinity Fe uptake mechanisms that are induced under iron limitation. The two major mechanisms of iron acquisition studied in plants are based either on reduction of organic ferric iron chelates, via a membrane associated ferrireductase (Strategy I plants), or on release of phytosiderophores that bind ferric ions and introduce it into the cell via a special receptor (strategy II plants) (Mori, 1999; Curie and Briat, 2003; Hell and Stephan, 2003). Algae appear to utilize similar mechanisms for iron acquisition. In the green algae Chlorella and Chlamydomonas and in several diatoms, iron deficiency induces a large enhancement of ferrireductase activity, associated with stimulation of high-affinity Fe uptake, suggesting a redox-driven iron uptake mechanism, similar to strategy I plants (Allnutt and Bonner, 1987; Lynnes et al., 1998; Eckhardt and Buckout, 1998). Other algae, such as Scenedesmus incrassatulus and Emiliania huxleyi, utilize siderophore-mediated iron uptake, similar to strategy II plants and bacteria (Benderliev and Ivanova, 1994; Boye and Berg, 2000). However, a comprehensive identification of proteins and genes associated with iron acquisition was made only in two species of algae, Chlamydomonas reinhardtii and Dunaliella salina. Several components of a copper-dependent iron uptake mechanism were identified and characterized in C. reinhardtii. They include a multicopper ferroxidase and an iron permease, suggesting that high-affinity iron uptake in C. reinhardtii is similar to that in Saccharomyces cerevisiae (La Fontaine et al., 2002, Herbik et al., 2002). A different high affinity Fe uptake mechanism was identified in the halotolerant alga D. salina, which is based on iron binding and internalization via a membrane associated transferrin-like protein, TTf (Fisher et al., 1997; 1998). In previous studies we demonstrated that Fe uptake and binding in D. salina resembles kinetic characteristics of animal transferrins, and that the process consists of two distinct stages, binding and internalization of Fe$^{3+}$ ions, which can be resolved by their different temperature dependence (Fisher et al., 1998; Schwarz, et al., 2003a). Iron deprivation or high salinity
induce in *D. salina* a small stimulation of Fe uptake that is correlated with the accumulation of the transferrin-like protein, TTf. However, the kinetic mechanism has not been investigated and the site of iron internalization was not identified. In a recent study we found that Fe deprivation induces in *D. salina* a large increase in Fe binding capacity and that this enhanced binding was correlated with accumulation of three additional plasma membrane proteins which were found to associate with TTf: a second transferin termed DTf, a multicopper ferroxidase, termed DFox and another 130 kDa protein (Paz et al, 2007).

In this work we studied the regulation of iron binding and uptake. Specifically, we characterized the kinetic changes in iron binding and uptake in response to iron deprivation or to saturation of intracellular iron stores. We show that iron deprivation or over-accumulation lead to large changes in iron binding suggesting that this is the critical parameter in regulation of iron uptake in *D. salina*. We also localized the site of Fe internalization within acidic vacuoles. The physiological significance of dynamic changes in Fe binding is discussed.

RESULTS

*Dunaliella* cells contain externally-bound iron

The time course of Fe uptake in *Dunaliella salina* reveals an initial time lag of 15-20 min. This time lag was apparent in control cells, which have been cultured with iron, but was absent in iron deficient cells (Fig. 1A). We found that pre-incubation of control *D. salina* cells for 30-60 min in iron-deficient medium, almost completely eliminated this apparent time lag (Fig. 1B). Conversely, pre-incubation of iron-deficient cells with iron, introduced a similar time lag in the onset of Fe uptake (Fig. 1C). These results suggest that the apparent time lag in Fe uptake in *D. salina* may result from pre-bound iron.

In order to test directly if *D. salina* cells contain pre-bound iron, we measured iron binding to cells before or after 1h pre-incubation treatments in Fe-deficient medium. Iron binding was measured at 4°C, to avoid internalization of bound iron which is strongly temperature-dependent (Fisher et al., 1998). As shown in Fig. 2, binding of iron to control
cells was greatly enhanced by the pre-incubation treatment. Iron-deficient cells have a 4-fold larger iron binding capacity, but in contrast to iron-sufficient cells the binding was hardly affected by the pre-incubation treatment. Conversely, preincubation of iron-deficient cells with iron, completely inhibited iron binding. These results confirm that the apparent inhibition of Fe uptake results from pre-bound iron. Pre-incubation in iron-deficient medium unloads the iron-binding site (Fe unloading), whereas pre-incubation with iron uploads the unoccupied sites (Fe uploading).

In earlier studies we have shown that the binding characteristics of iron to *D. salina* cells resemble transferrin iron binding sites (Fisher et al., 1998). Moreover, we found that isolated TTf from *D. salina* has very similar Fe$^{3+}$ binding parameters as intact cells and that dissociation of TTf from plasma membranes completely eliminated Fe binding activity to TTf-deficient membranes (Schwarz et al., 2003a). These studies demonstrated that most bound iron in *D. salina* is associated with this transferrin. To check if also Fe-deficient cell have similar iron binding characteristics, we compared the effects of characteristic parameters of TTf iron binding between Fe-sufficient and Fe-deficient *D. salina* cells (Table 1). A similar dependence was found on bicarbonate, a co-ligand for Fe binding to transferrins, on pH, and hardly any inhibition by excess of other metal ions suggesting a high specificity for Fe$^{3+}$. These results suggest that also in Fe-deficient cells, iron is mostly bound to transferrin.

A summary of the kinetic parameters of iron binding and uptake is presented in Table 2. The initial rate of iron binding was enhanced 8-fold in iron-deficient cells, as compared to control cells. This enhancement can be accounted for partly by the 4-fold increase in number of iron-binding sites, and partly by the 2-fold larger calculated binding rate constant ($k_b$). In contrast to the enhanced binding, the steady state rate of iron uptake was enhanced just by about 50% in iron-deficient cells. The large increase in iron binding capacity and binding rate constant suggests that iron deprivation induces accumulation of new iron binding sites, with slightly different kinetic properties. In order to test if iron deprivation affects also the kinetic properties of iron binding sites, we compared two kinetic parameters of iron binding between iron-deficient and iron-sufficient cells, the concentration dependence on iron and on bicarbonate anions which are co-ligands for iron binding to transferrins and are required for binding of Fe$^{3+}$ ions to *D. salina* transferrin, TTf
(Fisher et al., 1997;1998). As shown in Table 2, iron-deficient cells display a higher affinity for both iron and bicarbonate, suggesting changes in properties of the iron-binding sites.

**Tightly-bound iron exchanges slowly with medium Fe$^{3+}$**

The observation that *D. salina* cells contain pre-bound iron, that is not removed by extensive washing, is consistent with the high binding affinity of *D. salina* transferrin-like protein, TTf, for Fe$^{3+}$ ions that we reported in previous studies (Fisher et al., 1998; Schwarz et al., 2003a). To find out if iron is irreversibly bound or exchanges with medium Fe$^{3+}$ ions, we added excess of natural (non-radioactive) isotope mixture of Fe$^{3+}$, as Fe-citrate, after binding $^{59}$Fe$^{3+}$ to control or iron-deficient *D. salina* cells. As shown in Fig. 3 and in Table 3, there is less than 10% exchange within 1h at 4°C. The bound iron can be striped off effectively by ethylenediaminetetraacetic acid (EDTA) and to a lesser extent at acidic pH. These results explain why cells that were pre-cultured with iron do not bind $^{59}$Fe$^{3+}$, even after extensive washing, since all iron binding sites are occupied with tightly-bound iron.

**Bound iron is committed for internalization**

In a previous study we reported that bound iron in *D. salina* cells is internalized subsequent to the binding in a temperature-dependent process (Fisher et al., 1998). Since in iron-deprived cells we found a large increase in number and changes in kinetic parameters of iron binding sites, it seemed of interest to test how much of the bound iron is internalized in these cells. To test this, cells were first pre-incubated with $^{59}$Fe$^{3+}$ at 4°C, to saturate the iron binding sites, followed by washing and internalization in the presence of excess natural isotope mixture Fe-citrate, to exclude internalization of non-bound iron. As shown in Fig. 4, practically all the bound iron was internalized in both control and iron-deficient cells. The amount of internalized iron is largely enhanced in iron-deficient cells, corresponding to the much higher binding, but the process is slower, with a $t_{1/2}$ of about 30 min, compared to about 10 min in control cells. These results suggest that the massive amount of bound iron in iron-deficient cells is committed for internalization. About 30% of the bound iron in iron-deficient cells is not internalized after 1h of incubation without iron, but can be internalized by an extended incubation (not shown). Since excess of iron may be
toxic to cells, we wished to test if \textit{D. salina} cells can regulate their iron binding capacity to avoid over-accumulation of iron. To test this, cells were allowed to accumulate iron for 1.5 h or 4 h and tested for iron binding and uptake activities. To dislodge external binding sites from pre-bound iron, cells were exposed to two different treatments: acidic-ethylenediaminetetraacetic acid (EDTA) washes, which strip-off bound iron, or the 1h unloading treatment, to allow internalization of the pre-bound iron. As is shown in Table 4, these treatments gave different results: after 4 h of iron accumulation, ethylenediaminetetraacetic acid (EDTA) striping recovered about 90% of the original activity, whereas the unloading treatment recovered only 10-15%. These results suggest that as the internal iron stores fill up, the internalization is inhibited and more iron remains externally bound. This may represent a feedback mechanism to avoid iron overloading.

\textbf{Bound iron is internalized into acidic vacuoles}

In mammalian cells iron is internalized via the transferrin/transferrin receptor system into acidic vacuoles. In order to test if also in \textit{D. salina} iron in internalized into acidic vacuoles, we tested the effects of vacuolar inhibitors on iron uptake. In previous studies we have identified and characterized acidic vacuoles in \textit{D. salina} by staining with a fluorescent indicator quinacrine (Weiss and Pick, 1991) and by localization of intravacuolar polyphosphates which act as a pH buffering system (Pick et al.,1991; Pick and Weiss, 1991). We found that amines can induce an alkaline stress which is counterbalanced by massive polyphosphate hydrolysis within the vacuoles combined with amine accumulation. Thus, amines at alkaline pH can disrupt vacuolar activities in \textit{D. salina}. As shown in Table 5, the vacuolar \textit{H}⁺-ATPase inhibitor, bafilomycin, as well as ammonia at alkaline pH, suppressed iron uptake, but only partially inhibited iron binding. Under these conditions bafilomycin did not decrease cellular ATP, nor did it inhibit uptake of phosphate (data not shown) indicating that the inhibition is specific to vacuolar activities. In order to visualize internalized iron we stained \textit{D. salina} cells with acid ferrocyanide (Prussian blue), a stain utilized for sub-cellular localization of bound Fe\textsuperscript{3+} ions in different organisms, including plants (Parmley et al., 1978;Kim et al., 2003;Vasconelos et al., 2003;Arbab et al., 2006;Tallheden et al., 2006), and monitored stained cells by electron microscopy. Bound iron particles were clearly visualized at the extracellular cell
surface (Fig. 5A, inset). The remarkable difference before and after internalization, was the dense iron staining within vacuolar-like structures (compare Figs 5A and 5B). At high magnification, iron within some of the vacuoles appears to be associated with a delicate membrane-like reticulum (E-F) and in others is aggregated in dense plaques (C,D). This stained iron particles resemble in size and general appearance early ultrastructural localizations of iron within phagosomes of macrophage cells (Parmley et al., 1978). The significance of the different patterns of vacuolar iron staining is not clear. They may represent different stages in vacuolar maturation after endocytosis, possibly, the reticular structures may represent residual plasma membrane structures in immature vacuoles, shortly after endocytosis, which later mature to yield the aggregated iron morphology. In order to further characterize these iron-accumulating structures, we treated D. salina cells with the fluorescent indicator LysoSensor Green that specifically stains acidic vacuoles. As shown in Fig 6A, the stain accumulates in vacuolar structures similar in size and localization to the iron accumulating vacuoles. To further characterize these vacuoles, we immunolabeled D. salina cells with polyclonal antibodies against a vacuolar marker previously reported to label acidic vacuoles in the green alga Chlamydomonas reinhardtii, the H\(^+\)-transporting pyrophosphatase (Ruiz et al., 2001). The antibodies reacted with single component on Western immunoanalysis corresponding to its predicted molecular weight (not shown). As shown in Fig. 6B, anti H\(^+\)-pyrophosphatase labeled structures similar in size and localization to acidic vacuoles. Unfortunately, it is not possible to carry out co-localization of the iron stain with neither immunolocalization nor with the LysoSensor Green staining because of the different required preparation procedures (acid treatment, fixation and detergent permeabilization or vital stain, respectively). In order to test if iron internalization indeed results from endocytosis of iron-binding proteins, we tagged membrane surface proteins in iron-deficient cells with a membrane-impermeable biotin derivative and monitored biotin-tagged proteins labeled with fluorescein-avidin with a confocal microscope. We found that in iron-deficient cells, biotin tags almost exclusively three protein bands that were identified as the major iron-deprivation induced plasma membrane proteins (Paz et al., 2007): Two transferrin-like proteins, TTf (Fisher et al., 1997,1998) and DTf (Schwarz et al., 2003) and a 130 kDa band that was found to contain two proteins, a multicopper ferroxidase, termed DFox and a second unknown protein. In
stained cells that were kept at 4°C, biotin-tagged label was confined to the outer cell surface (Fig. 6C). However, after internalization of bound iron for 1h at 24°C, tagged vacuolar-like structures appeared at the apical side of the cells Fig 6D). Pre-incubation with the vacuolar inhibitor bafilomycin prevented the appearance of biotin-tagged vacuoles, but induced structural deformation that appears like thickening of the membrane surface at the apical side of the cells (Fig. 6E). Taken together, these results suggest that iron is internalized into acidic vacuoles by endocytosis of iron-binding proteins and that the process is inhibited by bafilomycin.

DISCUSSION

As shown in our previous work (Fisher et al., 1998) and here, *D. salina* binds large amounts of Fe$^{3+}$ ions. Iron-deficient cells can bind about 4 n mol Fe/10$^9$ cells, or 2.5x10$^6$ Fe$^{3+}$ ions/cell, yielding an exceptionally high calculated density of about 10$^5$ Fe/square micron (average cell diameter of 5 micron). Iron can be accepted from a variety of Fe$^{3+}$ ligands, including citrate, N-(2-Hydroxyethyl)ethylenediamine-N,N′,N′-triacetic acid, EDTA or desferal in the presence of bicarbonate (Pick, 2004) and is so tightly bound that it cannot be washed away or exchanged by excess medium iron and requires acidification and EDTA for effective release. In fact, the large increase in iron binding enables *D. salina* to bind in a short time sufficient iron to satisfy its whole iron budget, and then slowly internalize the bound iron. The tight binding of iron which is followed by an energy-dependant internalization (Fisher et al., 1998), render this process practically irreversible. These properties are characteristic to iron binding to transferrins, consistent with our previous demonstration that *D. salina* acquires iron via a surface-exposed transferrin, TTf.

The simplest kinetic model to describe iron uptake in *D. salina* according to our results is the following (see supplemental material):

$$
\text{Fe}^{3+} + \text{Tf} \xrightarrow{k_b} [\text{Fe-Tf}] \xrightarrow{k_{\text{int}}} [\text{Fe-V}]
$$
Where Fe$^{3+}$ represents free ferric ions, Tf- iron binding proteins, [Tf-Fe]- bound iron and [Fe-V]- internalized iron. $k_b$ and $k_{int}$ represent the rate constants for iron binding and internalization, respectively. $k_b$ is much larger than $k_{int}$, therefore the overall rate of Fe uptake is dominated by the rate of internalization, except at limiting Fe concentration. Over accumulation of iron according to this model, is predicted to inhibit $k_{int}$ leading to occupation of all iron binding sites (accumulation of [Fe-Tf]) and to inhibition of Fe uptake. Iron deprivation, as shown, increases primarily Tf (4-fold) and $k_b$ (2-fold) but not $k_{int}$ or [Fe-V], as manifested by the large increase in Fe binding capacity, Fe binding rate constant and the modest increase in Fe uptake, respectively. The 4-fold increase in Fe binding capacity indicates induction of more iron binding proteins at the plasma membrane. The increase in Fe-binding rate constant and the lower Km for iron and for bicarbonate indicate intrinsic changes in the iron binding sites. Conversely, the small increase in rate of internalization suggests that the number of Fe binding units, [Fe-Tf], does not increase very much. Our kinetic results can be accommodated with the above kinetic model, by inferring that in Fe-deficient plasma membranes, small iron binding units made of Fe-Tf, are converted to larger iron binding complexes with higher iron binding capacity, without major changes in the number of Fe binding units (or $[\text{Fe}^n_{\text{Tf}}]_x \rightarrow [\text{Fe}^m_{\text{Tf}}]_y$, where $m \gg n$ and $x \sim y$ ). As mentioned above, we found recently that the increase in Fe binding in Fe deficient cells is correlated with induction of three plasma membrane proteins DTf (Schwarz et al., 2003b) DFox and p130B that associate with TTf (Paz et al., 2007). These results are consistent with the proposed kinetic model. The reasons why Dunaliella selected transferrins for iron binding are not clear. One reason may be that iron binding to transferrins is quite resistant to high salt. Another reason may be the high affinity and selectivity of transferrins for ferric ions, which would exclude competition by common divalent and trivalent metal ions that are often abundant in hypersaline and brackish water solutions.

The kinetic response to Fe deprivation differs very much from plants, other algae, yeast and bacteria, where iron deprivation is usually associated with a dramatic increase in the rate of iron uptake.

However, such a mechanism is suitable for an organism that is exposed to large periodical changes in iron availability. The natural living habitats of Dunaliella are saline.
lakes and the open sea, which are often poor in bio-available iron and obtain their iron supply from winter floods or dust carried by storms. The proposed mechanism of iron acquisition in *D. salina* offers a competitive advantage in comparison to carrier-mediated uptake mechanisms under such conditions, since it enables to efficiently capture within a short time large quantities of iron from diverse iron binding ligands. Possible drawbacks of massive accumulation of proteins at the plasma membrane surface are the heavy energetic cost of protein synthesis and it may interfere with other membrane functions since it occupies a large area of the plasma membrane.

The finding that *D. salina* internalizes iron into acidic vacuoles resembles iron uptake via the transferrin/transferrin receptor system in mammalian cells, but is unusual for plants that store iron primarily in the chloroplast bound to ferritin (Briat, 2003). Nevertheless, plant seeds store iron in acidic vacuoles (Lanquar et al., 2005) and it was recently reported that in the presence of sufficient phosphate, *Arabidopsis* stores iron as phosphate/iron complexes within the vacuole (Hirsch et al., 2006). *Dunaliella* as well as other algae store phosphate primarily as insoluble polyphosphates within acidic vacuoles (Pick and Weiss, 1991; Ruiz et al., 2001). Vacuolar polyphosphates may serve as an excellent sink for iron storage by providing a high-capacity iron binding reservoir. Thus, acidic vacuoles may serve a central role in the regulation and storage of iron in *D. salina*. One manifestation of vacuolar regulation is the inhibition of iron binding and uptake as the vacuoles fill-up. It should be realized, however, that the vacuoles are not the final station of iron in *Dunaliella*. We estimated from $^{59}$Fe sub-cellular fractionation, that under steady-state growth conditions in Fe sufficient medium, 70-80% of the cellular iron in *D. salina* is localized within the chloroplast, partly stored in ferritin and partly incorporated into different proteins (Pick, unpublished observations). Thus, similar to plants, also in *Dunaliella* the chloroplast seems to be the major consumer and storage site for iron, and the acidic vacuoles are just an intermediary storage station. However, for an organism that takes up in a relatively short time enormous amounts of iron, vacuoles can serve as a safe buffering check-point that enables to control cytoplasmic levels of iron and to avoid iron toxicity. Thus, *Dunaliella* has evolved a very exceptional mechanism for iron acquisition and homeostasis that may have special advantages for adaptation to hyper-saline and to iron-deficient environments.
MATERIALS AND METHODS

Algal strain and growth condition

*Dunaliella salina*, a green species, was obtained from the culture collection of Dr. W. H. Thomas (La Jolla, CA). All culture glassware were washed in acid and thoroughly rinsed with Mili-Q water. Cells were cultured in Fe-sufficient or in Fe-deficient media as previously described (Fisher et al., 1997; 1998; Schwarz et al., 2003b). Standard growth medium contained 1M NaCl, with or without FeCl$_3$/ EDTA (1.5/6.0 μM) unless otherwise indicated.

Microscopic techniques

*Prussian blue staining of iron.* Ultrastructural localization of iron was made with acid ferrocyanide staining (Parmley et al., 1978). Iron-deficient cells were collected by centrifugation, washed once in 1M NaCl and suspended in iron uptake buffer containing growth medium without iron, 50 mM Na-N-(2-hydroxyethyl)-piperazine-N’-(2-ethanesulfonic acid) (HEPES), pH 8.0, and 5 mM NaHCO$_3$. Cells were incubated with 10 μM Fe-citrate for 30 min on ice (bound iron), or for 60 min at room temperature in the light under continuous shaking (internalized iron). Cells were fixed in 3.7% *para*-formaldehyde plus 1% glutaraldehyde and were next reacted for 30 min with a fresh Perl’s ferrocyanide solution, consisting of 0.5 g of potassium ferrocyanide in 49.5 ml of distilled water to which 0.5 ml of concentrated HCl (32%) was added. After extensive washing, fixed samples were rehydrated and embedded in Epon. Thin sections were examined in a Tecnai T12 (FEI, Eindhoven, Netherlands) electron microscope operating at 120 kV and images were recorded using a MegaView III CCD camera (SIS GmbH, Germany).

*Staining acidic vacuoles with LysoSensor Green.* *D. salina* cells (2x10$^7$ cells/ml in fresh growth medium, pH 8) were incubated for 10 min with 2 μM LysoSensor Green DND-189 (Molecular probes Inc., Eugene, Oregon) from a 1 mM stock solution in dimethylsulfoxide, washed twice, incubated for 10 min with 0.25% *p*-formaldehyde and placed on a poly-lysine-coated glass slide. Cells were viewed and photographed in a laser-scanning confocal microscope (Fluoview FV500, Olympus) using excitation wavelength of 458 nm and emission of 510-550 nm or of >660 nm to view LysoSensor Green and chlorophyll, respectively.
**Immunolocalization.** To immunolocalize acidic vacuoles, *D. salina* cells were labeled with antibodies against *C. reinhardtii* vacuolar H⁺-pyrophosphatase (V-H⁺-PPase), obtained from Dr. R. Decampo, University of Illinois, Urbana, Il. Cells were fixed for 1 h with freshly prepared 3.7% *p*-formaldehyde, permeabilized for 5 min with 1% NP-40, washed twice, incubated for 60 min with blocking medium containing 4% goat serum, 1% BSA and 0.05% Tween 20 in PBS. The preparation was incubated for 12 h with rabbit anti V-H⁺-PPase serum (at 1:200 dilution) in blocking solution at 5º, washed twice, incubated with fluorescein goat anti-rabbit polyclonal antibodies (Jackson ImmunoResearch Laboratories Inc., Baltimore, PA), washed twice and viewed and photographed in a confocal microscope as above at excitation wavelength 488 nm and emission wavelengths 505-525 nm or >660 nm to view fluorescein and chlorophyll, respectively.

**Iron uptake assay**

Fe uptake in *D. salina* cells was measured as previously described (Fisher et al., 1998). Cells (5×10⁷ cells/ml) were incubated with ⁵⁹Fe-citrate in the light for 1 h, the uptake was stopped by dilution into an acidified EDTA medium (pH 5.5), left for 15-30 min on ice, collected by centrifugation and washed once again in the same medium. This treatment was found to remove all externally-bound iron, but not internalized iron. Washed cells were counted in a β-counter.

**Iron Binding assay**

Before the binding assay, cells were treated to “unload” pre-bound iron by pre-incubation for 1 h at 23ºC in buffer containing growth medium without iron, 50 mM Na-N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 8.0, and 5 mM NaHCO₃ in the light. Iron binding to intact cells was measured essentially as previously described (Fisher et al., 1998). In brief, cells were incubated for 30 min at 4ºC with ⁵⁹Fe-citrate, washed in EDTA-free buffer (stop solution) and counted in a β-counter. Under these conditions >95% of the cell-associated iron was found to be externally-bound to transferrins (bicarbonate-dependent, EDTA-sensitive). Plasma membrane vesicles were incubated for 1 h on ice with ⁵⁹Fe-citrate in: 50 mM Na-HEPES 8.0, 0.2 M KCl and 5 mM NaHCO₃ and passed through semi-dry Sephadex G-50 columns to eliminate unbound ⁵⁹Fe as previously described (Schwarz et al., 2003a).
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FIGURE LEGENDS

**Figure 1.** Pre-bound iron causes the time lag in Fe uptake. A. The time lag in Fe uptake in *D. salina* cells. Samples of 5x10^7 iron-deficient or iron-sufficient cells were incubated with ^59^Fe^{3+}-citrate at room temperature for the indicated time-points, and analyzed for internalized iron contents. Arrow indicates the time lag in iron-sufficient cells. B. Time lag in Fe-sufficient cells. Samples of 5x10^7 cells were either not treated (Con.), or pre-incubated for 1h in the light in Fe-deficient medium, for “unloading” occupied iron binding sites (PI), or for 20 min on ice with saturating Fe-citrate, to “upload” unoccupied iron binding sites followed by washing (Fe-Cit.). After these treatments, cells were incubated with ^59^Fe^{3+}-citrate at room temperature for the indicated times, and analyzed for internalized iron contents. C. No time lag in Fe-deficient cells. Treatments as in B. Averages of 3 (A) or 2 (B,C) separate experiments.

**Figure 2.** Fe binding in *D. salina* cells is inhibited by pre-bound Fe. Cultures of iron-sufficient (+Fe, open symbols) or iron-deficient (-Fe, solid symbols) cells were either not treated (circles) or pre-incubated (PI, squares) to unload bound Fe or preincubated with Fe-citrate (Fe-Cit., triangles) as in Fig. 1. Samples were then washed and assayed for Fe binding at 4°C with ^59^Fe^{3+}-citrate for the indicated times. A representative experiment of 4 repetitions.

**Figure 3.** Dissociation and exchange of bound iron. Iron-deficient *D. salina* cell cultures were incubated with ^59^Fe^{3+}-citrate as in Fig. 2. After 20 min at 4°C, they were supplemented either with 200 µM Fe^{3+}-Citrate (natural isotope mixture) or with 5 mM EDTA. Samples were withdrawn at the indicated times, washed twice in Fe binding “Stop solution” and assayed for bound ^59^Fe. A representative experiment of 3 repetitions.

**Figure 4.** Bound iron is committed for internalization. Iron-sufficient (+Fe) or iron deficient (-Fe) cell cultures were incubated for 30 min with ^59^Fe^{3+}-Citrate on ice. Cells were washed twice in EDTA-free stop solution and 200 µM Fe-Cit (natural isotope
mixture) was added. Then cells were transferred to room temperature and illuminated, samples were taken at the indicated time points. Total: cell-associated (EDTA-resistant plus EDTA-sensitive); Bound (EDTA-sensitive); Internalized (EDTA-resistant). A representative experiment of 5 repetitions.

**Figure 5.** Fe localization with acid ferrocyanide staining. A. Cells before internalization. Inset: magnified outer surface sections. Black arrowheads indicate iron particles. B. Cells after internalization. C-F: Magnified Fe-loaded vacuoles after internalization. Arrows: Fe-loaded vacuoles. Ruler: 1 micron in A, B; 0.5 micron in A-inset and C-F.

**Figure 6.** Localization of acidic vacuoles and endocytosis of biotin-tagged surface proteins in *D. salina* cells. A. Staining of acidic vacuoles with LysoSensor Green. Cells were preincubated for 10 min with the fluorescent probe. Acidic vacuoles are stained in green (arrow) and chlorophyll, which marks the cup-shaped chloroplast, in red. B. Immunolocalization of acidic vacuoles with antibodies against vacuolar H\(^+\)-PPase. Cells were fixed, permeabilized and incubated for 12h with antibodies against *C. reinhardtii* vacuolar H\(^+\)-PPase and next with fluorescein goat anti-rabbit antibodies. Fluorescein indicating vacuolar H\(^+\)-PPase is stained in green (arrow) and chlorophyll in red. C-E. Biotin tagging of surface proteins. Cells were labeled with biotin, washed, and either fixed immediately (C) or treated to bind and internalize iron in the absence (D) or presence (E) of 5 μM Bafilomycin. Fixed cells were permeabilized and incubated with fluorescein avidin. Fluorescein, representing surface labeled proteins is stained in green (arrow) and chlorophyll in red.
Table 1
Iron binding characteristics in Fe-deficient and in Fe-sufficient D. salina cells.

<table>
<thead>
<tr>
<th>Addition or omissions</th>
<th>Bound Fe n mole Fe/10⁹ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Fe</td>
</tr>
<tr>
<td>none</td>
<td>0.9 +/- 0.2 (100)</td>
</tr>
<tr>
<td>- bicarbonate</td>
<td>0.1 +/- 0.05 (10)</td>
</tr>
<tr>
<td>pH 6</td>
<td>0.6 +/- 0.1 (70)</td>
</tr>
<tr>
<td>pH 9</td>
<td>1.15 +/- 0.2 (127)</td>
</tr>
<tr>
<td>+ ZnCl₂</td>
<td>0.5 +/- 0.1 (66)</td>
</tr>
<tr>
<td>+ MnCl₂</td>
<td>0.8 +/- 0.2 (89)</td>
</tr>
<tr>
<td>+ CuCl₂</td>
<td>0.75 +/- 0.2 (83)</td>
</tr>
<tr>
<td>+ AlCl₃</td>
<td>0.76 +/- 0.15 (85)</td>
</tr>
<tr>
<td>+ FeCl₃</td>
<td>0.05 +/- 0.02 (6)</td>
</tr>
</tbody>
</table>

Numbers in brackets represent percent of control (no additions) activities.
Table 2

Summary of Kinetic parameters of Fe binding and Fe uptake

<table>
<thead>
<tr>
<th>Parameter/ cells</th>
<th>+Fe</th>
<th>-Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tf (n mol Fe/10⁹ cells)</td>
<td>1.1 +/- 0.3</td>
<td>4.3 +/- 0.5</td>
</tr>
<tr>
<td>K₅₀ (HCO₃⁻) (mM)</td>
<td>0.7 +/- 0.2</td>
<td>0.3 +/- 0.1</td>
</tr>
<tr>
<td>K₅₀ (Fe³⁺) (µM)</td>
<td>0.8 +/- 0.25</td>
<td>0.2 +/- 0.06</td>
</tr>
<tr>
<td>Vₐ₀ (n mol Fe/10⁹ cells•min)</td>
<td>0.043 +/- 0.013</td>
<td>0.330 +/- 0.080</td>
</tr>
<tr>
<td>t₁/₂ (min)</td>
<td>17 +/- 4</td>
<td>6 +/- 1</td>
</tr>
<tr>
<td>kₗ (ml/n mol Fe•min)</td>
<td>56x10⁻³ +/- 11x10⁻³</td>
<td>110x10⁻³ +/- 14x10⁻³</td>
</tr>
<tr>
<td>Vₚ (n mol Fe/10⁹ cells•min)</td>
<td>69 +/- 8</td>
<td>109 +/- 11</td>
</tr>
<tr>
<td>Tlag (min)</td>
<td>No treatment: 17.3 +/- 3</td>
<td>4 +/- 1</td>
</tr>
<tr>
<td></td>
<td>+ pre-incubation: 1-3</td>
<td>2-4</td>
</tr>
<tr>
<td></td>
<td>+ Fe-citrate: 17-21</td>
<td>16-19</td>
</tr>
</tbody>
</table>

Tf - Number of Fe binding sites
K₅₀ (Fe³⁺) – Fe-citrate concentration required for 50% of maximal binding
K₅₀ (HCO₃⁻) – Bicarbonate concentration required for 50% of maximal binding
Vₐ₀ – initial rate of binding
t₁/₂ - time for 50% Fe binding
Vₚ – steady-state rate of Fe uptake
Tlag – initial time lag in Fe uptake

kₗ – Fe binding rate constant defined as:

Vₗ = kₗ • Feₗ • Tfₗ • (1- [Fe-Tf] / [Tf]ₗ)

The data represent 3-5 repetitions.

Further details are given in the text and in the Supplementary Material section.
Table 3

Dissociation and exchange of bound Fe

Binding of $^{59}$Fe to Fe-sufficient or Fe-deficient cells was carried out for 30 min at 4°C followed by two washes in EDTA-free stop solution. Subsequently, cells were incubated for 1h at 4°C as indicated in stop solution at pH 8 (none), at pH 8 with addition of 200 µM Fe-citrate (natural isotope mixture), in stop solution at pH 5 (pH 5), at pH 8 + 5 mM EDTA (EDTA) or at pH 5 + 5 mM EDTA (pH 5 + EDTA). Cells were washed and tested for bound $^{59}$Fe.

<table>
<thead>
<tr>
<th>Additions to incubation</th>
<th>Bound Fe n mole Fe/10⁹ cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe-sufficient cells</td>
<td>Fe-deficient cells</td>
</tr>
<tr>
<td>none</td>
<td>4.4 +/- 0.4</td>
<td>1.05 +/- 0.12</td>
</tr>
<tr>
<td>Fe-citrate</td>
<td>4.2 +/- 0.6</td>
<td>0.98 +/- 0.25</td>
</tr>
<tr>
<td>pH 5</td>
<td>3.8 +/- 0.5</td>
<td>0.85 +/- 0.17</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.8 +/- 0.8</td>
<td>0.44 +/- 0.16</td>
</tr>
<tr>
<td>pH 5 + EDTA</td>
<td>0.2 +/- 0.1</td>
<td>0.1 +/- 0.05</td>
</tr>
</tbody>
</table>
Table 4

Pre-accumulation of iron inhibits Fe binding and uptake

Accumulation of iron was performed by incubation of control (+Fe) or Fe-deficient (-Fe) cells for 0-4h in the light with 10 µM Fe-Cit. (natural isotope mixture). Cells were then treated either by pre-incubation for 1h in Fe-deficient medium (Inc. -Fe) or washed twice in “Fe uptake stop solution”, containing 5 mM EDTA (+EDTA) and twice in EDTA-free solution. All cell samples were analyzed for Fe binding and Fe uptake activities.

<table>
<thead>
<tr>
<th>Fe accumulation time h/ unloading</th>
<th>Fe binding n mole Fe/10⁹ cells</th>
<th>Fe uptake n mole Fe/10⁹ cells x h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Fe cells</td>
<td>-Fe cells</td>
</tr>
<tr>
<td>0 / Inc. -Fe</td>
<td>1.1 +/- 0.2</td>
<td>3.1 +/- 0.5</td>
</tr>
<tr>
<td>1.5 / Inc. -Fe</td>
<td>0.7 +/- 0.3</td>
<td>1.3 +/- 0.6</td>
</tr>
<tr>
<td>4.0 / Inc. -Fe</td>
<td>0.2 +/- 0.15</td>
<td>0.3 +/- 0.1</td>
</tr>
<tr>
<td>4.0 / +EDTA</td>
<td>1.0 +/- 0.3</td>
<td>2.7 +/- 0.6</td>
</tr>
</tbody>
</table>
**Table 5**

*Fe uptake is sensitive to vacuolar inhibitors*

Control or Fe-deficient cells were incubated with bafilomycin (5 µM) or with NH₄Cl (30 mM) for 45 min in light at pH 8 at room temperature prior to the uptake assay. The values represent three (NH₄Cl) or four (bafilomycin) repetitions.

<table>
<thead>
<tr>
<th>Added inhibitors</th>
<th><strong>Fe uptake</strong></th>
<th><strong>Fe binding</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Fe cells</td>
<td>-Fe cells</td>
</tr>
<tr>
<td>none</td>
<td>3.4 +/- 0.3</td>
<td>5.2 +/- 0.6</td>
</tr>
<tr>
<td>Bafilomycin</td>
<td>0.3 +/- 0.1</td>
<td>2.9 +/- 0.7</td>
</tr>
<tr>
<td>NH₄Cl, pH 8</td>
<td>0.3 +/- 0.2</td>
<td>1.6 +/- 1.2</td>
</tr>
<tr>
<td>NH₄Cl, pH 7</td>
<td>2.1 +/- 0.3</td>
<td>4.3 +/- 1.1</td>
</tr>
</tbody>
</table>
We assume the following kinetic model:

\[
\text{Fe}^{3+} + \text{Tf} \xrightarrow{k_b} [\text{Fe-Tf}] \xrightarrow{k_{\text{int}}} [\text{Fe-V}]
\]

Where \text{Fe}^{3+} represents free ferric ions, \text{Tf}- iron binding proteins/sites, \text{[Fe-Tf]} - bound iron and \text{[Fe-V]}- internalized iron. \(k_b\) and \(k_{\text{int}}\) represent the rate constants for iron binding and internalization, respectively, and \(k_b \gg k_{\text{int}}\).

The rate of iron binding to \text{Tf} can be expressed as:

\[
V_b = \frac{\partial [\text{Fe-Tf}]}{\partial t} = k_b \cdot (\text{Fe}_t - \text{Fe-Tf}) \cdot (\text{Tf}_t - \text{Fe-Tf})
\]

where \(\text{Fe}_t, \text{Tf}_t\) and \text{Fe-Tf} represent the concentrations of total \text{Fe}^{3+}, total iron binding sites and bound \text{Fe}^{3+}, respectively.

Assuming that \(\text{Fe}_t \gg \text{Fe-Tf}\) at any time (t):

\[
V_{b(t)} = k_b \cdot \text{Fe}_t \cdot \text{Tf}_t \cdot (1 - \text{Fe-Tf}(t)/\text{Tf}_t)
\]

Where \(\text{Fe-Tf}(t)/\text{Tf}_t\) represents the fraction of occupied Fe binding sites at time t.

The initial rate of iron binding is:

\[
V_{b(0)} = k_b \cdot \text{Fe}_t \cdot \text{Tf}_t
\]

And the time required to reach 50% binding \(t_{b1/2}\) is:

\[
V_{b1/2} = k_b \cdot \text{Fe}_t \cdot 0.5
\]
Figure 1
Figure 2

The figure shows the binding of Fe (iron) in relation to incubation time. The x-axis represents incubation time in minutes, ranging from 0 to 120. The y-axis represents Fe bound (n mol Fe/10^9 cells), ranging from 0 to 5. Different conditions are indicated by different symbols:

- Black squares: -Fe + PI
- Black circles: -Fe
- White squares: +Fe + PI
- White circles: -Fe + Fe-Cit
- White triangles: +Fe + Fe-Cit

The lines show the trend of Fe binding over time for each condition.
Figure 3
Figure 4

A. +Fe

B. -Fe

Bound or internalized Fe (cpm X 10^-3)

Time (min)