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Evaluation of Reductive Release as a Mechanism for Iron Uptake from Ferrioxamine B by *Chlorella vulgaris*

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ABSTRACT

The involvement of ferric reduction in the iron uptake mechanism of iron-stressed *Chlorella vulgaris* from ferrioxamine B was investigated. Some comparative data for ferric-citrate was also obtained. EPR and a spectrophotometric assay were used to measure Fe^{3+} reduction. These two methods differed in the absolute quantity but not in effectors of ferric reduction. The mechanism governing ferric reduction was investigated by use of respiratory inhibitors, uncouplers, alternative electron acceptors, and ATPase inhibitors. Reduction appears to play a role in iron uptake from both Fe^{3+}-deferrioxamine B and Fe^{3+}-citrate; however, the involvement of photoreduction in Fe^{3+}-citrate uptake implies multiple reductive mechanisms could be involved.

Reduction of ferric ion bound by HXAs[3] has been proposed as a mechanism by which organisms may utilize these extremely tight ferric complexes (13). The affinity of HXAs for the ferrous ion ($K_f = 10^8$) *versus* the affinity for the ferric ion ($K_f = 10^{28}$) (5), the decrease in the rigidity of the chelate on reduction (24), and the estimation that reduction of Fe^{3+}-HXAs may be possible within physiological limits (9) implies reduction may be involved in the mechanism for iron uptake. The Fe^{3+}-HXA could be taken up and reduced, then the Fe^{2+} utilized and free ligand released. This has been demonstrated for fungi (25) and enteric bacteria (17). Use of extracellular, ferrous chelators to follow ferric reduction has been applied to plant systems (7, 19). Alternative electron acceptors that permeate or do not permeate the plasma membrane have been used to determine where reduction occurs and define the manner of production of the reducing equivalents (21-23). Electron transport systems at the plasma membrane exist in a wide range of organisms (11, 18). A trans-plasma membrane electron transport system has been proposed as the mechanism for reduction of iron by iron-deficient plants (7, 22). The plasma membrane electron transporting system of oat seedlings has been shown to be composed of multiple systems (20). Peanut roots were unable to reduce iron from Fe^{3+}-DFOB (19).

We investigated the involvement of ferric reduction in utilization of iron by *Chlorella vulgaris* from ferrioxamine B (Fe^{3+}-DFOB) with some comparison to ferric-rhodotorulate (Fe^{3+}-RA) and Fe^{3+}-citrate. Reduction was determined by both EPR and spectrophotometric determination of Fe^{2+}-$BPDS_2$. The effects of metabolic inhibitors, ferrous chelators, and alternative electron acceptors were used to determine if reduction and iron uptake were correlated and to localize the site of ferric reduction. A preliminary report of some of these data has been presented elsewhere (1).

MATERIALS AND METHODS

Deferrioxamine B (DFOB) was a gift from Ciba Geigy Pharmaceuticals Co. Rhodotorulic acid (RA) was isolated from iron-stressed *Rhodotorula pilimanae* Hendrich *et* Burke (American Type Culture Collection strain 26423) by the method described by Atkin and Neilands (4). $^{59}FeCl_3$ and $^{55}FeCl_3$ were obtained from ICN Radiochemicals Division. Carbonyl cyanide *m*-chlorophenylhydrazone was obtained as a gift from Dr. Peter G. Heytler. All other chemicals were of reagent grade or better.

Iron was removed from glassware by an acid wash as described previously (2). Atomic absorption spectroscopy was used to determine the concentration of iron in stock solutions and medium as described previously (2). *Chlorella vulgaris* Beijerinck (ATCC strain 11468) was grown as previously described (2). Proteins were determined by the method of Lowry after a predigestion in 0.1 N NaOH at greater than 80°C for 30 min.

Iron uptake was assayed by use of the radioisotopic tracers ^{59}Fe or ^{55}Fe as previously described (2). EPR was used to follow reduction of ferric iron as described by Ecker et al. (12) with the modifications described previously (3). Peak to trough amplitude, which was shown to be representative of the concentration of Fe^{3+}-HXAs present when cell suspensions of constant cell density were maintained (3), was used to quantitate the ferric reduction.

The colorimetric determination of ferric reduction was modeled after that used previously (6, 8). Two types of experiments were performed with the ferrous specific chelator BPDS. In BPDS method I a 4 ml cuvette was used as the reaction vessel. Cells were harvested by centrifugation at 2700*g* for 15 min at 15 to 20°C, resuspended in GMD and placed in the same reaction vessel used for EPR and iron uptake assays. The cells were then kept at 23°C, under high light with water saturated air bubbled into the medium while they were stirred until used. Cells were removed to a 4 ml cuvette, with the cell suspension as 50% of the final reaction volume. Other additions were added to give a final concentration of 0.05% BSA and 2 mM Pipes, final pH 7.2. Any treatments were added at this time. The cells were mixed and allowed 2 min to equilibrate in the cuvette. The light from a dual wavelength scanning spectrophotometer at 535 and 610 nm was its only light source. BPDS was added to 400 μM then

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[3] Abbreviations: HXA, hydroxamic acid siderophore; BPDS, bathophenanthroline disulfonate; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DFOB, deferrioxamine B; EDDHA, ethylenediamine-di(*o*-hydroxyphenylacetic acid); EPR, electron paramagnetic resonance spectroscopy; GMD, modified Guillard's medium; N-BT, nitro-blue tetrazolium chloride; RA, rhodotorulic acid.

an additional 3 min equilibration allowed. At this point the iron-ligand was added to begin the experiment. At the end of 30 min the cuvette was less than 2°C above ambient temperature. Oxygen was measured by insertion of an oxygen electrode in the top of the cuvette and kept constant by aeration.

BPDS Method II was usually combined with iron uptake assays in the reaction vessel as described for the EPR assays. Cells were harvested immediately prior to use as described for method I. Cells and additions were added as described for method I except the final volume was increased to 10 to 15 ml. At timed intervals 3 ml were removed to a 4 ml cuvette and the A determined at 535 nm, with 610 nm as the reference beam. Temperature was controlled at usually 23 or 4°C. Quantification of iron reduced by these methods was made by addition of BPDS and sodium dithionite into the reaction mixture described above then addition of aliquots of $FeSO_4$.

RESULTS

Colorimetric determination of ferric reduction with BPDS was more rapid and sensitive than EPR determination, but relied on addition of a ferrous chelator to the reaction medium. Two variations of this method were used. BPDS method I was performed in a 4 ml cuvette in a dual wavelength spectrophotometer, while BPDS method II used the reaction vessel for the iron uptake assays with $A_{(535-610\ nm)}$ measured at timed intervals by removal of 3 ml aliquots to a cuvette. With BPDS method II, rapid photoreduction occurred when iron was provided as Fe^{3+}-citrate, while a small increase was observed when Fe^{3+}-EDTA was supplied (Fig. 1). No photoreduction was observed for the Fe^{3+}-HXAs (Fig. 1). When BPDS method I was used no photoreduction occurred within the 30 min reaction period, due in part to the decreased light levels provided by the spectrophotometer.

Iron uptake and ferric reduction (EPR method) of Fe^{3+}-RA and Fe^{3+}-DFOB correlated closely (Fig. 2) as was previously reported (1). Ferric reduction, as determined by BPDS method I, was rapid for the Fe^{3+}-HXAs, while reduction was much slower for Fe^{3+}-citrate (Fig. 3).

Correlation between iron uptake and ferric reduction was nearly perfect with the EPR method (Figs. 2 and 4), while the BPDS II method overestimated the reduction necessary to account for iron uptake (Fig. 4). When BPDS was added to the reaction vessel after a preincubation with Fe^{3+}-DFOB of 5 min no external pool of free ferrous ion was present as determined with BPDS method II.

When cells were grown with sufficient iron, no ferric reduction was observed with either the EPR or BPDS methods (Fig. 5). Both the EPR reduction and the excess reductive capacity seen with the BPDS method seem to be induced by iron stress.

Inhibition by uncoupler was observed for ferric reduction by use of either EPR or BPDS methods. Iron uptake and ferric reduction (EPR method) demonstrated a similar response to inhibition by 100 μM CCCP (Fig. 6). CCCP (100 μM) inhibited the initial rate of ferric reduction (BPDS method I) from Fe^{3+}-DFOB, Fe^{3+}-RA, and Fe^{3+}-citrate.

Antimycin A inhibited the initial ferric reduction rate (BPDS

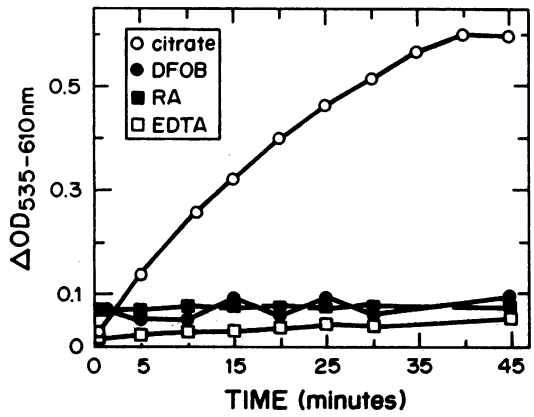

FIG. 1. BPDS method II reduction assay performed without cells to determine the contribution of autoreduction. Assayed at 24°C in GMD modified to contain 2 mM Pipes, 0.05% BSA, 100 μM Fe, 400 μM BPDS, and chelator (150 μM RA or DFOB, 300 μM citrate or EDTA).

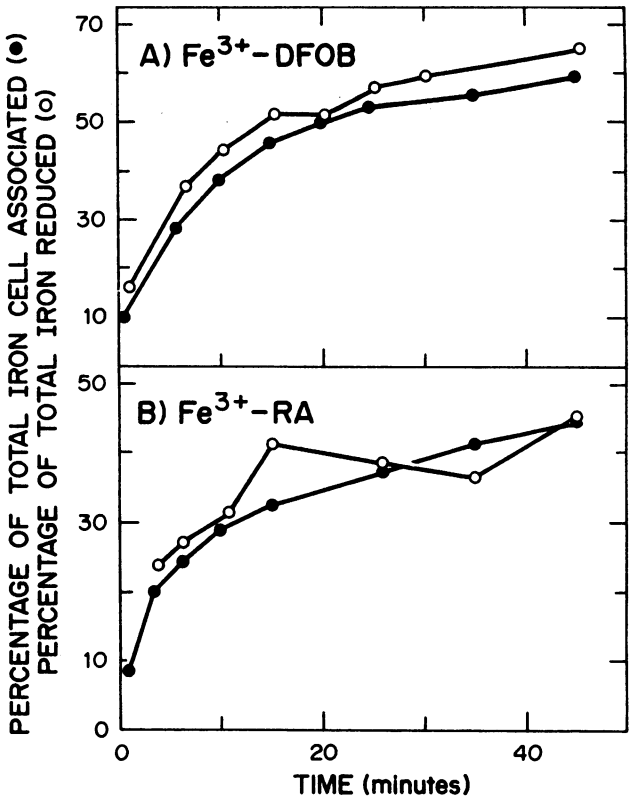

FIG. 2. Iron uptake compared to ferric reduction as measured by EPR by iron-stressed *C. vulgaris* of the iron chelated by (A) 25 μM Fe: 37.5 μM DFOB and (B) 25 μM Fe: 75 μM RA. Experiments were carried out at 24°C in GMD.

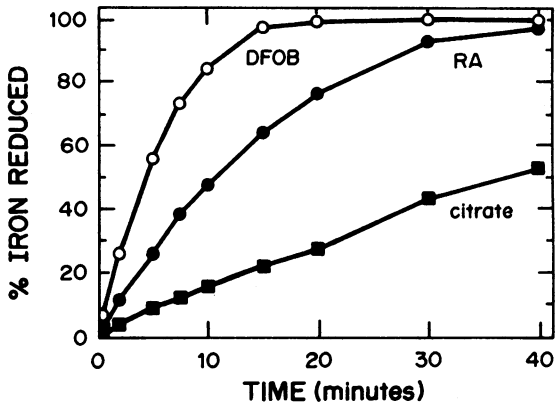

FIG. 3. Ferric reduction of 100 μM Fe: 150 μM DFOB, 100 μM Fe: 300 μM RA, and 100 μM Fe: 300 μM citrate by *C. vulgaris* as measured by BPDS method I. Reduction measured at ambient temperature as described in "Materials and Methods" but the reaction mixture was GMD modified to contain 2 mM Pipes (pH 7.0), 0.05% BSA, and 400 μM BPDS.

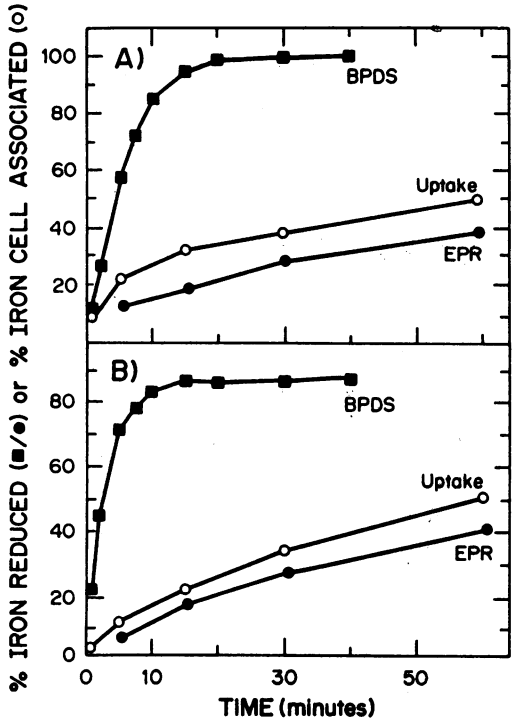

FIG. 4. Comparison of the BPDS method II and EPR methods for determination of ferric reduction by iron-stressed *C. vulgaris* from (A) 25 μM Fe: 37.5 μM DFOB and (B) 25 μM Fe: 75 μM RA. Assays were performed at 24°C as described in "Materials and Methods."

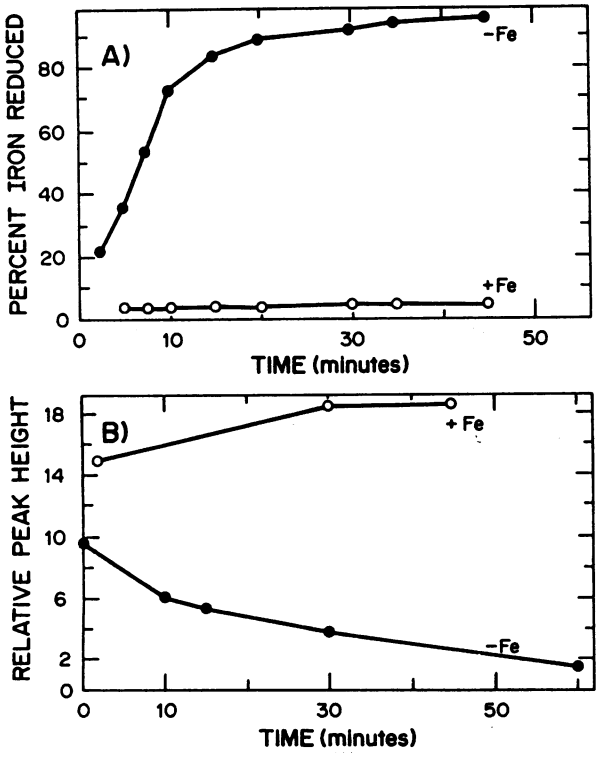

FIG. 5. Effect of iron-stress on ferric reduction by *C. vulgaris* as determined by (A) BPDS method I and (B) the EPR method. BPDS reduction assay was done with 100 μM Fe: 150 μM DFOB, while the EPR method was done with 10 μM Fe: 15 μM DFOB. The assay was carried out at 24°C as described in "Materials and Methods."

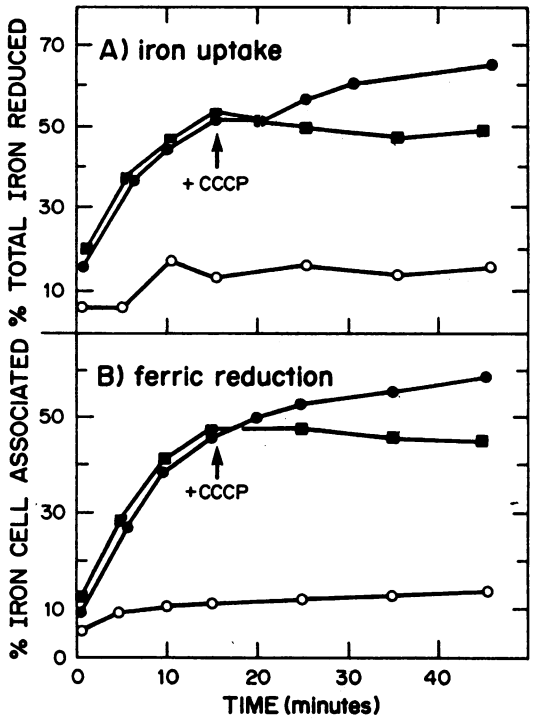

FIG. 6. Inhibition of (a) iron uptake and (B) ferric reduction by *C. vulgaris* as measured by the EPR method from 25 μM Fe: 37.5 μM DFOB. The assay was performed at 24°C as described in "Materials and Methods." (●), No CCCP added; (○), 100 μM CCCP added 5 min prior to addition of ligand; (■), 100 μM CCCP added at 16 min.

Table I. *Effect of Antimycin A on Ferric Reduction by Iron-Stressed C. vulgaris as Determined by BPDS Method I*

Chelator	Ferric Reduction[a]		
	Control	+ 25 μM antimycin A	Inhibition
		nmol Fe^{2+} mg^{-1} protein min^{-1}	%
RA	4.1 (0.6)	0.9 (0.1)	79
	13.1 (2.9)	1.9 (0.2)	86
		2.7* (0.5)	80
DFOB	6.0 (0.6)	1.4 (0.4)	77
	18.8 (3.7)	2.7 (0.4)	86
		5.4* (0.3)	72
Citrate	2.2 (0.3)	0.9* (0.0)	55
	2.1 (0.3)	1.5 (0.6)	26
		1.3* (0.2)	36

[a] For all runs except those with * antimycin A was added at 5 min after addition of the iron-ligand. Those with * were preincubated for 5 min with antimycin A prior to addition of the Fe^{3+}-ligand. Values in parentheses are the sample standard deviations of at least three determinations. The chelates had 50 μM Fe with either 75 μM DFOB or RA or 150 μM citrate.

method I) by greater than 70% for the Fe^{3+}-siderophores while about 50% inhibition occurred with Fe^{3+}-citrate (Table I). The addition of antimycin A to the BPDS reduction assay for Fe^{3+}-citrate gave an initial stimulation of reduction followed by a somewhat variable rate which was nearly identical to the initial rate. For this reason inhibition by 100 μM cyanide was examined for Fe^{3+}-citrate. Cyanide was found to inhibit the rapid initial rate of ferric reduction from 50 μM Fe: 150 μM citrate by 49.0%

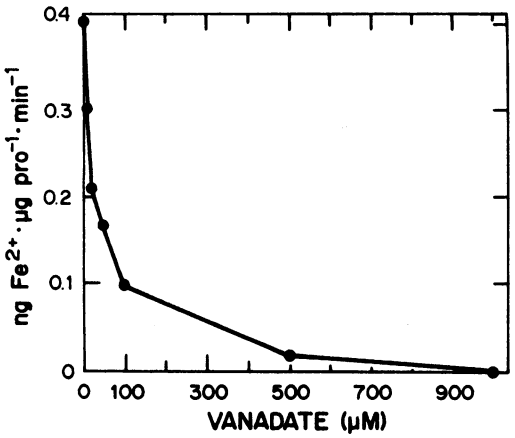

FIG. 7. Vanadate inhibition of ferric reduction, as measured by BPDS method I, by iron-stressed *C. vulgaris* from 100 µM Fe: 150 µM DFOB. Conditions as for Figure 3.

Table II. *Inhibition of Initial Rate of Ferric Reduction by Iron-Stressed C. vulgaris from Ferric Chelates by Vanadate at Various Concentrations*

Ferric reduction was determined by BPDS method I with 50 µM Fe supplied with either 75 µM DFOB or RA or 150 µM citrate. The maximum sample standard deviations for the DFOB, RA, and citrate treatment series were 0.7, 0.6, and 0.3, respectively.

Chelator	Reduction Rate with Vanadate Concentration:				
	Control	10 µM	50 µM	100 µM	500 µM
	nmol Fe^{2+} mg^{-1} protein min^{-1}				
Fe^{3+}-DFOB	7.2	5.4	3.0	1.7	0.3
Fe^{3+}-RA	9.9	6.2	4.2	3.5	1.7
Fe^{3+}-citrate	3.0	2.8	1.7	1.1	1.4

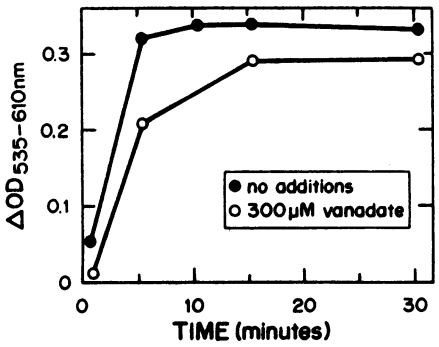

FIG. 8. Vanadate inhibition of ferric reduction, as measured by BPDS method II, by iron-stressed *C. vulgaris* from 100 µM Fe: 150 µM DFOB. Measurements made at 24°C with conditions as in Figure 3.

as determined by BPDS method I.

Because ATPases and proton symport have been implicated in models for iron uptake (26), the effect of vanadate was examined. Inhibition of the initial rapid rate of ferric reduction (BPDS method I) by vanadate was observed and had a K_i of about 25 µM for Fe^{3+}-DFOB (Fig. 7). Fe^{3+}-citrate and Fe^{3+}-RA responded similarly to vanadate (Table II). When longer term assays were carried out it was found that, while vanadate inhibited the initial rapid rate by 50% at 25 µM (Fig. 7), most or all of the iron was still reduced within 15 min (Fig. 8).

Since iron uptake and ferric reduction may be linked, we proposed that the external, Fe^{2+}-specific chelator BPDS might affect iron uptake. The presence of BPDS allowed either some initial iron uptake or adhesion to the cell but inhibited iron uptake entirely after about 8 min (Fig. 9).

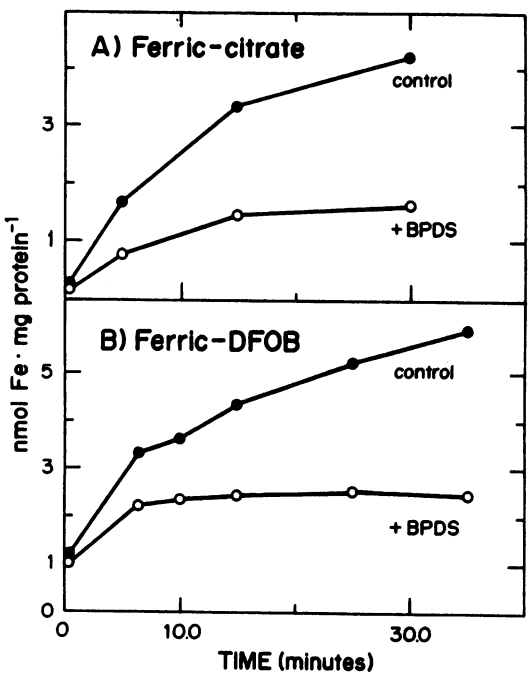

FIG. 9. BPDS inhibition of iron uptake by iron-stressed *C. vulgaris* from (A) 50 µM Fe: 150 µM citrate and (B) 50 µM Fe: 75 µM DFOB. Uptake was determined at 24 and 4°C with the values expressed as the difference of the average values at these two temperatures. Conditions as in Figure 6 except 400 µM BPDS was added 2 min prior to addition of Fe^{3+}-DFOB. The control had no added BPDS.

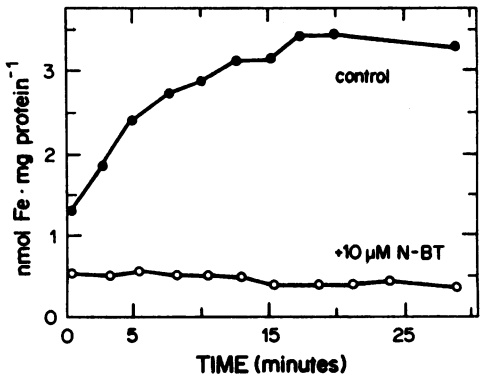

FIG. 10. Nitro-blue tetrazolium chloride (10 µM) inhibition of iron uptake by iron-stressed *C. vulgaris* from 50 µM Fe: 75 µM DFOB. Uptake was measured at 24°C with conditions as in Figure 6 except that 10 µM N-BT was added 5 min prior to addition of Fe^{3+}-DFOB.

BPDS method I was used to determine the effect of alternative electron acceptors on ferric reduction. N-BT, a membrane permeable electron acceptor (21), inhibited ferric reduction by the BPDS method (Fig. 10) and was also capable of inhibition of iron uptake (2). Reducing equivalents are then required for both iron uptake and ferric reduction. Ferricyanide is membrane impermeable (10, 16) and was used here as an exogenous electron acceptor for the ferric reduction system. Reduction of iron from the HXA siderophores was inhibited by ferricyanide, while Fe^{3+}-citrate reduction was stimulated (Table III).

DISCUSSION

Previous reports have indicated that photoreduction may play a role in plant utilization of iron (14) and other metals (15).

Table III. *Effect of Ferricyanide on Ferric Reduction by C. vulgaris as Measured by BPDS Method I*

Chelate	Ferricyanide Concentration	Reduction rate[a] average	s	Inhibited (stimulated)
	μM			%
Iron-stressed cells				
100 μM Fe^{3+}: 150 μM DFOB	0	7.0	0.7	0
	5	2.9	0.6	59
	10	1.7	0.0	76
	50	1.3	0.1	81
	100	0.6	0.0	91
	0	13.1	0.7	0
	50	2.5	0.4	81
100 μM Fe^{3+}: 150 μM RA	0	8.9	0.7	0
	5	5.0	0.3	44
	50	1.5	0.1	84
100 μM Fe^{3+}: 300 μM citrate	0	0.9	0.7	0
	5	1.2	0.0	(42)
	50	2.8	0.2	(215)
	0	1.2	0.1	0
	50	4.1	0.1	(257)
Iron-normal cells				
100 μM Fe^{3+}: 150 μM DFOB	0	0		
100 μM Fe^{3+}: 150 μM RA	0	0		

[a] The reduction data are reported as the mean of runs performed on a single day as nmol Fe min^{-1} mg^{-1} protein and s, the sample standard deviation, from four determinations is provided in the column to the right.

Determination of ferric reduction must account for interference from photoreduction of ferric iron when applied to some chelates (Fig. 1). The HXAs were not photoreduced during the time frame of these experiments, while Fe^{3+}-citrate was rapidly photoreduced.

The advantage of the BPDS method II was that the cells were exposed to light during the assay, which allowed a more natural environment for measurement of iron uptake and reduction. However, from these data (Fig. 1) caution is necessary when BPDS method II is applied in order to prevent a significant contribution from photoreduction (*i.e.* this method was not used to measure reduction of Fe^{3+}-citrate).

Comparison of data from the EPR and BPDS method I for measurement of ferric reduction indicate a discrepancy. Ferric reduction from the HXAs correlated closely with iron uptake when measured by EPR (Figs. 2 and 4), while the BPDS II method overestimated the reduction necessary to account for iron uptake (Fig. 4). All of the iron in the medium was reduced in less than 15 min *versus* the 25 to 50% ferric reduction observed in the EPR experiments in the same time period (Fig. 4). If ferrous ion was rapidly reoxidized without BPDS in the medium to stabilize it or if there was feedback regulation of the iron uptake mechanism, the large differences seen between these two methods could be explained. This could indicate that a reductive mechanism was involved to produce excess Fe^{2+}, which was then chelated (and thereby sequestered from the cell) when BPDS was present in the reaction medium. The excess Fe^{2+} may normally be reoxidized or not produced, but the presence of BPDS prevented this. No external pool of free Fe^{2+} accumulated after a 15 min incubation, with Fe^{3+}-DFOB, Fe^{3+}-RA, or Fe^{3+}-citrate already present, without BPDS added to the reaction medium. The EPR method may measure the physiological ferric reduction linked to iron uptake, while the BPDS method may reflect the total reductive capacity.

Unfortunately, the EPR data does not allow localization of the site of ferric reduction. It is unable to distinguish if the iron was taken up as a chelate then reduced in the cytoplasm or reduced then absorbed as the free ion by the cell, presumably to allow reuse of the chelator. An external ferrous chelator, BPDS, wasused for spectrophotometric determination of external ferrous iron concentration in order to localize the site of ferric reduction. BPDS method I determination of reduction showed that both HXAs were reduced much faster than the Fe^{3+}-citrate (Fig. 3). Since BPDS cannot penetrate the cell membrane (8), the ability of this ferrous chelate to scavenge Fe^{2+} indicated that reduction occurred on an exposed site, not in the cytoplasm. Previous researchers attributed the reduction of extracellular ferric salts to a trans-plasma membrane reductase in soybeans (22). The greater rate of reduction of Fe^{3+}-HXAs compared to Fe^{3+}-citrate indicated that a different mechanism may be employed for Fe^{3+}-citrate utilization or sufficient iron was released from Fe^{3+}-citrate *via* photoreduction or exchange such that no special uptake mechanism has been developed by the cell for this chelate.

Iron-stress induced cells initiate ferric reduction from the HXA siderophores regardless of the method of measurement used (Fig. 5). Previous data indicate that iron uptake from these chelates was also induced by iron-stress (2). This evidence supports a linkage between iron uptake and ferric reduction.

Ferric reduction from Fe^{3+}-DFOB was inhibited totally by 100 μM CCCP, an indication that a requirement for an electrochemical gradient existed (Fig. 6).

Low concentrations of vanadate inhibited the initial rapid reduction measured by use of BPDS method I while higher concentrations of vanadate drastically inhibited reduction (Table II; Fig. 7). The inability of 300 μM vanadate to inhibit long-term ferric reduction could be the result of multiple reduction mechanisms (Fig. 8). On the other hand the 50% inhibition by 25 μM vanadate of the rapid reduction rate shown with BPDS method I (Fig. 7) may indicate that while the rate was slowed, sufficient reductive capacity remained for removal of iron from these chelates and their eventual uptake. The incomplete inhibition of reduction was in contrast to the strong inhibition of iron uptake previously described (2).

Comparison of the reduction data (Fig. 4) to the uptake inhibition by BPDS (Fig. 9) indicated that the inhibition of iron uptake could be due to removal of ferrous ion (as Fe^{2+}-$BPDS_2$) rather than prevention of iron transport. The site of reduction would then, in either case, be exposed to the external solution. The initial uptake implies that ferric reduction must occur near to or on the cell surface otherwise the large excess of BPDS would prevent uptake. Since the initial iron content of cells at 4°C with BPDS present is lower than 4°C controls without BPDS, BPDS may prevent some nonspecific adsorption.

Respiratory inhibitors slow ferric reduction as measured by BPDS from iron chelates with citrate, DFOB, and RA. The linkage between respiration and iron uptake is unclear (2). With the inability to definitely show inhibition of iron uptake by respiratory inhibitors (2) while reduction was inhibited (Table I) raises some questions.

Alternative electron acceptors which can penetrate the plasma membrane (*e.g.* N-BT) and those which remain outside the membrane (*e.g.* ferricyanide) inhibit iron uptake by these cells from Fe^{3+}-DFOB. Reduction of the Fe^{3+}-HXAs can be prevented by an external electron acceptor while Fe^{3+}-citrate reduction was stimulated. It is unclear what occurred unless the reduction can be attributed to the reduction of Fe^{3+}-citrate by ferrocyanide (10). It appears that there is a mechanism for the reduction of the HXA siderophores which can distinguish between HXA chelates and other ferric chelates. Reduction of Fe^{3+}-HXAs may be a part of the mechanism for this tightly bound iron utilization by *C. vulgaris*. Iron was reduced at the cell surface, in an exposed site, then rapidly transported into the cell.

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