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Reorganization of the Photosystem II Unit in Developing Thylakoids of Higher Plants after Transfer to Darkness[1]

CHANGES IN CHLOROPHYLL *b*, LIGHT-HARVESTING CHLOROPHYLL PROTEIN CONTENT, AND GRANA STACKING

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ABSTRACT

A light-dependent reversible grana stacking-unstacking process, paralleled by a reorganization of thylakoid components, has been noticed in greening etiolated bean (*Phaseolus vulgaris*, var. red kidney) leaves upon transfer to darkness. The reorganization, based on biochemical and biophysical criteria, involves mainly the photosystem II (PSII) unit components: upon transfer to darkness, the light-harvesting chlorophyll protein (LHCP), its 25 kilodalton polypeptide and chlorophyll *b* are decreased, while the CPa and its 42 kilodalton polypeptide are increased and new PSII units of smaller size are formed. This reorganization of components occurs only in thylakoids still in the process of development and not in those present in steady state conditions.

It is proposed that this process does not reflect the turnover of the LHCP component *per se*, but a regulatory process operating during development, by which the ratio of light-harvesting to PSII reaction center components, determined by the environmental conditions, controls the photosynthetic rate.

The assembly of functional and structural components in the thylakoid during chloroplast development follows a step wise process (2, 20). For full growth and assembly of the membrane, exposure of the etiolated tissue in the light for a certain period of time is necessary, after which the thylakoid acquires all the characteristics and properties of the fully mature photosynthetic membrane.

Under conditions where the thylakoid is still in the process of development, its components seem to undergo a process of reorganization. Such a reorganization has been noticed earlier under certain experimental conditions: (a) in young etiolated leaves exposed to intermittent light for long periods of time after their transfer to CL[2] in the presence of the protein synthesis inhibitor chloramphenicol (5); (b) in etiolated leaves exposed to periodic light or to 2 h CL and transferred to darkness for some hours (1). In the first case (5), it was found that even though net Chl synthesis can no longer occur, the Chl *a*/Chl *b* ratio drops, new LHCP are detected in the thylakoid, the PSII unit size increases, and the PSII activity per mg Chl decreases. These results were proposed to reflect a reorganization of the PSII unit components: some units being destroyed (PSII activity per mg Chl decreased) and new ones organized (increased LHCP incorporation increasing the PSII unit size) (5). In the second case (1), it was found that upon transfer of 2 h CL leaves or intermittent light leaves to darkness the F_{max}/F_o as well as the DPC-DCIP activity per mg Chl increased in darkness by about 30% to 70%. This was again explained as reflecting the organization of unorganized Chl in new small sized PSII units during the subsequent dark period (1). The rationale behind this proposal was that in those cases where the thylakoid contains greater amount of PSII units than that of the mature green thylakoid (first case [5]) some of the PSII units are destroyed to reach the number found in the green plant; their Chl *a* thus liberated could be used for the growth of the remaining PSII units. On the other hand, in those cases where the content of PSII units is lower than that in the mature thylakoid (second case [1]), their LHCP is destroyed in darkness so that the liberated Chl *a* as well as the Chl *a* still not organized into units, could form new PSII units. This proposal, therefore, predicted that in the cases where the thylakoid contains equal concentration of PSII units as that of the mature thylakoid, no change in the LHCP content of the thylakoid would be noticed upon transfer to darkness or under other experimental conditions.

Recently, it has been shown by Bennett (12) that in etiolated pea leaves exposed to light for 24 h and then returned to darkness Chl *b* and the LHCP complex, accumulated in the light, were unstable in darkness. This was explained as reflecting the turnover of the LHCP protein after transfer to darkness (12). However, these results can also be explained as reflecting the reorganization process occurring in young etiolated leaves, previously described, rather than the turnover of this protein in the dark. To test this hypothesis, we tried to see whether the instability of the LHCP in the dark can be noticed under all experimental conditions or only during development of chloroplasts, where the thylakoid components are under a continuous process of reorganization. We thus compared the photochemical activity and thylakoid composition in young etiolated bean leaves exposed to continuous light for various periods of time and then transferred to darkness. We found that a reorganization can be noticed only in chloroplasts still in the process of development. Under these conditions, the PSII activity (on a Chl basis) increases in darkness by about 100% to 200% and high light intensity is required for saturation, total Chl per leaf is decreased by about 15%, with preferential decline of Chl *b* (60–80%), resulting in a pronounced increase in the Chl *a*/Chl *b* ratio. Parallel to these changes, the Chl distribution among the pigment protein complexes is altered in favor of CPa (the complex considered to originate in the PSII reaction center

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[2] Abbreviations: CL, continuous light; LHCP, light-harvesting chlorophyll protein(s); DCIP, dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; CPa, chlorophyll protein *a*; CPI, chlorophyll *a*-rich P700 pigment-protein complexes; FP, free pigment; MV, methyl viologen.

Table I. *Chl a and b Content of 6-Day Etiolated P. vulgaris Leaves Exposed First to CL and Then Transferred to Darkness (D)*

Chl were extracted with 80% acetone from leaves according to Argyroudi-Akoyunoglou and Akoyunoglou (8), and estimated spectrophotometrically according to MacKinney (16).

Sample	Leaf Fresh Wt.	Chl *a*	Chl *b*	Chl *a* + *b*	Chl *a*	Chl *b*	Chl *a* + *b*	Chl *a*/Chl *b*
	mg		*μg/g fresh wt*			*μg/leaf*		*ratio*
8.5 h CL	32	470.2	113.4	583.6	15.1	3.6	18.7	4.1
8.5 h CL + 25 h D	56	268.3	25.2	293.5	15.0	1.4	16.4	10.6
8.5 h CL + 50 h D	85	182.3	9.9	192.8	15.5	0.8	16.3	18.4
15 h CL	38	531.5	145.4	676.9	20.2	5.5	25.7	3.6
15 h CL + 25 h D	62	328.9	47.5	376.4	20.4	2.9	23.3	6.9
15 h CL + 50 h D	86	222.0	16.6	238.6	19.1	1.4	20.5	13.4
22.5 h CL	57	1047.8	359.1	1406.9	59.7	20.5	80.2	2.9
22.5 h CL + 25 h D	90	644.2	149.1	793.3	58.0	13.4	71.4	4.3
22.5 h CL + 50 h D	119	514.1	88.3	602.4	61.2	10.5	71.7	5.8
51 h CL	83	2671.6	959.6	3631.2	221.7	79.6	301.3	2.8
51 h CL + 25 h D	110	1910.2	605.4	2515.6	210.1	66.6	276.7	3.1
51 h CL + 50 h D	123	1911.7	511.2	2422.9	235.1	62.9	298.0	3.7

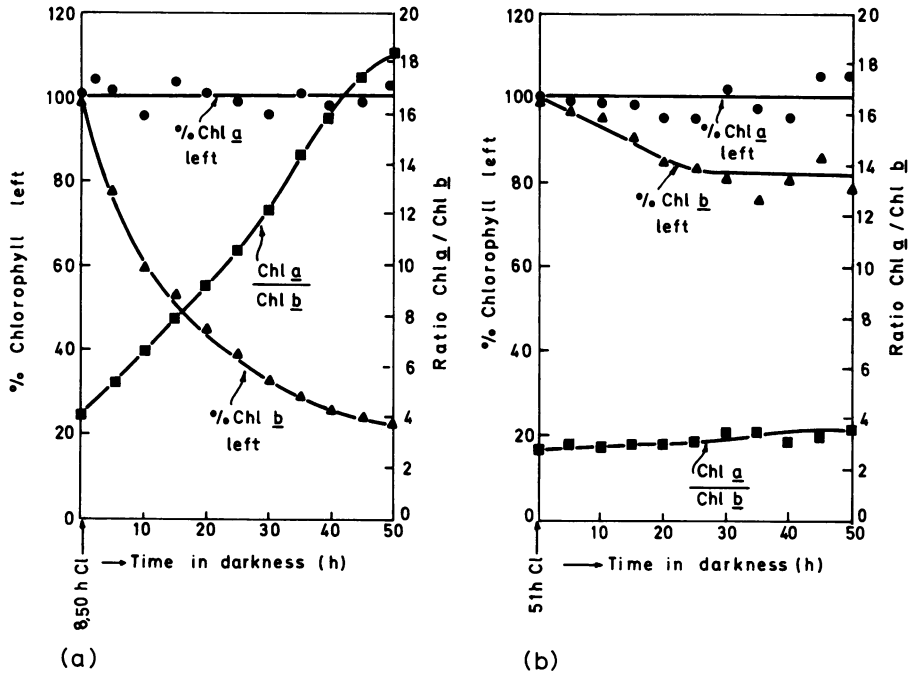

FIG. 1. Chl content in 6-d etiolated *P. vulgaris* leaves exposed to CL for 8.5 h (a), or 51 h (b), and then transferred to darkness. Exhaustive extraction of Chl as in (8) and percent values determined on the basis of the Chl/leaf values.

(6, 10, 13) and slightly in favor of CPI (the Chl *a*-rich P700-pigment protein complex); in the mean time, the LHCP complexes, as well as their 25 kD polypeptide decline drastically. Furthermore, the F_{max}/F_o and the $t_{1/2}$ of the Chl *a* fluorescence kinetics rise increase in darkness. The results, therefore, suggest that new PSII units of smaller size are formed in darkness, making use of the Chl *a* of the unstable LHCP. This instability can not be noticed in thylakoids which acquire the organization of the mature stage.

Parallel to these changes, an unstacking of the grana, preformed during preexposure to continuous light, occurs in darkness, resulting in the appearance of parallel arrays of single lamellae. This unstacking in darkness can not be noticed in chloroplasts exposed to light for more than 60 h.

We conclude that the instability of the LHCP does not reflect the protein turnover *per se*, but rather a control mechanism by which the plant alters the ratio of light harvesting to PSII reaction center components and thus regulates the photosynthetic rate.

MATERIALS AND METHODS

Etiolated bean leaves, *Phaseolus vulgaris* (var. red kidney), grown and handled as before (8) were exposed to CL for different periods of time and were then transferred to darkness.

Chloroplasts were obtained from 4-g fresh weight leaves ground in a Sorval Omnimixer for 15 s at 35% of the line voltage followed by 10 s at 58% (CL samples), or for 5 s at 50% of the line voltage followed by 25 s at 20% (samples transferred to darkness). The homogenization buffer (10 ml/g fresh wt) contained 0.3 M sucrose, 0.05 M phosphate (pH 7.2), 10 mM KCl, and 1 mg BSA/ml. The homogenate was filtered through six layers of cheese cloth and centrifuged for 2 min at 500*g*. Chloroplasts were collected by

Table II. *PSII Activity of Plastids Obtained from 6-Day Etiolated P. vulgaris Plants Exposed First to CL and Then Transferred to Darkness (D)*

The activity was measured as a DPC-DCIP reduction (23) at a light intensity of 300,000 lux, and 3 μg Chl/ml. The plastids prior to determination of activity were washed with Tris (24).

Sample	Leaf Wt.	Chl *a*	Chl *b*	Chl *a*	Chl *b*	DCIP Reduced	
	mg	*μg/g fresh wt*		*μg/leaf*		*μmol/mg Chl·h*	*μmol/leaf·h*
14 h CL	36	592	172	21.5	6.2	526	14.6
14 h CL + 24 h D	60	300.5	42.2	18.0	2.5	1193	24.4
14 h CL + 48 h D	92	222.1	16.6	20.4	1.5	1800	39.4
14 h CL + 48 h D + 24 h CL	128	1427.5	478.8	183.2	61.1	632	154.4
24 h CL	60	895.1	295.1	53.7	17.7	504	36.0
24 h CL + 24 h D	81	640.2	148.0	51.7	12.0	1081	68.8
24 h CL + 48 h D	119	445.0	73.6	53.0	8.8	1660	100.1
24 h CL + 48 h D + 24 h CL	136	1211.9	383.1	165.1	52.2	564	122.5
51 h CL	76	2182.2	858.6	166.4	65.5	392	90.9
51 h CL + 24 h D	91	1703.3	607.6	155.4	55.4	405	85.4
51 h CL + 48 h D	109	1506.3	420.3	163.8	45.7	474	99.3
51 h CL + 48 h D + 24 h CL	125	1490.7	626.6	186.3	78.3	399	105.6

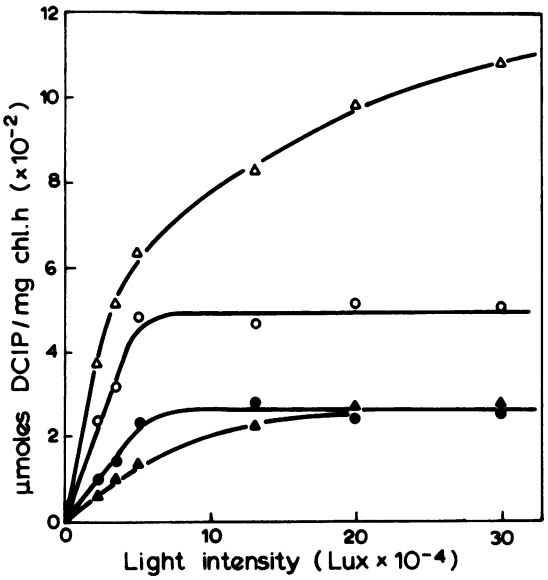

FIG. 2. Light saturation curves for PSII activity of chloroplasts obtained from etiolated *P. vulgaris* plants exposed to CL for 24 h (○, ●) and then transferred to dark for 24 h (△, ▲). (●, ▲), H_2O-DCIP reaction; (○, △), DPC-DCIP reaction in Tris-washed chloroplasts.

Table III. *Chl a Fluorescence Characteristics of Plastids Obtained from 6-Day-Old Etiolated Bean Leaves Exposed First to CL and Then Transferred to Darkness (D)*

Sample	$t_{1/2}$[a]	F_m/Chl	F_m/F_o
	ms		
14 h CL	8.8	44	3.6
14 h CL + 24 h D	19.0	48	4.3
24 h CL	7.0	62	4.2
24 h CL + 24 h D	14.0	48	5.5

[a] DCMU present. Five μg Chl/ml assay mixture. Fluorescence measurements as in "Materials and Methods." I = 550 μw/cm².

centrifuging the resultant supernatant for 10 min at 3,000*g*, and were resuspended in homogenization buffer, supplemented with 10 mg BSA/ml.

PSII activity was determined in chloroplasts prior to or after Tris washing (24), as the rate of DCIP reduction in the presence or absence of DPC (23).

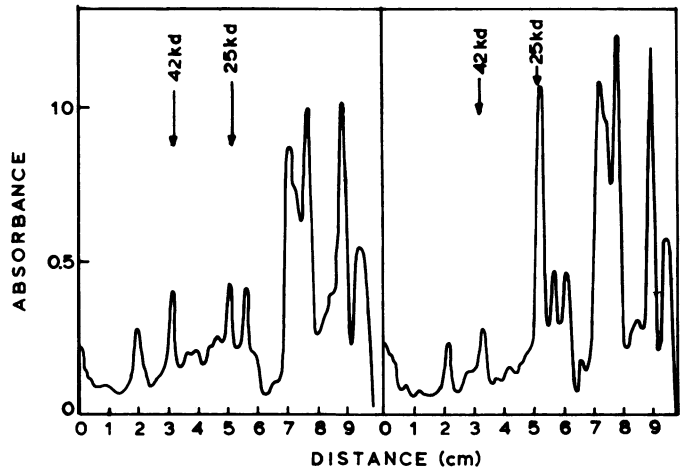

FIG. 3. Profiles of PAGE analysis of thylakoid polypeptides in 6-d etiolated bean leaves exposed to CL for 15 h (right) and then transferred to darkness for 46 more h (left). 400 μg SDS-solubilized thylakoid protein on each gel, after heating for 2 min in a boiling water bath. Electrophoresis in tubes.

Fluorescence measurements were done as previously described (9); the assay mixture (18) contained Tricine-NaOH buffer (pH 7.8), 0.4 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, and 1 mM $MnCl_2$. The Chl concentration was 5 μg/ml assay mixture.

The chloroplasts used for electrophoretic separations were prepared as in Argyroudi-Akoyunoglou and Akoyunoglou (10); the thylakoids were washed twice with 0.05 M Tricine-NaOH (pH 7.3) and solubilized in SDS. Solubilization of thylakoids and electrophoretic analyses of thylakoid polypeptides was done as in Hoober *et al.* (14); solubilization of thylakoids and electrophoretic analysis of pigment-protein complexes was done as in Anderson, *et al.* (6) and Argyroudi-Akoyunoglou and Akoyunoglou (10). Electrophoretic profiles were obtained in a Joyce-Loeble Chromoscan, and the distribution of Chl among the complexes was estimated on the basis of the area under each peak, by weight. Chl was determined spectrophotometrically according to MacKinney (16); it was extracted exhaustively from leaves according to Argyroudi-Akoyunoglou and Akoyunoglou (8).

RESULTS

Table I and Figure 1 show the accumulation of Chl in leaves exposed to light for various time periods and then transferred to

Table IV. *Distribution of Chl among the Pigment-Protein Complexes of SDS-Solubilized Thylakoids, Obtained from Etiolated Bean Leaves Exposed First to CL and Then Transferred to Darkness (D)*

Sample	Distribution of Chl					LHCP/CPa	LHCP/CPI
	CPI	CPa	LHCP	FP	LHCP + FP		
			%			*ratio*	
13 h CL	17.2	6.3	61.5	14.2	75.7	9.76	3.57
13 h CL + 22 h D	24.0	8.3	44.2	19.5	63.7	3.60	1.84
24 h CL	23.4	6.8	57.3	12.5	69.8	8.42	2.44
24 h CL + 22 h D	27.7	11.3	37.2	25.5	62.7	3.29	1.34
58 h CL	18.2	4.8	62.2	16.5	78.7	12.90	3.41
58 h CL + 22 h D	19.7	4.5	64.8	12.9	77.7	14.40	3.28
24 h CL	19.9	4.5	59.1	13.6	72.7	13.00	2.96
24 h CL + 48 h D	20.3	9.0	41.1	31.0	72.0	4.56	2.02
24 h CL + 24 h D + 24 h CL	18.8	4.8	52.0	16.0	68.0	10.60	2.76

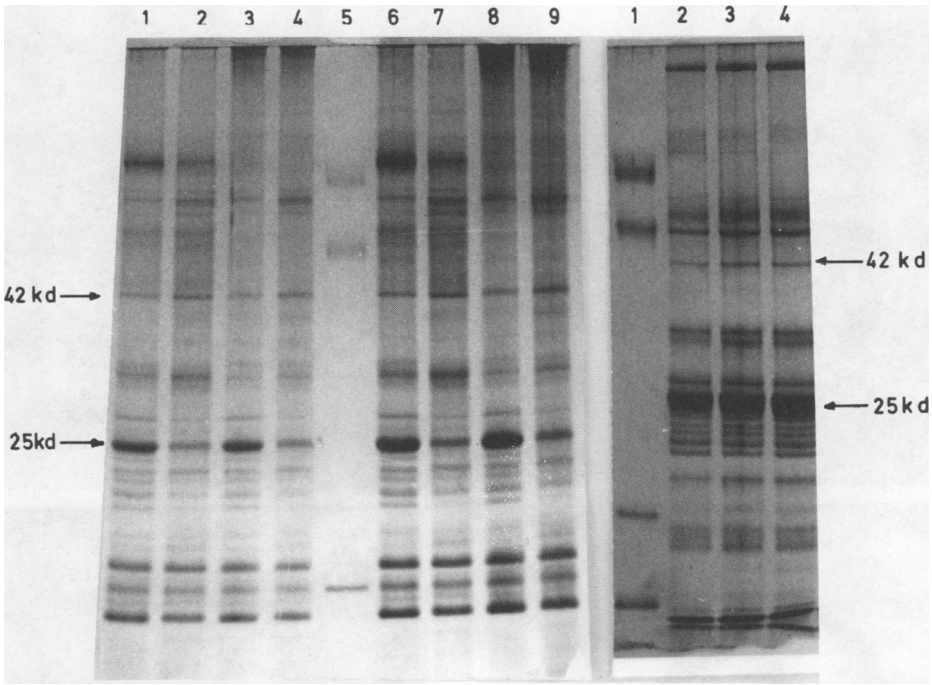

FIG. 4. Slab gel electrophoresis of thylakoid polypeptides of 6-d etiolated bean plants exposed to CL and then transferred to the dark. Left, exposure to CL for 14 h (slots 1 and 3 = 120 μg protein; slots 6 and 8 = 240 μg protein) and then transferred to darkness for 49 h (slots 2 and 4 = 120 μg protein; slots 7 and 9 = 240 μg protein). Samples in slots 1, 2, 6, and 7 were not heated; those in 3, 4, 8, and 9 were heated in a boiling water bath for 2 min. Slot 5, standard proteins (BSA, 67 kD; ovalbumin, 45 kD; myoglobin, 17 kD). Right, exposure to CL for 51 h (slot 2), transferred to darkness for 48 h (slot 3), and again exposed to CL for 24 h (slot 4). 120 μg protein on each slot. Slot 1, standard proteins (BSA, 67 kD; ovalbumin, 45 kD; myoglobin, 17 kD; and Cyt *c*, 13 kD). Polyacrylamide in the slab was 12.5%.

darkness. The results suggest that in the dark the leaves continue to expand and grow, the Chl *a* content per leaf remains constant, but that of Chl *b* decreases sharply. Thus, the Chl *a*/Chl *b* ratio increases drastically. The effect is more pronounced when the leaves are transferred to darkness, after short preexposure to CL. In cases where the preexposure to light is prolonged, the changes become smaller, or barely noticed, and the Chl *a*/Chl *b* ratio does not change.

Figure 2 and Table II show the PSII activity of the plastids under these conditions. The PSII activity (DPC-DCIP) per mg Chl or per leaf increases 2 to 3 times after transfer from light to dark. Since this occurs with no net Chl synthesis per leaf, as shown in Table I, the results suggest that the Chl *a* in the dark transferred plants is organized into new PSII units as well. These new PSII units seem to be of smaller size than those present in the leaves prior to transfer to darkness. This is evident from the light saturation curve of PSII in the two samples. As shown, the PSII activity of the plastids of leaves transferred to darkness (DPC-DCIP reaction) saturates at higher light intensities than that of plastids of leaves prior to transfer. The H_2O-DCIP activity, however, at high light intensity is equal in both samples. This was expected, since, as it has been previously shown (3, 15, 19, 21), the water-splitting enzymes, or their organization into active PSII units, are light-induced or activated. Thus, the H_2O-DCIP activity measures only these units of PSII that have H_2O splitting capacity (*i.e.* the ones formed in the light). The new units formed in the dark, on the contrary, have no such capacity, since their water-splitting enzymes require some time in the light for their activation. The new PSII units that are formed in the dark, therefore, are small in size and inactive in water splitting.

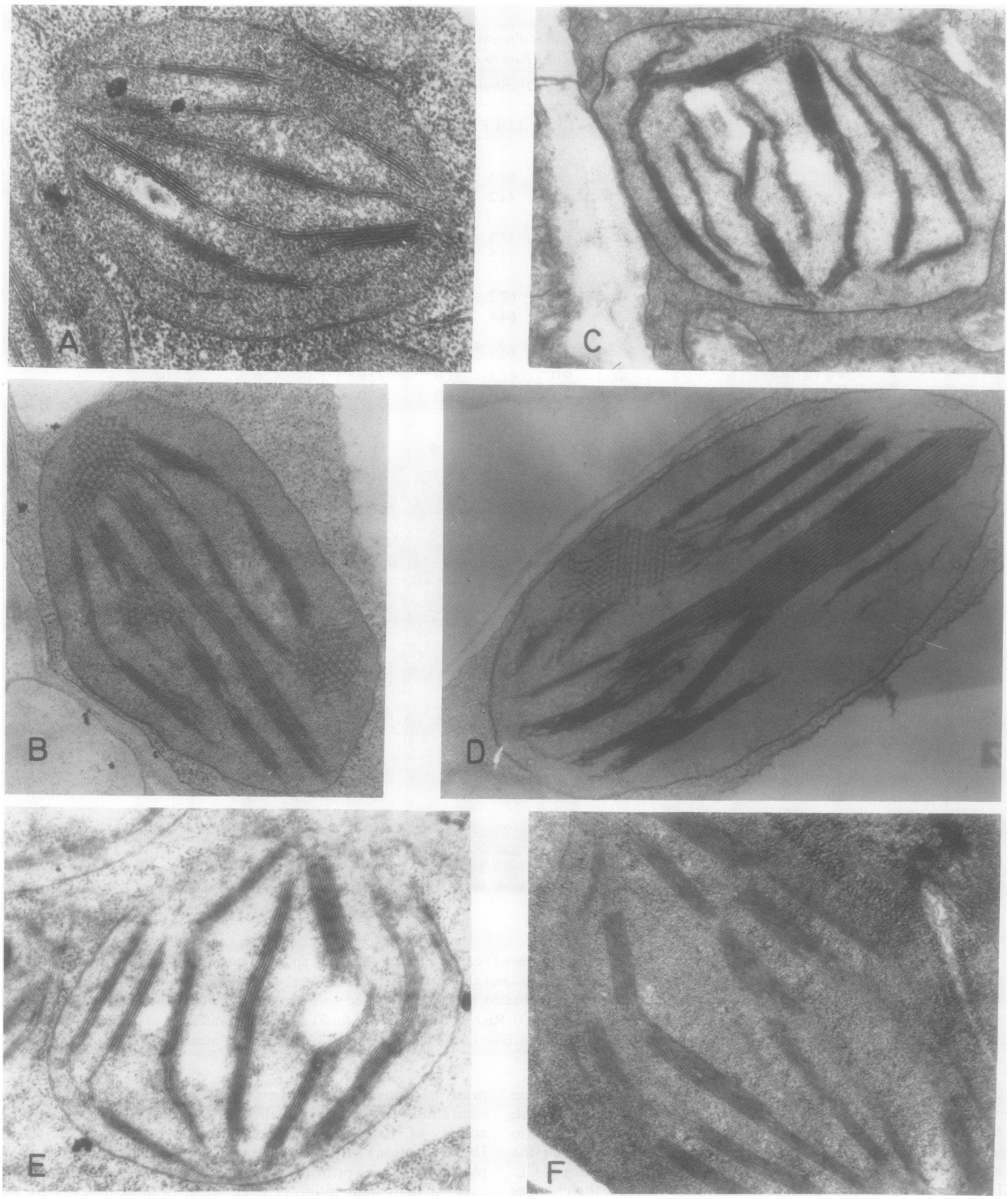

FIG. 5. Electron micrographs of chloroplasts present in 6-d etiolated bean leaves exposed to CL (A, C, E) and transferred to dark (D) (B, D, F). A, 13 h CL; B, 13 h CL + 48 h D; C, 24 h CL; D, 24 h CL + 60 h D; E, 60 h CL; F, 60 h CL + 60 h D. ×24,000; except in E where magnification was ×32,000. Electron micrographs obtained as in (4).

That the newly formed PSII units in the dark are of smaller size, is also supported by the fluorescence measurements. Table III shows representative results. It is evident that the F_m/F_o, as well as the $t_{1/2}$ of the fluorescence induction kinetics increase in the plants transferred to darkness. This indicates that the PSII units formed in the dark are smaller in size.

All these suggest that a reorganization of the PSII components occurs in thylakoids transferred to darkness after a short preex-

posure to CL, in which the LHCP and Chl *b* decrease, the Chl *a* liberated from LHCP is used for the formation of new PSII units (mainly the core of the units), and the average PSII unit size becomes smaller. This occurs only in plants transferred to darkness after short preexposure to CL and not after a long one.

To further check these possibilities, we estimated the pigment distribution among the pigment-protein complexes. As shown in Table IV, the LHCP decline in the leaves transferred to darkness after a short preexposure to light, while the relative amount of the CPa band is greatly increased. Thus, the LHCP/CPa ratio drops from a value of 10 to about 3. This effect is again less pronounced in samples preexposed to CL for longer time.

The effect of dark incubation on the decrease of the LHCP content in thylakoids is also reflected in the polypeptide composition shown in Figures 3 and 4. In Figure 3, the thylakoid polypeptide pattern of the leaves exposed to light for 14 to 15 h and those transferred to darkness is shown. As it is evident, the 25 kD polypeptide, component of LHCP, is completely reduced by the dark incubation. The 40 kD polypeptide (considered to be the component of CPa), on the other hand, is relatively increased. Parallel to the decrease of the 25 kD polypeptide, we also noticed a decrease in the 23 and 32 kD polypeptides in the dark.

The effect of dark incubation on the 25 kD polypeptide diminishes as exposure to light, prior to dark transfer, is prolonged. Thus, Figure 4 shows that in thylakoids of leaves exposed to 51 h CL and then transferred to darkness, the 25 kD polypeptide remains unaltered.

Since the LHCP content declines after the transfer of the leaves to darkness, and taking into consideration that the LHCP may act as the adhesive force holding the grana thylakoids into appressed stacks, we further studied the chloroplast appearance in the electron microscope, prior to or after transfer to darkness. Dark incubation results in unstacking of preexisting grana, as long as the transfer from light to dark occurs early in development (Fig. 5). Thus, one can find conditions where no grana stacks are present at all (13 h CL + 48 h D; Fig. 5B), or conditions where no morphological changes in the grana structures can be noticed (60 h CL + 60 h D; Fig. 5F).

DISCUSSION

The reorganization of thylakoid components noticed earlier under certain conditions during chloroplast development has been noticed again in this study. It became evident that the 'destruction' or instability of LHCP and Chl *b* during the dark incubation, as well as the reorganization of thylakoid components occurring under these conditions, can be demonstrated only in thylakoids still in the process of development. Early, during greening in continuous light, the thylakoids contain reduced amount of normal (large in size) PSII units, and thus, in order to increase their number in the dark, the reorganization process is set on; this mechanism involves destruction of LHCP and Chl *b* in the dark and reuse of the Chl *a* in the formation of new PSII units so that more reaction center components are now present with less LHCP.

Later, during greening in CL, when more PSII units have been formed, the reorganization in the dark is much smaller; and finally, when thylakoids of completely mature chloroplasts are transferred to darkness, no such reorganization is required since the number and size of their PSII units is that of the mature chloroplast. Thus, the shortest the preexposure to CL, the largest the number of new PSII units which are formed after transfer to darkness. The instability of LHCP, therefore, cannot be considered as reflecting the turnover of the protein *per se* (12), but rather a mechanism of the plant by which the ratio of LHCP/reaction center components controls the size and number of the PSII units.

The decrease in the size of the PSII units in the dark does not occur in all PSII units, but only to a proportion of them, and a mixture of small and large units seems to be present. This is clearly reflected by the results of the fluorescence characteristics and the light saturation curve of the PSII activity: saturation indicates very small PSII units in the dark (high light intensity required for saturation), while the $t_{1/2}$ of the fluorescence induction kinetics shows that the size of the PSII unit is half as that of the mature chloroplast. Experiments in our laboratory have shown that, when chloroplasts isolated from green leaves (which contain large PSII units) are mixed with plastids isolated from intermittent light leaves (which contain small PSII units) (1), then the light saturation curve of the PSII activity of the mixture is similar to that of the intermittent light plastids, but the $t_{1/2}$ reaches a value close to that of the mature chloroplast.

The dark effect found on the *in vivo* synthesis of the translatable mRNA for the polypeptide of the Chl *a*/*b*-protein complex, seems to be in agreement with the findings of this study (22). It was found that the amount of this translatable mRNA decreases relative to other mRNA, when *Lemna gibba* plants are placed in darkness.

The fate of the 25 kD polypeptide after transfer of the plants to darkness, as well as that of Chl *b*, remains to be studied. The instability of these components, however, reflect a process by which grana unstacking occurs in the dark. Formation of LHCP and grana stacking are clearly correlated during greening of higher plants (11), and LHCP has been proposed to act as the adhesive force holding the grana lamellae together. The process of grana stacking seems to be a reversible one, as long as the cementing components undergo a reversible synthesis and destruction. Further studies on this line are required to understand this reversible stacking-unstacking process. In addition, our results suggest that the inability to detect any Chl *b* or LHCP in thylakoids of leaves exposed for short periods to intermittent light (8, 10) may be due to the instability of both components during the 98-min dark phase of the cycle (2 min light/98 min dark); the deficiency of Chl *b* and LHCP in these plants probably underlies the need of the plant to form reaction center components, using the reduced amount of Chl present under these conditions.

Preliminary work on the effect of dark transfer on the activity of PSI, expressed as the rate of O_2 uptake in a DCIPH$_2$-MV reaction, and as P700/Chl ratio, in the chloroplast samples of leaves prior to or after transfer to darkness, shows that the PSI is also changing during the transfer (Table V). In the dark-transferred plants, the PSI activity at high light intensity is increased by about 1.5 times, as compared to that in the chloroplasts of plants prior to transfer. Similarly, the P700/Chl ratio in the dark transferred plants is twice as high as that in the plants prior to

Table V. *PSI Activity, P700/Chl Ratio, and Chl a/Chl b Ratio in Plastids of 6-Day Etiolated Bean Plants Exposed First to CL and Then Transferred to Darkness (D)*

PSI activity was determined as the rate of O_2 uptake in a DCIPH$_2$-MV reaction as in Reference 7. The assay mixture contained in a final volume of 3 ml: 10–20 μg Chl, 2 mM NaN_3, 0.2 mM MV, 0.01 mM DCMU, 0.2 mM DCIP, 5 mM Na ascorbate, and 2.8 ml buffer (7). P700 was determined by the absorption difference of the ferricyanide oxidized minus ascorbate reduced samples (17), on the basis of $\Delta\epsilon = 64$ meq^{-1} cm^{-1}.

Sample	Chl*a*/Chl*b*	O_2 Uptake	P700/Chl
	ratio	*μeq/mg Chl·h*	*ratio*
14 h CL	2.5	330	1:500
14 h CL + 24 h D	7.2	530	1:300
23 h CL	2.5	342	1:550
23 h CL + 24 h D	4.7	507	1:360
Green	3.0	495	1:500
Green + 24 h D	2.9	500	1:590

transfer. Here again the effect is pronounced in plants preexposed to short CL treatment, and can not be noticed at later stages of greening. We do not know yet whether the two photosystem activities (PSI and PSII) stop from changing at the same time or not, and whether they stop independent from each other.

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