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Membrane Development in the Cyanobacterium, *Anacystis nidulans*, during Recovery from Iron Starvation[1]

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ABSTRACT

Deprivation of iron from the growth medium results in physiological as well as structural changes in the unicellular cyanobacterium *Anacystis nidulans* R2. Important among these changes are alterations in the composition and function of the photosynthetic membranes. Room-temperature absorption spectra of iron-starved cyanobacterial cells show a chlorophyll absorption peak at 672 nanometers, 7 nanometers blue-shifted from its normal position at 679 nanometers. Iron-starved cells have decreased amounts of chlorophyll and phycobilins. Their fluorescence spectra (77K) have one prominent chlorophyll emission peak at 684 nanometers as compared to three peaks at 687, 696, and 717 nanometers from normal cells. Chlorophyll-protein analysis of iron-deprived cells indicated the absence of high molecular weight bands. Addition of iron to iron-starved cells induced a restoration process in which new components were initially synthesized and integrated into preexisting membranes; at later times, new membranes were assembled and cell division commenced. Synthesis of chlorophyll and phycocyanins started almost immediately after the addition of iron. The absorption peak slowly returned to its normal wavelength within 24 to 28 hours. The fluorescence emission spectrum at 77K changed over a period of 14 to 24 hours during which the 696- and 717-nanometer peaks grew to their normal levels, and the 684 nanometer peak moved to 687 nanometers and its relative intensity decreased to its normal level. Analysis of chlorophyll-protein complexes on polyacrylamide gels showed that high molecular weight chlorophyll-protein bands were formed during this time, and that low molecular weight bands (related to photosystem II) disappeared. The origin of the fluorescence emission at 687 and 696 nanometers is discussed in relation to the specific chlorophyll-protein complexes formed during iron reconstitution.

Photosynthetic pigments (*e.g.* Chl and phycobilins) are associated with specific membrane proteins to form functional complexes either embedded in or associated with the thylakoid membranes of higher plants, algae, or cyanobacteria (2, 8, 12). Studies directed toward the understanding of the organization, composition, and biogenesis of these pigment-protein complexes involve various *in vivo* and *in vitro* approaches. Biogenesis of these complexes have classically been studied by following a developmental sequence during membrane synthesis. In higher plants and eucaryotic algae, greening of dark-grown cells under light has been studied to analyze such developmental processes (18). The thylakoid membranes from the chloroplasts of these organisms contain polypeptides encoded by their nuclear as well as chloroplast genome. As a result, the development of the thylakoid membranes during greening involves the coordinated sequential synthesis of different gene products from both the nuclear and the chloroplast genomes, and their insertion into the preexisting membranes. Despite this complexity, such analyses have been very useful in the understanding of the organization of functional complexes in the thylakoid membranes.

Cyanobacteria are the only procaryotic organisms that perform oxygenic photosynthesis. Their single genomes control the synthesis of all the components of the thylakoid membranes, and thus make these organisms ideal for the study of the biosynthesis and organization of the functional complexes. However, developmental studies using light-induced 'greening' experiments typically cannot be performed with these organisms. For example, *Anacystis nidulans* is an obligate photoautotroph which does not grow in the dark and does not lose its thylakoid membranes under these conditions (26). As a result, some other approach is needed to study the development of the thylakoid membranes in these organisms.

Previous results of Oquist (19–21) and this laboratory (9, 10, 25) indicate that iron deficiency in cyanobacteria may provide one such approach. Cyanobacterial cells grown in iron-deficient media are perfectly viable (9). However, the number of thylakoids per cell decreases at least 2- to 3-fold under iron-stress (25). Iron deficiency affects a number of photosynthetic characteristics of these cells, including the room-temperature absorption spectrum, 77K fluorescence emission spectrum, and the kinetics of fluorescence yield at room temperature. The analysis of Chl-protein bands of these iron-starved cells by LDS[4]-PAGE have indicated that iron-deficient membranes substantially lack the high mol wt Chl-protein complexes (9). Ultrastructural studies also showed that the iron-starved cells lose most of their phycobilisomes, the phycobilin containing pigment-protein complexes that are now known to serve exclusively as antenna of PSII complexes of cyanobacteria (16).

In this report, we have investigated the spectral characteristics and banding profiles of the Chl-protein complexes from membranes of iron-starved cells during iron-induced recovery. We have shown that the first 24 h after the addition of iron to iron-starved cells represented the time period for development of the thylakoid membranes and their constituent complexes. We have also demonstrated that fluorescence emission spectra (77K) from intact cells could be used to monitor different stages during this developmental process. Analysis of the Chl-protein complexes

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[4] Abbreviations: LDS, lithium dodecyl sulfate; CP, chlorophyll-protein.

indicated that stable, large Chl-protein bands were absent in iron-deficient cells, and were formed during specific time intervals throughout the reconstitution process. These data suggest that the iron-induced reconstitution process represents a true developmental system in *A. nidulans* cells, and can be used for studies on the assembly of thylakoid membranes and their constituent complexes.

MATERIALS AND METHODS

Cyanobacterial Strain and Culture Condition. *A. nidulans*, strain R2, was originally characterized by Grigorieva and Shestakov (7) and was kindly provided to us by Dr. G. A. van Arkel of the University of Utrecht, The Netherlands. Cells were grown axenically in shaking liquid cultures at 28°C (24), using BG-11 growth medium (1). Illumination was provided by cool-white fluorescent light at a continuous intensity of 6 mw/cm^2. Iron-deficient medium was prepared by replacing ferric ammonium citrate in BG-11 with equivalent amounts of ammonium citrate. Prior to use, all the glassware for iron-deficient media were thoroughly washed with 0.1 M EDTA to remove iron. Furthermore, all the monovalent cationic stock components of BG-11 were passed through Chelex 100 (BioRad) columns to remove trace quantities of iron. Effects of iron-deficiency were observed 4 to 5 d after inoculation of the culture. Cultures were propagated in iron-deficient media for at least 6 cycles of growth (2–3 generations each) prior to use. Iron was restored in stable iron-deficient cultures by adding 6 mg of ferric ammonium citrate per liter of medium. Aliquots of reconstituting samples were collected at specific time intervals after the addition of iron. The number of cells per ml was counted using a Petroff-Hauser counter.

Spectral Analyses. Room temperature absorption spectra of whole cells were obtained on an Aminco-Chance DW2 dual wavelength spectrophotometer. The data were corrected for scattering, and the simultaneous equations of Arnon *et al.* (3) were used to calculate the concentrations of Chl and phycocyanin. Fluorescence emission spectra at liquid N_2 temperature (77K) were obtained on an SLM 8000 spectrofluorimeter (SLM Instruments Inc., Urbana, IL), provided with an attachment for low temperature measurements. The digital output from the fluorimeter was recorded directly on a Hewlett-Packard 85 microcomputer and subsequently processed using the software supplied by SLM, and later modified in this laboratory. For low temperature measurements, samples were prepared in a 50% glycerol suspension to a Chl concentration of 25 μg/ml. Samples were illuminated with 435 nm light (8 nm bandwidth) to excite Chl molecules. All emission spectra were corrected for the unequal response of the photomultiplier tube at different wavelengths by using the correction factors supplied by the manufacturer. The final spectra were plotted on an HP 7225A plotter.

Membrane Preparation and Analysis of Chl-Proteins. Cells were harvested at different time points after addition of iron to iron-starved cultures. Thylakoid membranes from these cells were isolated by Braun homogenization. Aliquots of cultures (100 ml) were collected at different times and immediately pelleted down by centrifugation at 5000*g* for 5 min. Each pellet was then resuspended in 10 ml of BG-11 medium and mixed with an equal volume of glass beads and homogenized in a Braun homogenizer (B. Braun, Melsungen, W. Germany) for 90 s. The samples were kept cold by controlled flow of liquid CO_2. The homogenized samples were centrifuged first at 4,500*g* for 5 min to get rid of residual glass beads and whole cells, and the resultant supernatants were then centrifuged at 35,000*g* for 20 min. Each membrane pellet was then resuspended (at a final Chl concentration of 2 mg/ml) in buffer consisting of 10 mM Tricine-NaOH (pH 7.5), and 1 mM each of the protease inhibitors, phenylmethyl-sulfonyl fluoride, benzamidine, and ε-aminocaproic acid.

The CP composition of the membranes was examined by PAGE according to the procedure of Delepelaire and Chua (5) as modified in this laboratory (8). Slab gels were formed with 5 to 15% polyacrylamide gradients and with a 5% stacking region. Samples were prepared by adding sucrose to a final concentration of 6%, and LDS to a detergent to Chl ratio of 10:1 (w/w). Samples containing 5 μg of Chl were loaded in each well and run at a constant current of 12.5 mamp for 4 to 6 h at 6°C. Upon termination of electrophoresis, the green CP bands on the gels were photographed through a blue filter. The same gels were then transilluminated by long wavelength UV light on a Chromatovue transilluminator (Ultraviolet Products Inc., San Gabriel, CA) to examine the red fluorescence emission from the Chl-containing bands, and photographed through a red filter. Individual lanes cut out from the gels were scanned at 670 nm on a Gilford 240 spectrophotometer, and the area under each peak was measured to compare the relative amounts of different CP bands in each lane.

RESULTS

Growth and Pigment Changes upon Iron-Induced Recovery. *A. nidulans* R2 cells initially grew slowly under severe iron-limited conditions. As indicated previously (25), cells started dividing at a normal rate during reconstitution of iron-deprived cells only after an initial lag of about 12 h. Thus, we examined several features of the reconstituting cells during the first 24 h of reconstitution; *i.e.* during the lag period and for an additional generation (12 h).

Room temperature absorption spectra of iron-deficient cells were significantly different from those of the normal cells. As noted earlier (9), the ratio of absorption peaks of phycocyanin (625 nm) and Chl (between 670 and 680 nm) was significantly decreased in iron-deprived cells, indicating the relative decrease of phycocyanin compared to Chl. The more interesting change was observed in the position of the Chl absorption peak, since it was blueshifted in the iron-starved cells. The extent of the blueshift depended on the age of the culture in the iron-starved conditions, and the peak reached a steady value of 672 nm after 5 d of incubation under iron-deficient conditions. The position of this peak did not change in the first 6 to 8 h after the addition of iron (Table I). However, it reached a value of 676 nm at 14 h and 679 nm at 24 h after the addition of iron. The gradual changes in the position of the Chl peak over 24 h indicated that there were sequential changes in the organization of the Chl molecules in the thylakoid membranes of the cells recovering from iron starvation.

The changes in the concentrations of Chl and phycobilin pigments of iron-starved cells after the addition of iron are documented in Table I. As observed earlier (9), the levels of Chl and phycocyanin were decreased in iron-starved cells; in addition, the ratio of phycocyanin to Chl was also decreased in these cells. Addition of iron induced the synthesis of both of these pigments immediately. However, at the initial stages of the recovery process, the rate of synthesis of phycocyanin molecules was much higher than that of Chl molecules (Table I, line 3). Ultrastructural studies had suggested that phycobilisomes, the phycocyanin-containing, light-harvesting structures of PSII in cyanobacteria, are formed around 6 to 8 h after the addition of iron to iron-starved cells (25), and the results in Table I are in accordance with these morphological findings. The rate of Chl synthesis increased within the next 10 to 12 h, such that, by 24 h after the addition of iron, the ratio of the concentrations of phycocyanin and Chl was very similar to that found in normal cells.

Fluorescence Emission Spectra. The liquid N_2 temperature (77K) fluorescence emission spectra of normal cells, iron-deprived cells, and cells at different stages during iron-induced

Table I. *Absorption Peaks and Pigment Content of Iron-deficient and Iron-restored A. nidulans R2 cells*

Room-temperature absorption spectra of whole cells were measured as described in "Materials and Methods". The concentrations of Chl and phycocyanin were determined from these spectral data as described by Arnon *et al.* (3).

Sample	Time after Addition of Iron	Chl Absorption Peak	Chl	Phycocyanin	C_p/C_c
	h	*nm*	*μg C_c/ml*	*μg C_p/ml*	*ratio*
Normal		679	4.57	29.90	6.54
Iron deficient	0	672	2.99	10.02	3.35
	6	672	3.46	20.56	5.94
	10	673	3.67	22.80	6.21
	14	676	3.79	27.37	7.22
	24	679	4.60	31.58	6.87

recovery are shown in Figure 1. All of these spectra were corrected for the characteristics of the photomultiplier tube used and normalized with respect to the fluorescence from an external standard (Rhodamine solution). As described earlier, the fluorescence emission spectra of iron-deprived cells were dramatically different from those of the normal cells (9, 21, 25). Figure 1A shows the characteristic fluorescence emission spectra of normal and iron-starved *A. nidulans* R2 cells at 77K. Normal cells had three characteristic Chl emission peaks at 687, 696, and 717 nm (11), whereas iron-deprived cells had only one prominent peak at 684 nm. This latter peak was broad on the red side, indicating the presence of species fluorescing in the 696-nm region. Another important feature of the spectra concerned the relative height of the fluorescence peaks under normal and iron-deprived conditions. The amount of fluorescence at 696 nm as well as that in the 717 nm region were substantially decreased compared to those in normal cells. The height of the 684-nm emission peak from iron-deprived cells, on the other hand, was almost twice that of the 687-nm peak from normal cells, indicating the presence of highly fluorescent Chl-containing complexes in iron-starved cells. This observation correlated very well with the fluorescence properties of CP complexes from iron-starved cells as described in a later section.

The sequential changes in the fluorescence emission spectra of iron-deprived cells after the addition of iron are shown in Figure 1, B and C. Within the first 6 h, the 696- and 714-nm peaks began forming and the 684-nm peak shifted to 686 nm (Fig. 1B). In addition, the height of the 686-nm peak was slightly decreased from that observed in iron-deprived cells. The situation changed much further during the next 4 h. At 10 h after the addition of iron, the 696-nm peak had become most prominent and the height of the 686-nm peak was considerably decreased. During these 4 h, the 713-nm peak height also increased. The spectrum at 14 h was most interesting, since the 698-nm peak clearly had the greatest intensity (Fig. 1C). Examination of this peak indicated that the Chl species fluorescing at this wavelength had a very broad peak and probably shifted the position of the 686-nm peak by a few nm towards the red. The last peak shifted to 716 nm at this time. By 24 h, the three peaks were finally found at 687, 696, and 715 nm. The height of the 696-nm peak decreased considerably between 14 and 24 h, whereas that of the 687-nm peak increased slightly during this time. The spectrum looked remarkably similar to that of the normal cells (Fig. 1A). Judging by the profiles of these fluorescence emission spectra, we considered the iron-induced reconstitution of iron-deprived *A. nidulans* R2 cells to be essentially completed by 24 h after the addition of iron. Indeed, 77K fluorescence emission spectra were the best monitors of different stages during this reconstitution process, and were reproducible during 12 different reconstitution experiments.

Chl-Protein Complexes. Previous studies have shown that iron-deficiency changes the CP composition of thylakoid membranes of cyanobacteria as well as of higher plants (9, 15). We analyzed the CP organization of iron-starved cells during iron-induced reconstitution by PAGE. As described by Guikema and Sherman (8), solubilization of thylakoid membranes of normal cells with LDS and subsequent analysis on a 5 to 15% gradient polyacrylamide gel revealed six Chl-protein bands (Fig. 2, CP I–VI). Bands I to IV have large apparent mol wt (360–100 kD), and represent the association of reaction center proteins with additional polypeptides in the thylakoid to form large CP aggregates. Band V is associated with the PSI reaction center, whereas band VI is associated with PSII only. Under the conditions used here, bands V and VI are often not visible or, at best, poorly visible in preparations from normal cells (Fig. 2A, lane A).

Iron deficiency resulted in a significant loss of bands I and II (lane B). At the same time, bands V and VI were present in greater amounts. The relative proportion of Chl in each CP band is summarized in Table II. The amount of Chl present in any band varied after storage of the membrane samples at −20°C for prolonged period of time. However, the total amounts of Chl present in bands I plus II, as well as that in bands III plus IV were fairly constant for samples prepared after different lengths of storage. Two distinct bands were usually seen in both the CP V and CP VI region of the gel (lane B). Characterization of these bands will be presented in a later communication (Pakrasi, Riethman, and Sherman, in preparation).

Figure 2A also shows the profiles of the Chl-protein bands at 6 h (lane C), 9 h (lane D), 12 h (lane E), 18 h (lane F), and 24 h (lane G) after the addition of iron. The amount of CP I started increasing at 9 h and returned to its normal value by 12 h. During this time, the amount of CP V and CP VI decreased markedly. Examination of the Chl-protein bands indicated that stable, high mol wt Chl-proteins were formed between 9 and 12 h after the addition of iron to iron-deficient cells. The data presented here is from one reconstitution experiment, but very similar patterns were observed in more than six different experiments.

Figure 2B shows the red fluorescence emission from the Chl-protein bands during transillumination of the gel with UV light. Whereas the most prominent fluorescent band was CP VI, none of the higher mol wt bands (CP I–IV) were fluorescent. The amount of this fluorescence was very high in iron-deficient cells (lane B) and started declining with time during iron-induced recovery. By 24 h after the addition of iron, there was almost no detectable fluorescence from the CP VI region. The presence of highly fluorescent CP VI in iron-deficient cells, and the time course of its disappearance during iron-induced recovery correlated well with the level of 77K fluorescence in the 684 to 687 nm region (Fig. 1).

DISCUSSION

The major goal in this study was to show that iron-induced reconstitution of iron-deficient *A. nidulans* cells provides a useful

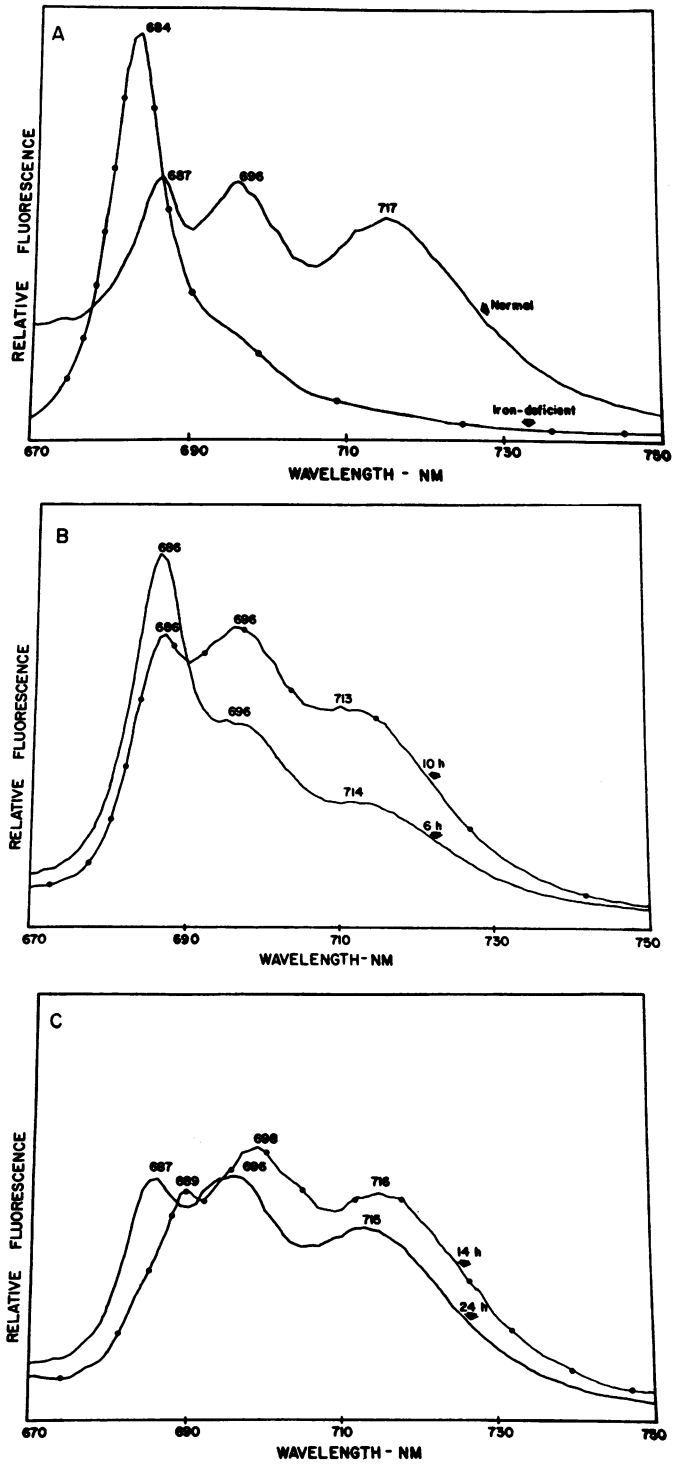

FIG. 1. Fluorescence emission spectra of normal, iron-deficient, and reconstituting *A. nidulans* cells. The spectra were obtained on a SLM 8000 spectrofluorimeter at 77 K and corrected for the response of the detecting photomultiplier tube at different wavelengths. The samples were excited at 435 nm, and the fluorescence intensity is presented in a relative and arbitrary scale. Number of hours next to a trace indicates the time after the addition of iron to an iron-deficient culture. A, Spectra of normal and iron-deficient cells; B, spectra of reconstituting cells 6 and 10 h after the addition of iron; C, spectra of reconstituting cells 14 and 24 h after the addition of iron.

developmental system to study the assembly of the photosynthetic apparatus in the thylakoid membrane. Recently, Nishio and Terry (17) have shown that in sugar beet plants, iron-induced recovery of iron-starved plants provides a system for the study of chloroplast development. Similarly, our studies have shown that the addition of iron to iron-starved cyanobacterial cells initiates a developmental process in the synthesis of photosynthetic membranes (25) and the assembly of their functional complexes. There are two stages in this iron-induced restoration process: (a) different components of the thylakoid membranes are synthesized, and then inserted into the membranes during the first 12 h, and (b) new membrane synthesis and cell division begin between 12 and 24 h (see Sherman and Sherman [25]). The reconstitution process is essentially complete by 24 h when almost all the cells regain their full complement of thylakoid membranes.

During the first stage of recovery, the pigment levels in the cells start changing almost immediately after the addition of iron. During the first 6 h, phycocyanin was preferentially synthesized as compared to Chl, and Chl synthesis proceeded at a moderate rate during the first 12 to 14 h (Table I). Since iron-deprivation is known to inhibit the synthesis of Chl in higher plants (22), the addition of iron might have helped in the formation of precursors in the biosynthetic pathway of Chl or phycobilins. Room temperature absorption studies indicated that the blueshifted Chl absorption peak at 672 nm gradually moved to its normal position at 679 nm within 24 h after the addition of iron to iron-starved cells (Table I). Discrete changes took place between 10 and 14 h, indicating that the structural organization of the bulk of the membrane Chl changed significantly during this time.

Analysis of the low temperature (77K) fluorescence emission spectra of iron-starved and reconstituting cells can provide significant information about the presence and the *in vivo* organization of Chl-containing complexes inside the thylakoid membranes (24). For example, Katoh and Gantt (13) have indicated that most, if not all, of the fluorescence at 687 nm originates from allophycocyanin B, the terminal pigment in phycobilisomes. On the other hand, Rijgersberg (23) has shown that most of the fluorescence in the 687-nm region originates from the Chl molecules associated with PSII. Iron-starved *A. nidulans* cells had a very low level of phycocyanin (Table I), and very few phycobilisomes per unit membrane area (25). However, excitation at 435 nm, a wavelength absorbed exclusively by Chl generated a very pronounced emission peak at 684 nm (Fig. 1A). These data argue against allophycocyanin B being the only origin of the 684-nm peak.

Even more controversial is the origin of the emission peak at 696 nm. Breton (4) has recently proposed that fluorescence at 696 nm originates from pheophytin a, the primary acceptor molecule in PSII reaction centers. According to his hypothesis, excitation energy does not return to the PSII antenna pigment bed after charge recombination at the reaction center, and, thus, the antenna Chl have no role in the fluorescence at 696 nm. Data in this report, on the other hand, suggest that the presence of the antenna bed may be important for 696 nm emission. During recovery from iron-starved conditions, the emission in the 695-nm region became most prominent between 10 and 14 h (Fig. 1, B and C). Electrophoretic analysis of the Chl-protein complexes from reconstituting cells indicated that stable, high mol wt complexes, *e.g.* CP I and CP II, were formed around this time (Table II). This correlation between the *in vivo* fluorescence and the *in vitro* Chl-protein data suggests that the presence of large Chl-protein aggregates is required for much of the fluorescence emission at 696 nm (8). Larkum and Anderson (14) have recently shown that proteoliposomes containing either PSII core complexes or LHC units from higher plant thylakoids give rise to 696 nm fluorescence emission only when the ratio of proteins

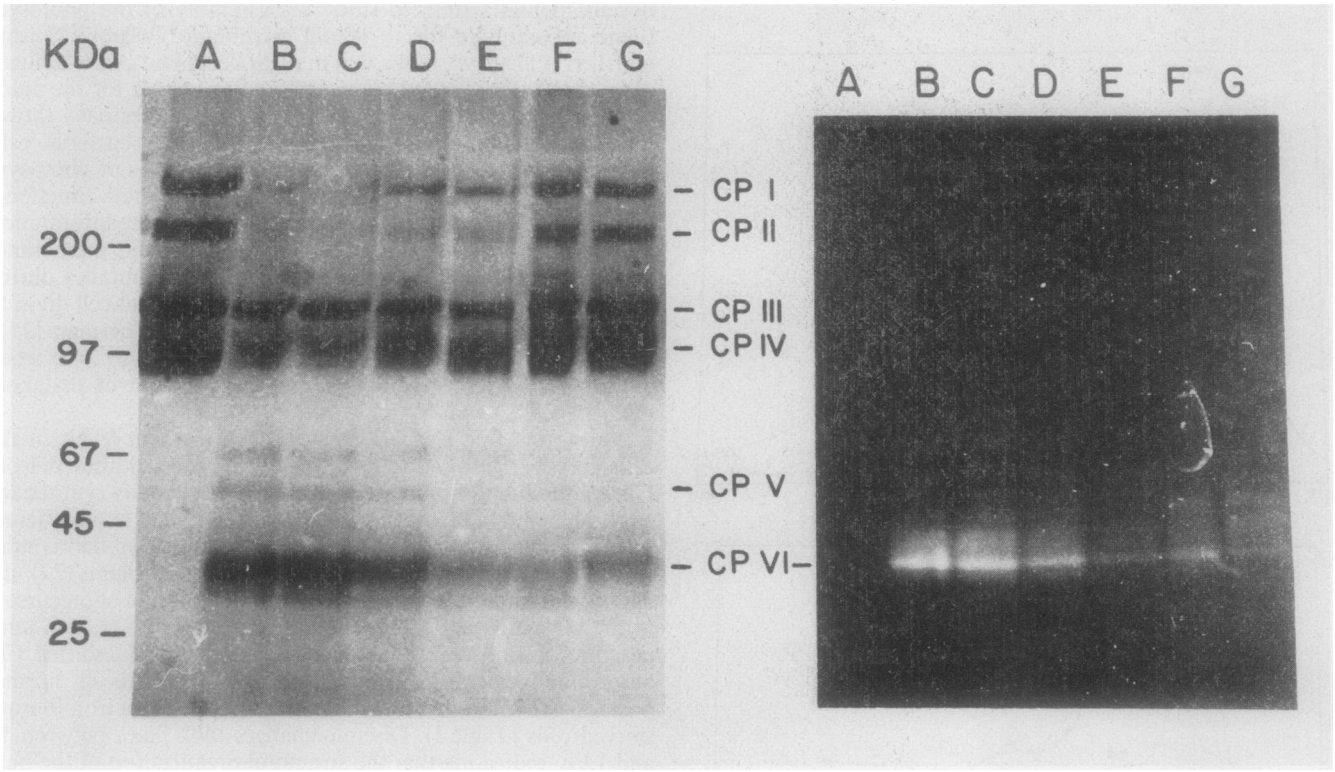

FIG. 2. Electrophoretic profiles of the CP complexes of membranes from normal, iron-deficient, and reconstituting *A. nidulans* cells. Membranes were solubilized in LDS and electrophoresed on a 5 to 15% polyacrylamide gradient gel. The CPs are labeled as CP I–VI. The panel on the left shows the profile of green Chl-protein bands, whereas the panel on the right shows the profile of the fluorescent bands when the same gel is transilluminated with UV light. Lanes A, normal; B, iron-deficient; and C to G are reconstituting cells at 6, 9, 12, 18, and 24 h after the addition of iron, respectively.

Table II. *Analysis of CP Complexes*

CP complexes from thylakoid membranes were analyzed as described in the legend to Figure 2. Individual lanes from gels were cut out and scanned at 670 nm for Chl-containing bands. The area under each peak was measured and used as an indicator of the amount of the Chl in each band. The numbers in the table indicate the percentage of Chl in the band compared to the total Chl in that lane.

Sample	Time after Addition of Iron	CP Bands					
		I	II	III	IV	V	VI
Normal		22	12	36	30		
Iron-deficient	0	5		40	19	6	30
	6	5		38	20	7	30
	9	17		35	28	3	17
	12	22	7	32	27		12
	18	24	11	31	25		9
	24	25	12	33	28		2

to lipids is high. The role of pheophytin in the fluorescence emission at 696 nm could not be analyzed in our experiments. However, recovery of iron-deprived cells, especially around 14 h after the addition of iron, provides a unique situation where the predominant fluorescence emission from intact cells is at 696 nm. It will be interesting to determine if pheophytin molecules are oriented differently inside the membrane during this time.

Analysis of Chl-protein complexes and their fluorescence emission during iron-induced recovery revealed some important information about the changes in the organization of the Chl molecules within the thylakoid membranes. As shown in Table II, the relative amount of lower mol wt CP V and CP VI bands decreased between 9 and 12 h after the addition of iron, and there was a parallel increase in the amount of high mol wt CP I and CP II during this time. Work done by Ohad and his coworkers (6, 18) on light-induced regreening of dark-grown *Chlamydomonas reinhhardii* cells suggests that each photosystem has three main functional parts: the reaction center, the main light-harvesting antenna, and an interconnecting or immediate antenna that links the light-harvesting complex with the respective reaction center. Similar to the iron-deprived *A. nidulans* cells, dark-grown *C. reinhardii* cells have a single uncorrected fluorescence emission peak at 682 nm, and their Chl-protein profiles show the absence of high mol wt bands. During light-induced recovery, higher mol wt CP bands are formed along with the fluorescence emission peaks at 696 and 716 nm. To explain this phenomenon, the authors suggested that the interconnecting antennae and their constituent polypeptides are formed during the regreening process. Similar kinds of synthesis and assembly processes might take place during the recovery of iron-deficient cells. Analysis of the synthesis of proteins in *A. nidulans* R2 cells during iron-induced recovery suggests that certain small mol wt polypeptides that are synthesized rapidly between 7 and 13 h after the addition of iron may be candidates for the apoproteins of these immediate antennae (10).

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