A Multicopper Ferroxidase Involved in Iron Binding to Transferrins in Dunaliella salina Plasma Membranes*

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The halotolerant alga Dunaliella salina is unique among plants in that it utilizes a transferrin (TTf) to mediate iron acquisition (Fisher, M., Zamir, A., and Pick, U. (1998) J. Biol. Chem. 273, 17553–17558). Two new proteins that are induced by iron deprivation were identified in plasma membranes of D. salina as follows: a multicopper ferroxidase termed D-Fox and an internally duplicated glycoprotein (p130B). D-Fox and p130B are accessible to glycolytic, proteolytic, and biotin surface tagging treatments, suggesting that they are surface-exposed glycoproteins. Induction of D-Fox was also manifested by ferroxidase activity in plasma membrane preparations. These results are puzzling because ferroxidases in yeast and in Chlamydomonas reinhardtii function in redox-mediated iron uptake, a mechanism that is not known to operate in D. salina. Two lines of evidence suggest that D-Fox and p130B interact with D. salina triplicated transferrin (TTf). First, chemical cross-linking combined with mass spectroscopy analysis showed that D-Fox and p130B associate with TTf and with another plasma membrane transferrin. Second, detergent-solubilized D-Fox and p130B comigrated on blue native gels with plasma membrane transferrins. $^{59}\text{Fe}$ autoradiography indicated that this complex binds Fe$^{3+}$ ions. Also, the induction of D-Fox and p130B is kinetically correlated with enhanced iron binding and uptake activities. These results suggest that D-Fox and p130B associate with plasma membrane transferrins forming a complex that enhances iron binding and iron uptake. We propose that the function of D-Fox in D. salina has been modified during evolution from redox-mediated to transferrin-mediated iron uptake, following a gene transfer event of transferrins from an ancestral animal cell.

Iron is an essential element for all photosynthetic organisms because it is a cofactor of multiple components in photosynthetic electron transport. Iron deficiency is a common limitation for plant and algae proliferation because of its low availability in aerobic aqueous solutions. On the other hand, an excess of iron is highly toxic because it can elicit the formation of reactive oxygen species causing oxidative damage. Therefore, all photosynthetic organisms had to develop efficient regulatory mechanisms to control the uptake and storage of iron in order to maintain optimal iron metabolism.

Two major strategies of high affinity iron uptake have been demonstrated in plants and in algae as follows: siderophore-mediated Fe$^{3+}$ uptake and a redox-mediated mechanism. The siderophore-mediated Fe$^{3+}$ uptake was first described in bacteria and is characteristic to several algae (1, 2) and to grasses (strategy II plants). Redox-mediated iron acquisition, initiated by reduction of ferric to ferrous ions by a plasma membrane ferrireductase, is characteristic to strategy I plants, yeast, and algae (3–6). The mechanism of redox-mediated iron uptake in plants differs from that in yeast and in the alga Chlamydomonas reinhardtii in that plants transport the reduced ferrous ions directly via a divalent metal transporter, whereas in yeast and in C. reinhardtii ferrous ions are reoxidized to ferric ions. Reoxidation is mediated by a membranal multicopper ferroxidase, FeT3 or Fox1, belonging to the multicopper oxidase (MCO) superfamily (7, 8). Ferric ions are next transported through a coupled ferric-specific transporter. The MCO Fox1 and the iron permease Ftr1 are believed to form a complex at the plasma membrane as demonstrated in yeast (9, 10). Both siderophore and redox-mediated iron uptake are up-regulated under iron deprivation, thus compensating for the decreased iron availability by a more efficient high affinity iron uptake.

The halotolerant alga Dunaliella salina is well adapted to iron deprivation, as manifested by its proliferation and by maintenance of photosynthetic activity under iron deprivation (11). We found that D. salina has an unusual mechanism of iron uptake, mediated by binding and internalization of Fe$^{3+}$ to a surface transferrin-like protein, TTf (12, 13). TTf serves as a housekeeping mechanism for iron uptake, but its expression level and activity are enhanced under iron deprivation or at high salinity, which limits iron availability. The origin of transferrins in Dunaliella is not clear, because no other transferrins have been described so far in other plants or in related organisms. More recently we identified another transferrin, DTf, that is induced under iron deprivation in D. salina plasma membrane. The function of DTf has not been elucidated yet (14).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY987035 and AF450137.

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2 The abbreviations used are: MCO, multicopper oxidase; D-Fox, D. salina ferroxidase; Fox1, C. reinhardtii ferroxidase; TTf, D. salina triplicated transferrin; DTf, D. salina transferrin; PNGase, peptide N-glycosidase; BN, blue native gels; MES, 4-morpholineethanesulfonic acid; RACE, rapid amplification of cDNA ends; IEF, isoelectric focusing; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; DTT, dithiothreitol; TMM, tetraethylmolybdate; DST, disuccinimidyl tartrate; EGS, succinimidylsuccinate; Bis-tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxyethyl)propane-1,3-diol; UTR, untranslated region; MS, mass spectrometry.
Because TTF is an iron-binding protein that is loosely associated with the outer plasma membrane surface (15), it may be expected that iron uptake in *D. salina* requires additional plasma membrane proteins. In an attempt to identify such proteins, we compared the protein composition of plasma membranes derived from iron-deficient and from iron-sufficient *D. salina* cells. The comparison revealed two major induced proteins of about 130 kDa in the plasma membrane of iron-deficient cells. Based on their cDNA-deduced sequence, one of the proteins was identified as a member of the MCO family, and hence termed *Dunaliella* ferroxidase (D-Fox), whereas the other protein (p130B) showed no sequence homology to any known protein. Unexpectedly, we found that D-Fox is not involved in redox-mediated iron uptake, like MCO in yeast or in *C. reinhardtii*, but it is associated with transferrins in *D. salina* plasma membranes and functions in enhancing iron binding. Thus, enhancement of iron binding appears to be a critical parameter for iron acquisition in *D. salina*.

**EXPERIMENTAL PROCEDURES**

**Algal Strain and Growth Conditions**—*D. salina* was obtained from Dr. W. H. Thomas, La Jolla, CA. All culture glassware was washed in acid and thoroughly rinsed with Milli-Q water. Cells were cultured in iron-sufficient or in iron-deficient media as described previously (12, 13). Standard growth medium contained 1 M NaCl, with or without 1.5 mM EDTA, unless otherwise indicated.

**Plasma Membrane Preparation**—Plasma membranes were prepared by differential centrifugation as described earlier (14). In brief, cells were washed in glycerol medium and osmotically lysed; chloroplasts were then removed by sedimentation at 12,000 × *g* for 10 min, and plasma membranes were sedimented at 12,000 × *g* for 60 min, washed, and stored in liquid nitrogen.

**Cloning of D-Fox and p130B**—Total RNA was prepared from 5 × 10⁷ cells harvested 20 h after transfer to iron-deficient medium with the RNeasy kit (Qiagen GmbH, Hilden, Germany). Synthesis of cDNA using 1 μg of RNA was carried out with the superscript reverse transcriptase (Clontech). The cDNA was used as template in PCR performed with degenerate primers designed from peptide sequences obtained from MS analysis (D-Fox 5′-sense, GTNCCNGARRAYGCGNC; D-Fox 5′-antisense, GTNGCNGTRTTNCCNCC; p130B 5′-sense, AYACNGAYGARCARTTYTG; and p130B 5′-antisense, TCTYGCTATRTTNCTGNT-CCCC). The 600-bp PCR product was subcloned into pTZ57R vector using the InstAclone™ cloning kit (Fermentas UAB, Vilnius, Lithuania). Specific primers (D-Fox 5′-sense, CTGGATCTCATGATCCCCC; D-Fox 5′-antisense, GTAGGGTTTGTATCGCTGC; p130B 5′-sense, CTCAGCTACTAACAAATGTTGGG; and p130B 5′-antisense, CAAGCTTGGCAGTGTCTGCC) were used in 3′- and 5′-RACE reactions to clone the full-length cDNA using the SMART™ RACE cDNA amplification kit (Clontech).

**Polyclonal Antibodies**—Polyclonal antibodies were prepared in rabbits against synthetic peptides derived from the amino acid sequences of D-Fox and p130B (C-TTFEEVERQPEDAY and PADGNKEELRNdL-C, respectively). The peptides, containing an extra terminal cysteine, were coupled to a carrier protein, maleimide-activated keyhole limpet hemocyanin, according to the manufacturer’s instructions (Pierce), and were injected into rabbits. On Western blot analysis with protein extracts from intact cells or plasma membrane, both antibodies reacted with a single band of about 130 kDa.

**Trypsin Digestion**—Iron-deficient cells (2 × 10⁷ cells/ml) were suspended in iso-osmotic suspension buffer (100 mM NaCl, 1.4 M glycerol, 10 mM Tris-HEPES, pH 7.2, 10 mM KCl, 2 mM MgCl₂) and were then incubated with 3 mg/ml trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) for 30 min at room temperature. The digestion was terminated by addition of 12 mg/ml trypsin inhibitor. An aliquot of 2 × 10⁶ cells was analyzed on SDS-PAGE.

**Deglycosylation with PNGase F**—Plasma membrane preparations from iron-deficient *D. salina* cells were denatured in glycoprotein denaturing buffer (0.5% SDS, 1% B-mercaptoethanol) at 100 °C. Next, the deglycosylation reaction was carried out in 50 mM sodium phosphate buffer, pH 7.5, supplemented with 1% Nonidet P-40. After addition of 1 unit of N-glycosidase PNGase F, the mixture was incubated at 37 °C for 60 min. The digestion was arrested by addition of loading sample buffer. Samples containing 20 μg of protein were resolved on 7.5% SDS-PAGE, stained with Coomassie Blue, or analyzed by immunoblotting.

**Biotin Labeling of Surface Proteins**—The biotinylation procedure was performed essentially according to Ref. 14. In brief, cell samples were washed and incubated with 0.5 mg/ml NHS-LC-biotin (Pierce). Cells were lysed, and plasma membranes were prepared as described above. After protein separation by SDS-PAGE and electroblotting, biotinylated proteins were identified with streptavidin.

**Chemical Cross-linking**—Plasma membrane preparations from iron-deficient *D. salina* cells were suspended in 1.4 M glycerol, 20 mM Na-HEPES, 10 mM KCl, and 2 mM MgCl₂. Disuccinimidyl tartrate (DST) in Me₂SO was added to a final concentration of 5 mM followed by incubation for 30 min at room temperature. The reaction was terminated with 50 mM unbuffered Tris and incubated for another 10 min on ice.

**Protein Analysis by Two-dimensional Gel Electrophoresis**—Separation and analysis of proteins on 7.5% SDS-PAGE was performed as described previously (16). Analysis of plasma membrane proteins by two-dimensional isoelectric focusing (IEF)/SDS-PAGE was as follows. Plasma membrane protein samples (350 μg of protein) were incubated in standard IEF buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 5% glycerol, 0.5% IPG buffer (GE Healthcare), and 20 mM DTT for 1 h at room temperature, loaded onto an 18-cm IPG dry strip, pH 3.0–5.6 (GE Healthcare), by active rehydration (for 12 h), and subjected to IEF for 65,000 V-h using IPG-phor instrument (GE Healthcare). Focused gel strips were equilibrated in SDS equilibration buffer (50 mM Tris-Cl, pH 8.8, 30% glycerol, 2% SDS, 6 M urea), first in 1% DTT (w/v) and next in 4% iodoacetamide. Strips were washed briefly with running buffer and loaded onto 7.5% SDS-PAGE. Gels were stained with Coomassie Blue or electroblotted to polyvinylidene difluoride paper for reaction with antibodies. Liquid chromatography/tandem mass spectrometry analysis of excised protein spots
was performed by the MS service unit at The Hebrew University, Jerusalem, Israel.

**Two-dimensional BN/SDS-PAGE**—Membrane samples (75 μg of protein) were solubilized in 30 μl of buffer containing 1% Triton X-100, 50 mM Bis-tris, pH 7.0, 0.5 mM e-aminocaproic acid, 2 mM EDTA, 10% glycerol, and plant protease inhibitor mixture. After 20 min at 4 °C, unsolubilized material was precipitated, and the solubilized proteins were mixed with BN sample buffer (final: 0.1 mM 5-aminocaproic acid, 0.5% Coomassie Blue G-250). The samples were resolved on 4.5–13% gradient BN gel at 4 °C for 4 h. For resolution on a second dimension, lanes were cut out and equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, and 30% glycerol, supplemented consecutively with 1% DTT or with 2% iodoacetamide and resolved on 7.5% SDS-PAGE. Gels were stained with Coomassie Blue and analyzed by immunoblotting as described above.

**Ferroxidase Activity**—Ferroxidase activity was determined by following the oxidation of ferrous ions using ferrozine as a specific chelator of ferrous ions (17). Cells were washed once and suspended in a buffer containing 1 M NaCl, 50 mM Na-HEPES, pH 7.0, 10 mM KCl, and 2 mM MgCl₂. Ferrous sulfate was dissolved in 100 mM sodium acetate, pH 5.5, under N₂ gas (to avoid ferrous spontaneous oxidation), prior to use. The reaction was initiated by adding ferrous sulfate to a final concentration of 50 μM at a cell density of 10⁷ cells/ml. Incubation was under continuous shaking in the dark to avoid generation of O₂ in the oxidation assay. Reactions were terminated by addition of ferrozine to a final concentration of 600 μM, followed by sedimentation, and the rate of ferrous oxidation was calculated from the decreased absorbance at 562 nm (ε₅₆₂ of 27,900 mol liter⁻¹ cm⁻¹).

**Iron Binding and Uptake**—Iron binding and uptake assays were performed as described previously (13). Cells (5 × 10⁷ cells/ml) were suspended in growth medium buffer without iron, containing 50 mM Na-HEPES, pH 8.0, and 5 mM NaHCO₃. The binding/uptake assays were initiated by addition of ⁵⁹Fe citrate and incubated for 30 min in the dark at 4 °C (iron binding) or for 1 h in the light under continuous shaking at 24 °C (iron uptake). Incubations were terminated by a 5-fold dilution into ice-cold stop solutions, containing 1 M NaCl, 50 mM Na-MES, pH 5.3, without (iron binding) or with (iron uptake) 5 mM Na-EDTA. The cells were washed twice in the same solutions and counted in a β-counter.

**RESULTS**

**Identification and Cloning of Iron Deficiency-induced Proteins**—A comparison of plasma membranes polypeptide composition was carried out between iron-deficient and iron-sufficient *D. salina* cells. As shown in Fig. 1A, three major proteins accumulate under iron deprivation as follows: two transferrin-like proteins, TTf and DTf (idi-100), and a broad pro-
tein band of about 130 kDa, which was absent from plasma membranes derived from iron-sufficient cells. Separation of plasma membrane proteins from iron-deficient cells on two-dimensional IEF/SDS-polyacrylamide gels, resolved the 130-kDa band into two distinct proteins of similar apparent molecular weight that were termed p130A and p130B (Fig. 1B).

Treatment of plasma membranes from iron-deficient cells with a peptidyl N-glycosidase, also resolved the 130-kDa bands into two distinct components on one-dimensional SDS-polyacrylamide gels (see Fig. 4C). Both protein bands from the two different gels were excised, cleaved with trypsin, and analyzed by nanoelectrospray MS/MS. A partial sequence of about 30 peptides was obtained. A search in protein data bases revealed that 14 of these sequences show homology to MCO. Based on these sequences, forward and reverse degenerate nucleotide primers were designed and utilized in different combinations for PCR amplification on cDNA from iron-deficient cells. A PCR product of 600 bp was initially cloned, and the sequencing was completed by 3′-RACE and 5′-RACE PCR extensions. A DNA product of 4275 bp was obtained, containing an open reading frame of 3225 bp, encoding a deduced polypeptide of 1075 amino acids, flanked by 45 bp at the 5′-UTR and by 1003 bp at the 3′-UTR. A polyadenylation signal (TGTAA), located 14 bp upstream from the poly(A) tail, was identified. All 14 amino acid sequences obtained by the MS analysis, covering about 12% of the total protein, were identified within the deduced amino acid sequence (data not shown). The highest sequence similarity (40% identity and 57% similarity) was obtained by FOX1, an iron-deficiency induced protein from the green alga *C. reinhardtii* (Fig. 2; compare with Fig. 3 in Ref. 8). The protein was therefore termed D-Fox (*Dunaliella* ferroxidase; GenBank™ accession number AY987035).

**Sequence Analysis of D-Fox**—By comparing amino acid sequences of D-Fox to Fox1, we found 20% higher abundance of acidic residues, a characteristic feature of proteins in halophiles, which was also found previously in extracellular proteins of *Dunaliella* (18). Analysis of the deduced amino acid sequence of D-Fox revealed that it consists of three internal homologous repeats with 28–31% sequence identity (amino acids 61–350, 400–715, and 764-end), a typical feature in the MCO family, probably result-
ing from a gene duplication event. Moreover, we identified several putative copper-binding sites, which are conserved in the MCO protein family and in particularly in *C. reinhardtii* Fox1. The copper-binding sites included three type I motifs, between amino acids 331–351, 694–714, 1049–1070, and one type II copper-binding motif, between amino acids 336 and 347. In addition, several His-X-His copper-binding motifs were found, as reported also in *C. reinhardtii* Fox1 (Fig. 2). Interestingly, type I copper-binding motifs were perfectly aligned in all three domains within the internal triplication. A hydrophy plot of D-Fox revealed a large hydrophilic protein with only one clear hydrophobic stretch at the N-terminal end. Sequence analysis predicted a signal peptide in the N-terminal hydrophobic stretch, which is most likely cleaved between amino acids 25 and 26. Three potential N-glycosylation sites were identified at amino acid residues 382, 814, and 934.

**Cloning and Sequence Analysis of p130B**—To identify p130B, degenerate nucleotide primers were designed corresponding to the amino acid sequences of peptides that did not correspond to D-Fox, and we utilized them in different combinations for PCR amplification on cDNA from iron-deficient cells. This resulted in cloning of a second DNA product of 3948 bp, containing an open reading frame of 3163 bp, encoding a deduced polypeptide of 1054 amino acids, flanked by 93 bp at the 5'-UTR and by 692 bp at the 3'-UTR (Fig. 3A). All 17 amino acid sequences obtained from the MS analysis, covering about 10% of the total protein, were identified within the deduced amino acid sequences as shown in Fig. 3A. The sequence of p130B (GenBank™ accession number AY987036) showed no significant homology to any known protein in the data bases. It should be noted that the sequence of p130B revealed an internal duplication with 38% sequence identity and 59% similarity. Hydropathy plot demonstrated two putative membrane-spanning helices, one at the N-terminal end and another at the C-terminal end of the protein (Fig. 3B). The N-terminal helix was predicted to be a leader peptide helix, which is most likely cleaved between residues 23 and 24 (computed by the CBS pre-
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D. Fox and p130B Are Surface-exposed Glycoproteins—If D-Fox is involved in iron acquisition, it is expected to be localized at the extracellular surface of the plasma membrane. Two approaches were selected to test the orientation of D-Fox and p130B as follows: exposure to proteolysis and biotin surface tagging. As demonstrated in Fig. 4A, exposure of intact cells to trypsin treatment resulted in digestion of TTF, p130B, D-Fox, and DTf, as shown by the disappearance of the corresponding protein bands and by Western analysis. Labeling intact cells with a membrane-impermeable biotin tag, followed by detection with streptavidin, revealed a selective labeling of four major components corresponding to TTf, p130B, D-Fox, and DTf (Fig. 4B). These results suggest that p130B and D-Fox, similar to TTf and DTf (14), are also exposed to the extracellular side of the plasma membrane of D. salina. Treatment of plasma membrane preparations from iron-deficient cells with PNGase F increased the electrophoretic mobility of D-Fox and of p130B and resolved them into two distinct bands on SDS-PAGE (Fig. 4C). These results indicate that D-Fox and p130B are N-glycosylated proteins, consistent with their putative N-glycosylation sites. We also found that both proteins, as well as TTf, are stained with the glycoprotein stain DIG-Glycan detection kit (Roche Applied Science), (not shown). Together, these results clearly indicate that D-Fox and p130B are N-glycosylated proteins located at the outer surface of the plasma membrane.

D-Fox Is a Plasma Membrane Ferroxidase—To test if D-Fox functions as a ferroxidase, we measured ferroxidase activity in intact cells and in plasma membrane preparations derived from iron-deficient cells. The colorimetric assay is based on the oxidation of Fe²⁺ to Fe³⁺, measured with the Fe²⁺-specific chelator ferrozine. As shown in Table 1, the measured ferroxidase activity in iron-deficient cells, as well as in the corresponding plasma membrane, was greatly enhanced, in correlation with the accumulation of D-Fox. Kinetic characterization of ferroxidase activity in plasma membrane preparations yielded an optimal pH of 7.0 and a slight stimulation by salt, with optimal activity between 0.5 and 1.0 M NaCl (data not shown). A notable factor in the regulation of multicopper ferroxidase biosynthesis in yeast and in C. reinhardtii is the availability of copper, a cofactor in all MCO members. Depletion of copper from the growth medium of iron-deficient cells partially inhibited the accumulation of D-Fox protein and suppressed ferroxidase activity (Table 1). Tetramethylthiuram disulfide (TTM) is a characteristic inhibitor of MCO proteins (21). Incubation with 250 μM TTM strongly inhibited ferroxidase activity of both cells and plasma membranes from iron-deficient cells, but it only slightly affected the activity of control or high salt cells (<15%, not shown). These results indicate that D-Fox is a multicopper ferroxidase and that it may be involved in iron uptake in iron-deficient D. salina cells.
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**Induction of D-Fox, p130B, and DTf Are Correlated with Elevated Iron Binding and Uptake**—We next tested by immunoanalysis whether the inductions of D-Fox, p130B, DTf, and TTf are kinetically correlated. Accordingly, we monitored the changes in expression levels of these proteins by an immunoanalysis during the progress of adaptation to iron deficiency and following iron re-supplementation. As shown in Fig. 5A, similar time dependences of induction and degradation of D-Fox, p130B, and DTf were observed. All three proteins were induced between 12 and 24 h from the onset of iron deprivation and disappeared within 12 h after re-supplementation of iron. However, the level of TTf, the housekeeping transferrin in *D. salina* (13), deviated from the other three proteins, being induced faster upon iron deprivation and suppressed slower upon iron re-supplementation.

To test if the induction of these proteins is correlated with changes in iron binding or iron uptake activities, we monitored in parallel the expression level of the proteins and the specific iron binding and iron uptake under the same conditions. As shown in Fig. 5B, there is a large increase in iron binding and a smaller increase in iron uptake, starting 24 h following iron deprivation, which are correlated with the induction of D-Fox, p130B, and DTf but not with the faster elevation in level of TTf. Similarly, re-supplementation of iron after 36 h suppressed iron binding and uptake within 12 h in correlation with disappearance of D-Fox, p130B, and DTf but not TTf. Induction of ferroxidase activity followed the same time dependence (not shown). These results suggest that the large increase in iron-binding capacity in iron-deprived cells is not induced by TTf but may be associated with the induction of DTf, D-Fox, and/or p130B. RNA transcript analysis revealed that the transcript levels of the new plasma membrane proteins, D-Fox and p130B, increased in parallel, preceding the accumulation of the corresponding proteins (data not shown). These results suggest that the induction of D-Fox, p130B, and DTf may be coregulated.

**DISCUSSION**

Multicopper ferroxidases are crucial components in Redox-mediated iron uptake in yeast and in the algae *C. reinhardtii*. These proteins are induced under iron deprivation in coordi-
nation with a membrane-associated ferrireductase and with a specific Fe$^{3+}$/H$^{1001}$ transporter, which together mediate high affinity iron uptake (9, 10, 23–25).

*D. salina* D-Fox resembles in many respects Fox-1 from *C. reinhardtii*. Both proteins are induced in the plasma membrane under iron deprivation and have ferroxidase activity that is dependent on copper availability and is sensitive to TTM (8, 26). Moreover, the high sequence similarity between Fox-1 and D-Fox suggests that these proteins probably had a common evolutionary origin, implying a similar physiological function.

Unexpectedly, we could find no evidence for redox-mediated iron uptake in *D. salina*; iron-deficient *D. salina* cells have no detectable ferrireductase activity, neither in intact cells nor in plasma membranes, indicating that no plasma membrane ferrireductase is induced in this alga under iron deprivation (data not shown). This finding contradicts a previous report of induction of a weak ferrireductase activity in iron-deprived *Dunaliella bardawill* (27). However, upon testing iron uptake in *D. bardawill*, we found that this species acquires iron primarily via transferrins and that redox-driven iron uptake and iron reductase activities are hardly detectable and by far lower than in *C. reinhardtii*. Also, we found that ferrozine, a chelator of Fe$^{2+}$/H$^{1001}$, does not inhibit iron uptake in *Dunaliella*, suggesting that ferrous ions are not intermediates in iron uptake in *D. salina*.

These results suggest that D-Fox in *D. salina* is not involved in redox-mediated iron uptake. In previous studies we have demonstrated that TTf is an Fe$^{3+}$/H$^{1001}$-binding protein and concluded that all Fe$^{3+}$ uptake in *D. salina* is mediated by internalization of Fe$^{3+}$ bound to TTf (13, 15). Also in iron-deficient *D. salina* cells, we found that the iron binding shows characteristic features of transferrins. For example, elimination of bicarbonate, a co-ligand for Fe$^{3+}$/H$^{1001}$ binding to transferrins, completely inhibits iron uptake and binding. Moreover, we have previously shown that dissociation of TTf from the plasma membranes of iron-deficient cells completely eliminated iron binding indicating that TTf is the major contributor to iron binding activity (14, 15). The $^{59}$Fe$^{3+}$ binding analysis on BN-PAGE shows that the iron-binding components are TTf and DTf monomers as well as protein complexes that contain both TTf and DTf. Our previous unsuccessful attempts to show iron binding to DTf (14) may be due to the weaker binding affinity of this protein compared with TTf. D-Fox and p130B are unlikely to bind Fe$^{3+}$ because their predicted sequences have no typical iron-binding motifs and because on BN-PAGE neither monomeric D-Fox nor oligomeric p130B shows $^{59}$Fe binding activity. Taken together, these results suggest that TTf and/or DTf are the iron-binding proteins in *D. salina* plasma membranes. It is likely that the major iron-binding element in the native mem-

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3 U. Pick, unpublished observations.

4 Y. Paz and U. Pick, unpublished results.
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brane is a complex of TTf and DTf with D-Fox and/or p130B, which partially dissociated during resolution on the BN-PAGE.

The kinetic analysis of protein induction and of iron binding and uptake shows that the induction of TTf precedes the maximal iron binding/uptake activities, reached 24 h after the onset of iron deprivation. If indeed TTf is the major iron-binding protein in iron-deficient cells, how is it possible that the large increase in iron binding activity is not correlated with a parallel increase in level of TTf? A possible explanation may be that in iron-sufficient cells TTf does not exhibit its maximal iron-binding activity. According to its predicted amino acid sequence, TTf possesses three potential iron-binding sites (12). Yet, we found that isolated TTf binds substoichiometric amounts of Fe$^{3+}$ of less than 1 iron/TTf (15, 29). It is therefore possible that the interactions of TTf with DTf, D-Fox, and/or p130B in iron-deficient cells induce a conformational change in TTf that opens up all three iron-binding sites thus increasing its iron-binding capacity. This interpretation is consistent with our recent finding that the affinity for Fe$^{3+}$ and for bicarbonate is significantly increased in iron-deficient D. salina cells, indicating modification of the iron-binding sites.4 We therefore propose that the function of DTf, D-Fox, and p130B is to create a high affinity and stable iron-binding complex with TTf in D. salina plasma membranes that manifests its maximal iron-binding capacity.

How did Dunaliella evolve an MCO ferroxidase that is not linked to redox-mediated iron uptake? A possible course of events may be that Dunaliella switched during evolution from redox-mediated to transferrin-mediated iron acquisition, as a consequence of a gene transfer event of a transferrin-like gene from an ancestral animal cell. The low sequence similarities between Dunaliella TTf and DTf to mammalian transferrins suggest that such an event occurred early in the evolution of this alga. It may be expected that acquisition of an efficient iron uptake mechanism, perfectly suited for iron uptake in anaerobic conditions, such as sulfate limitation (28), which could give D-Fox a direct functional significance for iron acquisition. Accordingly, we propose that D-Fox changed its physiological role during the evolution of Dunaliella from redox-mediated to transferrin-mediated iron uptake by association with transferrins at the plasma membrane surface.

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