A Chlorophyll a/b-binding Protein Homolog That Is Induced by Iron Deficiency Is Associated with Enlarged Photosystem I Units in the Eucaryotic Alga Dunaliella salina*

Received for publication, October 11, 2005, and in revised form, February 9, 2006. Published, JBC Papers in Press, February 9, 2006, DOI 10.1074/jbc.M511057200

Tal Varsano†, Sharon G. Wolf‡, and Uri Pick§,‡

From the †Department of Biological Chemistry and ‡Electron Microscopy Unit, Weizmann Institute of Science, Rehovot 76100, Israel

Adaptation of the halotolerant alga Dunaliella salina to iron deprivation involves extensive changes of chloroplast morphology, photosynthetic activities, and induction of a major 45-kDa chloroplast protein termed Tidi. Partial amino acid sequencing of proteolytic peptides suggested that Tidi resembles chlorophyll a/b-binding proteins which compose light-harvesting antenna complexes (LHC) (Varsano, T., Kaftan, D., and Pick, U. (2003) J. Plant Nutr. 26, 2197–2210). Here we show that Tidi shares the highest amino acid sequence similarity with light-harvesting I chlorophyll a/b-binding proteins from higher plants but has an extended proline-rich N-terminal domain. The accumulation of Tidi is reversed by iron supplementation, and its level is inversely correlated with photosystem I (PS-I) reaction center proteins. In native gel electrophoresis, Tidi co-migrates with enlarged PS-I-LHC-I super-complexes. Single particle electron microscopy analysis revealed that PS-I units from iron-deficient cells are larger (31 and 37 nm in diameter) than PS-I units from control cells (22 nm). The 77 K chlorophyll fluorescence emission spectra of isolated complexes suggest that the Tidi-LHC-I antenna are functionally coupled to the reaction centers of PS-I. These findings indicate that Tidi acts as an accessory antenna of PS-I. The enlargement of PS-I antenna in algae and in cyanobacteria under iron deprivation suggests a common limitation that requires rebalancing of the energy distribution between the two photosystems.

Iron is recognized as a major limiting element for photosynthesis, both in higher plants and in marine phytoplankton. Consequently, iron limitation can cause serious losses of agricultural productivity and affect oceanic carbon assimilation (1, 2). The photosynthetic apparatus represents one of the most iron-enriched cellular systems and therefore is highly vulnerable to iron limitation. Iron deficiency in photosynthetic organisms is manifested by the development of chlorosis (loss of chlorophyll), which is accompanied by loss of photosynthetic components and by inhibition of photosynthetic electron transport reactions (3). Recent studies, mostly carried out in cyanobacteria and in algae, show that photosystem I (PS-I) is the primary target to be affected by iron limitation, probably because of its relatively high iron content (12–13 Fe/PS-I units).

In cyanobacteria, iron deficiency induces a large drop in PS-I/PS-II units ratio (reviewed in Ref. 4) and the accumulation of a major chlorophyll a-binding protein, the isiA gene product CP43′ that forms an 18-subunit ring around the trimeric PS-I core. Spectroscopic evidence suggests that CP43′ is functionally coupled to PS-I reaction centers, indicating that it may act as a peripheral light-harvesting antenna (5, 6). In the green alga Chlamydomonas reinhardtii, iron deprivation is associated with remodeling of PS-I, resulting in changes in the polypeptide composition of LHC-I and in a decreased efficiency of energy transfer between the light harvesting and reaction center (RC) (7, 8).

Changes in the structure and composition of PS-I under iron deprivation have been reported also in algae. In the green alga C. reinhardtii and in the red alga Rhodella violacea, iron deficiency induced uncoupling of the light-harvesting antenna, LHC-I, from PS-I reaction centers, as indicated by chlorophyll fluorescence emission measurements and by physical dissociation of PS-I units (7, 9). In C. reinhardtii, antenna proteins in the two photosystems appear to be differentially affected by iron deprivation, and although LHC-II overall abundance and composition remain fairly constant, LHC-I is down-regulated; Lhca3 undergoes N-terminal processing, and other LHC-I polypeptides are either increased or suppressed (8).

The observations that PS-I antenna are being modified under conditions of iron deprivation, which are associated with loss of chlorophyll and/or phycobiliproteins, are intriguing, and their significance is not clear. It has been proposed that the extra CP43′ may compensate for the decrease in light harvesting efficiency because of degradation of phycobiliproteins (10). Alternatively, CP43′ and the functional dissociation of LHC-I from RC-I in C. reinhardtii may dissipate excessive absorbed light to protect against photoinhibition (11).

In a previous work we reported that the halotolerant alga Dunaliella salina maintains photosynthetic activity and cell division even under extreme iron deprivation that causes extensive chlorosis, indicating that its photosynthetic system adapts well to conditions of iron limitation. We also reported the identification of an iron deficiency-induced thylakoid protein of 45 kDa (Tidi), which was tentatively identified as a chlorophyll a/b-binding protein (CAB) (12).

In this work we describe the cloning and characterization of Tidi, the first iron-regulated member of the CAB protein family that was cloned in eucaryotes. Tidi contains a unique proline-rich N-terminal domain, is associated with PS-I-LHC-I units, and based on chlorophyll fluorescence characteristics is energetically coupled to PS-I RCs. These findings, together with the reports from cyanobacteria and other algae, suggest that modulation of the outer antenna system around PS-I is an important step for the adaptation to iron deficiency. The possible physi-

---

* This work was supported by the Avron Minerva Center for Photosynthesis and the Charles and Louise Gartner fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093-0651.

‡ To whom correspondence should be addressed. Fax: 972-8-9344118; E-mail: uri.pick@weizmann.ac.il.

§ The abbreviations used are: PS, photosystem; LHC, light-harvesting chlorophyll; BN, Blue Native; MS, mass spectrometry; CAB, Chl a/b-binding; Chl, chlorophyll; PBS, phosphate-buffered saline; DM, dodecyl maltoside; ELIPs, early light-induced proteins; Tricine, N-(2-hydroxyethyl)glycine; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol; MOPS, 4-morpholinopropanesulfonic acid; RC, reaction center.
LHC-I Protein Induced Under Low Iron

![Image](Image-100.png)  
**FIGURE 1.** Resolution of Tidi by two-dimensional gel electrophoresis of thylakoid membrane proteins. Protein samples from thylakoid membranes of iron-deficient (–Fe) and control (C) cells were separated two-dimensional gels. Proteins were stained with Coomassie Blue. Boxes mark Tidi.

Biological significance of LHC-I reorganization under iron deprivation will be discussed.

**EXPERIMENTAL PROCEDURES**

**Culture Growth and Chlorophyll Measurements**—D. salina cells were cultured as described previously (13). To induce iron deprivation, cells were pre-cultured for 48 h in complete growth media containing 1.5 μM iron/4 μM EDTA and next were transferred either for 48 h to fresh iron-deficient media or for 30 h to media containing 50 nM iron (mild iron depleion, 1.30 from standard conditions). For iron re-supplementation, cells were transferred to fresh growth medium containing 1.5 μM iron/4 μM EDTA. Starting cell concentrations were 10^5 or 5 × 10^5 cells/ml for iron deprivation or re-supplementation, respectively. Chlorophyll contents were determined according to Arnon (14). Low temperature (77 K) chlorophyll fluorescence emission was measured in an SLM 8000 spectrofluorimeter (SLM-Aminco-Chance, Urbana, IL) equipped with a low temperature attachment (12).

**Protein Analysis and Antibodies**—Protein concentrations were determined using the bicinechinonic acid assay kit (BCA-1, Sigma). Analysis of proteins on SDS-PAGE was according to Fisher et al. (15). Protein extraction in phenol and analysis by isoelectric focusing two-dimensional PAGE was according to Liska et al. (16). Proteolytic cleavage of Tidi by V8 protease and separation of peptides was as described previously (17). Mass spectrometry analysis of protein samples was performed at the mass spectrometry service units at the Hebrew University in Israel. Polyclonal antibodies against Tidi protein were prepared in rabbits by excising the protein band from polyacrylamide gels and mixing with Freund’s adjuvant. Antibodies against a keyhole limpet hemocyanin-coupled synthetic peptide P31 (PEPKKGSAFKGY, amino acids 88–99; see Fig. 4) were prepared similarly and subsequently purified on a Sepharose-P31 affinity column. Antibodies against PsaD, PsAE, PsAF, PsAG, PsAH, and ribulose-bisphosphate carboxylase/oxygenase were a gift from Prof. Rachel Nechushtai at the Hebrew University in Israel. Antibodies against light-harvesting proteins Lhca3, 17.2, and LHC-II were a gift from Prof. Michael Hippler at the Friedrich-Schiller University of Jena, Germany. Immunolocalization was done according to Ref. 15.

**Preparation of Thylakoid Membranes from D. salina**—Cultures containing 2–3 × 10^6 cell/ml were filtered through Miracloth®, and cells were washed and osmotically lysed essentially as described earlier (18). Briefly, cells were washed in an isosmotic glycerol buffer and osmotically lysed by a 3-fold dilution. Thylakoid membranes were collected by centrifugation (15 min at 4,000 × g at 4 °C) and further purified by centrifugation (12,000 × g, 20 min 4 °C) on a 0–25% Percoll gradient in suspension buffer (0.4 M glycerol, 10 mM Tris-MOPS, pH 7.2, 10 mM KCl, 2 mM MgCl2 plus protease inhibitors). The upper green band was collected and washed twice with glycerol buffer, and aliquots were stored in liquid nitrogen until further use.

**Cloning and Northern Blot Analysis**—Total RNA was prepared from 5 × 10^6 cell samples, harvested 38 h after transfer to iron-depleted medium, with the RNeasy maxi kit (Qiagen, Hilden, Germany). Poly(A)^+ mRNA was isolated using the poly(A)^+ tract mRNA isolation kit (Promega, Madison, WI). Synthesis of cDNA using 4 μg of mRNA was with the thermostable avian myeloblastosis virus-reverse transcriptase (Sigma). The cDNA was used as template in PCR performed with degenerate primers designed from peptide sequences (5′-sense GCC AAG GYC GGN GTN ATH CC; 5′-antisense AC GGA GCC GGG YTC CTT RTA YTC YTG). Specific products were enriched using a second PCR with nested primers. PCR products were extracted from the gel by GenElute™-agarose spin column (Superco, Bellefonte, PA), subcloned into pTZ57R vector using the InstAclone™ PCR product cloning kit (Fermentas, Vilnius, Lithuania), and sequenced. Specific primers were synthesized from the positive clone and used in 3′- and 5′-rapid amplification of cDNA ends reactions using the Smart™ rapid amplification of cDNA ends cDNA amplification kit (Clontech). Northern blot analysis was done according to Schwarz et al. (19) using an 813-bp probe from the 5′ end of Tidi cDNA, which was labeled using the random-primed DNA labeling kit (Roche Applied Science).

**Separation of Protein Complexes by Blue Native (BN)-PAGE**—Separation of thylakoid membrane complexes was according to Kugler et al. (20), with some modifications. Protein samples of the thylakoid membrane fraction (1200 μg of protein) were pelleted and resuspended in 500 μl of BN buffer (25 mM BisTris, pH 7.0, 20% glycerol, 2 mM EDTA, 0.2 mg/ml DNase I, and protease inhibitors as above). After 30 min of incubation at 4 °C, membranes were pelleted and subsequently solubilized in 0.1 μl of BN buffer supplemented with 4% DM. The samples were incubated for 15 min at 4 °C, and unsolubilized material was precipitated (100,000 × g, 20 min at 4 °C). Ten microliters of 10× concentrated BN solubilization buffer (5% Serva Blue G, 100 mM BisTris, pH 7.0, 0.5 M aminocaproic acid, 30% sucrose) were added to 90 ml of
solubilized samples and after 5 min loaded on BN-polyacrylamide gels. Complexes were separated at 4 °C, at 5 mA constant current (with maximum 200 V) for 6 h. Second dimension separation on SDS-PAGE was according to Ref. 21. Protein complexes were electro-eluted from BN gels using GeBAflex-tube (GeBA, Kfar-Hanagid, Israel) in Elution buffer (25 mM Na-Tricine, 7.5 mM BisTris, 10% glycerol, 0.001% Serva Blue G, and 0.03% DM), at a constant current of 10 mA for 6 h at 4 °C. Protein complexes were concentrated in Centricon® YM-100 (Centricon HR, Houston, TX).

Transmission Electron Microscopy—Concentrated D. salina cells were frozen in cellulose capillary tubes (inner diameter, 200 μm), sandwiched between 100-μm-spaced aluminum platelets, in a Bal-Tec (Bal-Tec, APRIL 14, 2006•VOLUME 281•NUMBER 15

JOURNAL OF BIOLOGICAL CHEMISTRY

10307

FIGURE 2. Comparison of proteolytic products of Tidi spots 1–5 by SDS-PAGE and by mass spectrometry. A, the two-dimensional gel spots (Fig. 1, 1–5) were excised from the gel and separately digested in-gel by V8 protease (1 mg). Peptides were separated on a 10–20% gradient SDS-polyacrylamide gel. Lane designations are as follows: 1–5, spots corresponding to Fig. 1, right; V8, protease control; M, molecular weight markers. B, MS electrospray analysis of tryptic peptides obtained from spots 2 (upper panel) and 3 (lower panel).
Images were collected on a TVIPS TemCam CCD camera (1,024 × 1,024 pixel, 20 mm × 20 mm) equipped with a field emission gun at 200 kV. The frozen samples were freeze-substituted utilizing an automatic freeze substitution system device (Leica, Lienz, Austria) in anhydrous acetone containing 0.01% glutaraldehyde + 0.2% uranyl acetate at −90 °C for 3 days, after which the temperature was raised to −30 °C during 24 h, washed twice with anhydrous ethanol, and infiltrated for 7 days in a series of increasing concentration of Lowicryl HM-20 in ethanol as follows: 10, 20, 40, 60, and 80% and six exchanges of pure resin during 24 h, washed twice with anhydrous ethanol, and infiltrated for 7 days in a series of increasing concentration of Lowicryl HM-20 in ethanol as follows: 10, 20, 40, 60, and 80% and six exchanges of pure resin (adopted from Ref. 23). Samples were UV-polymerized, and thin sections were obtained using a Leica Ultracut microtome (Lienz, Austria). Sections were incubated for 30 min in a blocking solution (0.5% gelatin, 0.5% bovine serum albumin, 0.2% glycine, 1% Tween 20 in PBS) and transferred for a 2-h incubation in rabbit antibodies against Tidi diluted 1:30 with the same blocking solution containing 1% normal goat serum. The sections were then washed with 0.2% glycine in PBS, incubated for 5 min in blocking solution, and transferred for incubation with mouse anti-rabbit antibodies conjugated to colloidal gold particles. After two washes with 0.2% glycine in PBS followed by two washes in double distilled water, sections were stained with 2% aqueous uranyl acetate followed by Raynold’s lead citrate. Sections were observed in a Tecnai T-12 (FEI, Eindhoven, The Netherlands), and images were recorded using a MegaView III CCD camera (SIS, Gröbenzell, Germany).

Single Particle Electron Microscopy—Suspensions of solubilized PS-I particles in elution buffer were prepared for EM using the droplet method. Samples (containing about 0.8 mg of Chl/ml) were applied to glow-discharged, carbon-coated electron microscope grids for 1 min, washed once with washing buffer (25 mM Na-Tricine, 7.5 mM BisTris, pH 7.0), and stained with 2% uranyl acetate for 1 min. Grids were examined on a Tecnai F20 electron microscope equipped with a field emission gun at 200 kV. Images were collected on a TVIPS TemCam CCD camera (1,024 × 1,024 pixels) at nominal pixel size of 0.32 nm, with defocus values between 0.8 and 2.0 μm. Particles were picked from images using the “Boxer” routine contained in the “EMAN” program (24). A total number of 2,372 and 1,822 particles of PS-I-LHC-I were chosen for analysis from control (complex 3) and from iron-deficient cells (complex e), respectively. Particles were classified using the k-means algorithm within EMAN, after low pass filtration to 2.5 nm. Cross-correlations between individual classes were calculated with SPIDER (25).

**RESULTS**

**Tidi Is Localized in Thylakoid Membranes and Is Resolved into Five Components on Two-dimensional Gels**—In a previous study we reported that iron deficiency induces the accumulation of a 45-kDa protein in a thylakoid-enriched preparation from *D. salina*. Based on a partial amino acid sequence homology of two proteolytic fragments, obtained by Edman degradation, we proposed that the protein is a chlorophyll *a/b*-binding protein (12). To validate this observation, purified thylakoid membranes were prepared from iron-deprived and from iron-sufficient *D. salina* cells, and their protein composition was analyzed by two-dimensional gel electrophoresis. The analysis revealed a very similar pattern of proteins except for the appearance of five distinct spots of about 45 kDa, with isoelectric points 5–6, in thylakoids from iron-deficient cells (Fig. 1). To clarify whether these spots represent the same protein, spots 1–5 were cleaved with V8 protease, resulting in a practically identical peptide fragmentation pattern on SDS-PAGE (Fig. 2A). Analysis of a tryptic digest of spots 2 and 3 by mass spectrometry (MS) shows a very similar peptide composition (Fig. 2B). These results suggest that components 1–5 represent the same 45-kDa protein. The protein was termed Tidi, for thylakoid iron deficiency-induced protein. The differences between spots 1 and 5 are not clear. Comparisons of the MS/MS data indicate that they do not result from carbamylation of lysine or arginine side chains. Differential phosphorylation can also be ruled out, because Tidi is not labeled with 32P and does not cross-react with antibodies against phosphoserine, phosphothreonine, or phosphotyrosine (data not shown). They may result either from a different post-translational modification or from alternative processing of N- or C-terminal charged amino acids as demonstrated for Lhcb proteins of *C. reinhardtii* (26). Polyclonal antibodies against purified Tidi were raised in rabbits and utilized for immunocytochemical localization of Tidi in intact cells. Tidi was found to co-localize with the Chl fluorescence in iron-deficient cells, confirming the exclusive localization in thylakoid membranes (confocal microscopy; data not shown). Electron micrographs of immunogold-labeled cell slices revealed that Tidi appears in small clusters between stacked thylakoids at the early stages of iron deprivation (Fig. 3). These results verify that Tidi is confined to chloroplast thylakoids.

![FIGURE 3. Localization of Tidi by immunogold labeling.](image)

*Fig. 3. Localization of Tidi by immunogold labeling. D. salina* cells cultured for 30 h with limiting iron were frozen, cryo-sectioned, labeled with anti-Tidi rabbit polyclonal antibodies and then with gold-labeled goat anti-rabbit antibodies, and analyzed by electron microscopy. A, section of the chloroplast; B, particle density in different cellular compartments. GP, gold particles; Chl, chloroplast; Cyt, cytoplasm, Str, starch granule; N, nucleus; V, vacuoles. Scale bar = 200 nm.
FIGURE 4. Alignment of the predicted amino acid sequence of Tidi with similar CAB proteins. Aligned with Tidi are the first hits from a BLAST search (ClustalW) that includes five type III (T3) and two type II (T2) CAB proteins. 

tom, tomato, P27522; pin1 and pin2, Scotch pine, S17696 and S17695, respectively; pea, garden pea, T06411; ara1 and ara2, Arabidopsis thaliana, AAA18206 and S46295, respectively; chlamy, C. reinhardtii, AAA03734. Sequences were aligned and shaded using ClustalW1.82 and PrettyBox programs.
**LHC-I Protein Induced Under Low Iron**

**FIGURE 5. Time course of induction of Tidi mRNA and protein.** *D. salina* cells were cultured for 73 h in iron-depleted growth medium and then transferred for 24 h to fresh iron-containing medium (1.5 μM iron). At the indicated time points, samples were taken either for protein extraction from purified thylakoids, followed by resolution on SDS-PAGE, or for RNA extraction. Protein level of Tidi was analyzed by Western blot analysis using anti-Tidi antibodies. RNA levels were determined by Northern blot hybridization, taking ribosomal RNA (16S) mRNA as a standard for mRNA level.

---

**Tidi Is a Chlorophyll a/b-binding Protein Homolog with an Extended N-terminal Domain**—To clone the gene encoding Tidi, we utilized the amino acid sequence information, obtained by the partial amino acid sequence of two proteolytic products of Tidi (12), to design degenerate primers, and by using PCR we amplified a DNA fragment from the cDNA of iron-deficient cells. The full-length cDNA was obtained by 5′ and 3′ amplification of cDNA ends (GenBank™ accession number AY965258). The 1,556-bp transcript contained a 1,050-bp open reading frame, encoding a 350-amino acid polypeptide with a calculated molecular mass of about 38 kDa. The open reading frame is flanked by a 102-bp 5′-untranslated region and a 404-bp 3′-untranslated region including a polyadenylation site (TGTTAA) 13 bp upstream from the poly(A) tail. Six peptide sequences, obtained either by N-terminal Edman degradation or by MS analysis of tryptic fragments, covering 28% of the putative protein sequence, were identified within the open reading frame. The predicted amino acid sequence of Tidi includes the conserved domain of CAB proteins (amino acids 127–323). The best six hits in a BLAST search were LHC-I, type III, and CAB proteins from various higher plants, with a maximum of 53% identity and 68% similarity. Most surprisingly, CAB proteins from *C. reinhardtii*, a closely related green alga, show significantly lower sequence homology with Tidi (Fig. 4). Tidi differs from other LHC-I proteins in the extended N-terminal domain of 126 amino acids. The N-terminal domain of Tidi is unusual not only in its length but also in the high abundance of prolines (23% of the first 90 amino acids), bordered mostly by basic amino acids. This domain does not resemble any CAB or other known protein sequences in the protein data bases. The 30 first N-terminal amino acids have a predicted structure resembling plant transit peptides, a random coil followed by a helix (not shown). The N-terminal amino acid of the mature protein could not be determined experimentally, because it turned out to be chemically modified, but the theoretically predicted cleavage site may be either after amino acid 31 or 33 (programs MitoProII or SignalP, respectively). These results demonstrate that Tidi is a chlorophyll a/b-binding protein homolog, which contains an unusual N-terminal domain that is preceded by a signal peptide.

**Transcriptional Analysis and Stress Specificity**—We studied the time courses of Tidi transcript and protein accumulation following iron deprivation by Northern blot and by Western blot analyses, respectively (Fig. 5). The comparison showed that the transcript of Tidi precedes the accumulation of the protein and peaks 2 h after the onset of iron depletion and decreases thereafter. Twenty four hours following iron re-supplementation, Tidi transcript completely disappears, and the protein level markedly decreases mostly because of dilution (5–7-fold increase in cell number). These results suggest that Tidi is regulated mainly at the mRNA level. To check if Tidi is specifically induced by iron deficiency or is a general stress protein, polyclonal antibodies were utilized to test its accumulation under different stress conditions by Western analysis. We found that high light, low temperature, high salinity (3 M NaCl), oxidative stress (methyl viologen, H₂O₂), or deficiency of other nutrients did not induce the accumulation of Tidi (not shown), indicating that it is a specific response to iron stress.

**Induction of Tidi Is Correlated with a Decrease in PS-I RC Proteins**—To test if the induction of Tidi is interrelated with other changes in the chloroplast, we compared its accumulation with the levels of other chloroplast proteins. As reported previously (12), iron deprivation does not inhibit cell division within 72 h, but it induces a remarkable shrinkage of the chloroplast and in parallel suppresses the Chl level, reaching about 80% inhibition after 72 h (Fig. 6, I and II). We found that irrespective of the large drop in Chl, the ratio of Chl per protein in thylakoids, isolated from iron-sufficient or iron-deprived cells, remains fairly constant (1.6–1.8; data not shown). Therefore, the Chl level can be taken as a representative basis for quantitation of thylakoid proteins during progressive iron deprivation. A quantitative immunoblot analysis shows that the Tidi/Chl ratio rapidly increased (within 36 h) or decreased (within 24 h) following iron depletion or re-supplementation, respectively. In parallel with the induction of Tidi, we observed a large decrease in PsaA/B, PsaG (not shown), and PsaD subunits of PS-I RC, which was reversed by iron supplementation. In contrast, the relative abundances of LHC-I (represented by Lhca3) as well as LHC-II CAB proteins per Chl did not decrease during iron deficiency (Fig. 6, A and B). These results suggest the induction of Tidi is correlated with a decrease in number of PS-I units, which precede the decrease in Chl synthesis, consistent with reorganization of PS-I under iron deficiency.

**Association of Tidi with PS-I**—To find out if Tidi is associated with PS-I or with PS-II units, we solubilized thylakoid membranes in DM and resolved protein complexes by BN-PAGE. In membranes from iron-insufficient cells, eight major Chl-containing bands ranging in size from 150 to 700 kDa could be identified (Fig. 7A). Resolution of these bands on a second SDS gel revealed that each band represents distinct protein complexes (Fig. 7B). Western immunoblot, combined with MS analyses, identified bands 1 and 2 (red, 700–750 kDa) as PS-II-LHC-II complexes and bands 3 and 5 (blue, 500–600 kDa) as PS-I-LHC-I complexes (Fig. 7C). Band 6 (~380 kDa) represents free PS-II reaction centers, whereas band 7 represents free LHC-II monomers. In iron-deficient cells, significant changes were observed in the PS-I but not in PS-II protein complexes, in comparison to the iron-sufficient cells. Although bands 3 and 5 were substantially reduced, larger complexes (α-ε) appeared (Fig. 7, A and B). Analysis of the protein composition of these new complexes revealed that they contain Tidi as well as proportional amounts of 9–11 proteins that appear also in bands 3 and 5 (Fig. 7C). The immunoblot analysis indicated that these complexes contain two PS-I reaction center components PsaA/B and PsaD and two components of LHC-I, Lhca3, and Lhc-17.1 (Fig. 7D). MS-MS analysis identified two proteins of PS-I-LHC-I; the 26-kDa protein was identified as Lhca5, and the 23-kDa protein was identified as PsaD. One protein of LHC-II (Lhcb1, about 30 kDa) was also identified in these complexes.
These results demonstrate that Tidi is associated with PS-I-LHC-I units in iron-deficient D. salina cells.

Fluorescence Emission Spectra of Isolated Chl-Protein Complexes—In a previous study we reported that iron deprivation of Dunaliella cells enhanced 77 K Chl fluorescence emission around 710 nm, which was attributed to PS-I RC (12). To test if the 710 nm emission indeed represents PS-I, we electroeluted PS-I and PS-II complexes and tested their Chl fluorescence emission at 77 K. Complexes 1 and 2 from iron-deficient and from control cells have similar Chl fluorescence emission spectra, with two peaks at 685 and 696 nm, which correspond to PS-II RC (Fig. 8, top) (27, 28). Complex 3 from control cells emits maximally around 712 nm, which corresponds to PS-I core complexes (27–32).
The fluorescence emission maxima of PS-I/LHC-I complexes from iron-deficient cells (designated \(/H9251, /H9252, /H9254, \text{ and } /H9280\)) was similar. Addition of a low concentration of Triton X-100 to these complexes completely abolished the emission at 711–713 nm and induced an emission peak at 675 nm (Fig. 8, bottom). Similarly, we observed that elution of PS-I complexes in the presence of DM inhibited the 77 K emission at 712 nm and elevated the emission at 675 nm. We attribute the 711–713 nm emission to the PS-I core and the 675 nm peak to the LHC-I antenna, and we

**FIGURE 7.** Identification of Tidi in PS-I units by BN-gel electrophoresis. Thylakoid membranes were isolated from iron-deficient (–Fe) and from iron-sufficient (C) *D. salina* cells. Proteins (12 mg/ml) were solubilized in 4% DM, loaded (130 μg of protein/lane), and separated by electrophoresis (A). Each lane was scanned without fixation or staining and analyzed by densitometry using the ImageQuant software (B). Green bands were cut out from BN gels, extracted with SDS, and resolved by SDS-PAGE (C). The proteins were blotted onto a nitrocellulose membranes and analyzed by Western blotting with antibodies against subunits of PS-I reaction centers (PsaA/B and PsaD), PS-II reaction center (D1), LHC-I (Lhca3 and Lhca17.1), LHC-II, and Tidi (D). Greek letters, complexes induced by iron deprivation. Roman letters, complexes present in control cells. Red, PS-II; blue, PS-I.
suggest that the detergent-induced changes in Chl fluorescence emission reflect reversible uncoupling of energy transfer from LHC-I antenna from PS-I RCs. This interpretation is consistent with a previous analysis of isolated PS-I units from C. reinhardtii, which demonstrated detergent-induced dissociation of LHC-I from the PS-I core, with corresponding 77 K fluorescence emissions at 675 and 715 nm, respectively (33), and with similar observations in cyanobacteria (5). The results clearly confirm that the enhanced 77 K chlorophyll fluorescence emission at >700 nm in iron-deficient cells indeed results from PS-I, and they also suggest that energy is efficiently transferred from LHC-I to PS-I core in the isolated complexes.

**DISCUSSION**

Adaptation of the halotolerant alga *D. salina* to iron deprivation is associated with remodeling of PS-I, which was manifested by an increase in size of PS-I particles, by an increase in PS-I chlorophyll fluorescence emission (12), and by the induction of a new Chl a/b-binding protein homolog, termed Tidi. The sequence similarity between Tidi and CAB proteins and the electron micrograph single particle density maps suggest that Tidi is most probably localized at the peripheral antenna of PS-I.

Tidi is the first reported CAB-like protein that is specifically induced by iron deprivation in a photosynthetic eucaryote. It differs from any known CAB protein in its extended N-terminal domain that bears no sequence homology to other chlorophyll-binding proteins. Another CAB-like protein, which contains proline-rich N-terminal domains, is Cbr from *Dunaliella bardawill*, which is a homolog of early light-induced proteins (ELIPs) (37). Interestingly, ELIPs are also stress-related CAB-like proteins that are induced by high light and have been associated with protection against photoinhibition (38). However, the evolutionary origin of ELIPs is probably unrelated to that of Tidi, because they show no sequence similarity. Also, the proline clusters in ELIPs are interspersed mainly by serines and threonines, whereas in Tidi, they are bordered mostly by basic amino acids.

The size heterogeneity of PS-I units from iron-deprived *D. salina* cells resolved on BN gels appears to result from the different relative abundance of Tidi, as reflected by the intensity of staining/immunoreactions.
of Tidi relative to other PS-I subunits (Fig. 7, C and D). However, it is not clear if this apparent heterogeneity results from partial dissociation of Tidi from PS-I during the solubilization by DM or during the electrophoretic resolution, or whether it reflects structural variations of PS-I units in the native state in thylakoid membranes from iron-deprived cells. Notably, IsiA was reported to create an astonishing variation of supercomplexes with PS-I in cyanobacteria, including single and double rings, monomeric and trimeric RC units, and empty shells (10). It is not clear, however, if these structures have a physiological significance under native conditions.

Another open question is how are PS-I units in *D. salina* remodeled under iron deprivation. Specifically, does newly synthesized Tidi associate with pre-existing PS-I units or are PS-I units completely reassembled? In *C. reinhardtii*, PS-I remodeling involves processing of Lhca3, changes in the relative abundance of other LHC-I components, and a decrease in Psak, which suggests dissociation and reassembly of new PS-I subunits. In contrast, in *D. salina* isolated PS-I units, there are no evident changes in the relative abundance of LHC-I or RC-I components, other than the accumulation of Tidi (Fig. 7, C and D). The observation that Tidi accumulation was correlated with a large decrease in PS-I RC polypeptides suggests that the decrease in the number of PS-I units is coordinated with their increase in size, which may be brought about by the synthesis of Tidi. However, because these changes in PS-I are relatively slow and take place during active cell division, they may represent synthesis and assembly of new PS-I units, rather than reorganization of pre-existing units.

What are the functional implications of PS-I remodeling in *D. salina*? Generally, the changes in the photosynthetic system of *D. salina* in response to iron deprivation appear to resemble those in cyanobacteria and in other algae. The large drop in PS-I units, indicated by the decrease in abundance of PS-I reaction center proteins, is characteristic of iron-deprived cyanobacteria, green algae, and diatoms (summarized in Refs. 7, 10, 11, 13).
The increase in 77 K PS-I Chl fluorescence has also been reported in the green alga C. reinhardtii (7) and in the red alga R. violacea (9) and has been interpreted as a decrease in efficiency of energy transfer between the light harvesting and reaction centers of PS-I.

However, a closer comparison between the responses of D. salina PS-I and that of C. reinhardtii PS-I to iron deprivation reveals several differences. In C. reinhardtii and in R. violacea, the enhancement of 77K PS-I Chl fluorescence is associated with significant blue shifts in chlorophyll emission spectra, consistent with perturbation of energy transfer from LHCl-I to PS-I RCs (7, 9). The functional uncoupling was proposed to result from dissociation and remodeling of PS-I, initiated by the disappearance of PsAK (7) and by the N-terminal processing of Lhca3 (8). In contrast, in iron-deprived D. salina cells there are no indications for structural or functional dissociation of LHCl-I from PS-I reaction centers; there were no significant chlorophyll emission spectral shifts either in intact cells (12) or in isolated PS-I complexes (Fig. 8). Also we did not detect any decrease in relative abundance of Lhca3 or of any other LHCl-I component in isolated PS-I units from iron-deprived cells.

In fact, we have indications that PS-I in D. salina becomes even more efficient upon iron deprivation; we found that the rates of PS-I electron transport per chlorophyll from ascorbate/dichlorophenolindophenol to methyl viologen + 3-(3,4-dichlorophenyl)-1-dimethylylurea in chloroplasts from iron-deprived cells are 35 ± 16% faster at saturating light but 2–2.5-fold faster at low light intensities in comparison to iron-sufficient cells.4 These results suggest an increase in PS-I functional antenna size in iron-deprived D. salina, consistent with the larger PS-I single particle units observed by electron microscopy.

In this respect D. salina appears to resemble cyanobacteria more than C. reinhardtii in its response to iron deprivation. Spectroscopic measurements in cyanobacteria have shown that CP43′ efficiently transfers light energy to PS-I reaction centers and increases the light harvesting capacity of PS-I by 70–100% (5, 40). The intriguing observation that evolutionarily unrelated chlorophyll-binding proteins, Tid and CP43′, are induced under iron limitation in PS-I of green alga and of cyanobacteria, respectively, suggest a common evolutionary pressure on the photosynthetic systems, possibly to rebalance energy distribution between PS-I and PS-II. A recent comparison between coastal and oceanic diatoms revealed that the latter, which are adapted to iron limitation, manage to decrease their iron requirements without compromising photosynthetic capacity. This is achieved by a large reduction in the relative abundance of the iron-rich PS-I and of cytochrome b6f complexes, which presumably saves iron (39). The results imply an increase in absorption cross-section for PS-I in oceanic diatoms. Thus, D. salina may resemble oceanic diatoms in being able to maintain high growth rate and photosynthetic activity under iron limitation, by creating fewer but more efficient PS-I units.

What can be the possible reasons for the different responses of C. reinhardtii and of D. salina PS-I remodeling under iron deprivation? A possible clue may be that C. reinhardtii is a heterotroph that can survive in darkness on different carbon sources, whereas D. salina is an obligate photosynthetic organism. It is conceivable that mobilization of iron from iron-rich photosynthetic complexes to the respiratory system offers an evolutionary advantage for survival under iron limitation. Thus, it is possible that C. reinhardtii switches from photosynthesis to respiration as the major energy generator under iron limitation, whereas D. salina, oceanic diatoms, and cyanobacteria maintain efficient photosynthesis by creating fewer and larger PS-I units.

Acknowledgments—We thank Dr. Eyal Shimoni from the Electron Microscopy Unit at The Weizmann Institute of Science for help with the immunogold localization studies. We thank Prof. Izhak Ohad from The Hebrew University, Jerusalem, Israel, for helpful discussions and help with the photosynthesis experiments. We also thank Dr. Michael Hippler from Jena, Germany, and Prof. Rachel Necashtai from The Hebrew University, Jerusalem, Israel, for providing antibodies.

REFERENCES

1. Watson, A. J., Bakker, D. C. E., Ridgwell, A. J., Boyd, P. W., and Law, C. S. (2000) Nature 407, 730–733
2. Falkowski, P. G., Barber, R. T., and Smetacek, V. V. (1998) Science 281, 200–207
3. Spiller, S., and Terry, N. (1980) Plant Physiol. 65, 121–125
4. Strauss, N. (1994) in Iron Deprivation: Physiology and Gene Regulation (Bryant, A. D., ed., Kluwer Academic Publishers Group, Dordrecht, Netherlands
5. Bibby, T. S., Nield, I., and Barber, J. (2001) J. Biol. Chem. 276, 43246–43252
6. Bibby, T. S., Nield, I., and Barber, J. (2001) Nature 412, 743–745
7. Moseley, J. L., Allinger, T., Herzog, S., Hoehrt, P., Wehinger, E., Merchant, S., and Hippler, M. (2002) EMBO J. 21, 6709–6720
8. Ben-Shem, A., Frolow, F., and Nelson, N. (2003) Nature 426, 630–635
9. Boekema, E. J., Jensen, P. E., Schlodder, E., van Gorkom, H. J., Dekker, J. P., and Boekema, E. J. (2005) FEBS Lett. 579, 3253–3257
10. Sandstrom, S., and Okada, I. (2001) Photoschem. Photobiol. A, 74, 431–437
11. Varsano, T., Kaftan, D., and Pick, U. (2003) J. Plant Bio. 26, 2197–2210
12. Morgan-Kiss, R., Ivanov, A. G., Williams, J., Khan, M., and Huner, N. P. A. (2002) Biochim. Biophys. Acta 1649, 190–200
13. Fleischmann, M. M., Ravanel, S., Delosme, R., Olive, J., Zito, F., Wollman, F. A., and Scheller, H. V., and Dekker, J. P. (2001) Plant Physiol. 128, 1029–1036
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Hohenerberg, H., Manneweiler, K., and Muller, M. (1994) J. Microc. (Org.) 175, 34–43
16. Hummel, B. M., Kornoni, M., Takagi, T., Kamawsa, N., Ishijima, S. A., and Onuki, M. (2001) Yeast 18, 433–444
17. Ludtke, S. J., Baldwin, P. R., and Chiu, W. (1999) J. Struct. Biol. 128, 82–97
18. Frank, J., Radermacher, M., Penzcek, P., Zhu, J., Li, Y. H., Ladjadi, M., and Leith, A. (1996) J. Struct. Biol. 116, 190–199
19. Stauber, E. J., Eink, M., Markert, C., Krause, O., Jochsningsmeier, U., and Hippler, M. (2005) Eukaryot. Cells 4, 978–994
20. Morgan-Kiss, R., Ivanov, A. G., Williams, J., Khan, M., and Huner, N. P. A. (2002) Biochim. Biophys. Acta 1561, 251–265
21. Vassilev, I. R., Kolzer, Z., Wymans, M. D., Mauzzerall, D., Shulka, V. K., and Falkowski, P. G. (1995) Plant Physiol. 109, 963–972
22. Murakami, A. (1997) Photosynth. Res. 53, 141–148
23. Moseley, J. L., Page, M. D., Alder, N. P., Eriksson, M., Quinn, J., Soto, F., Tegh, S. M., Hippler, M., and Merchant, S. (2002) Plant Cell 14, 673–688
24. Fleischmann, M. M., Ravanel, S., Delosme, R., Olive, J., Zito, F., Wollman, F. A., and Rochaix, J. D. (1999) J. Biol. Chem. 274, 30987–30994
25. Cardol, P., Giloro, G., Havaux, M., Remacle, C., Matagne, R., and Franck, F. (2003) Plant Physiol. 133, 2016–2020
26. Kargul, J., Nield, J., and Barber, J. (2001) J. Biol. Chem. 276, 16135–16141
27. Germano, M., Yakushwetva, A. E., Kegisti, W., van Gorkom, H. J., Dekker, J. P., and Boekema, E. J. (2002) FEBS Lett. 525, 121–125
28. Ben-Shem, A., Frolow, F., and Nelson, N. (2003) Nature 426, 630–635
29. Boekema, E. J., Jensen, P. E., Schlodder, E., van Breemen, J. F. L., van Roon, H., Scheller, H. V., and Dekker, J. P. (2001) Biochimie 83, 1029–1036
30. Lee, A., Levy, H., and Zamir, A. (1991) J. Biol. Chem. 266, 13698–13705
31. Braun, P., Blanot, G., Tal, T., Malkin, S., and Zamir, A. (1996) Plant Physiol. 110, 1405–1411
32. Strzepek, R. F., and Harrison, P. J. (2004) Nature 431, 689–692
33. Andrizhiyevskaya, E. G., Schwabe, T. M., Germano, M., D’Haene, S., Krup, J., van Grondelle, R., and Dekker, J. P. (2002) Biochim. Biophys. Acta 1556, 265–272

4 T. Varsano, S. G. Wolf, and U. Pick, unpublished observations.