Thylakoid Polypeptides of Light and Dark Aged Chloroplasts[1, 2]

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ABSTRACT

Spinach (*Spinacia oleracea*) chloroplasts were aged at 4°C under red light and in the dark. The electron transport activity was monitored together with the thylakoid polypeptide patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The light-induced decay of photosystem II (PSII) activity (half-life, about 4 hours) was correlated with a decrease in polypeptides with apparent molecular weights of 36, 48, and 50 kilodaltons. There was very little decay of photosystem I (PSI) activity until after 8 hours illumination. Prior freezing of the chloroplasts enhanced the decrease in PSI activity which was correlated with chlorophyll-protein complex I (CPI) disappearance and an increase in a polypeptide with apparent molecular weight of 60 kilodalton. No variations were detected in the light-harvesting chlorophyll *a/b* protein. In the dark, the decay of PSII started at 4 to 6 hours and showed a half life of about 30 hours. PSI activity decay (half life about 6 days) occurred simultaneously with the disappearance of CPI. The use of bovine serum albumin (30 mg/mg of chlorophyll) in the light-induced decay experiments increased the stability of PSII more than 2-fold; in the dark experiments, the stability of both photosystems was also more than doubled and the stability of the CPI complex was considerably improved. Comparative electrophoresis of the purified proteins indicated no changes in the cytochrome *f* band or in the subunits of the ATPase coupling factor during the light-induced decay experiments. Heating of purified PSI particles prior to electrophoresis showed that the 60 kilodaltons polypeptide increased with the disappearance of CPI.

It is well known that chloroplasts lose their electron transport and O_2 evolution activities rapidly after isolation. Several authors have tried to stabilize electron transport activity using different buffer systems (8), high osmotic strength media with sugars (13, 15) adding proteins like BSA which are able to chelate free fatty acids (29) or egg albumin (12) which acts as an antimicrobial agent, or treatments with cross-linking reagents (for a review see [21]).

At present, there are only a few reports trying to correlate degradation of the thylakoid membrane and the events which lead to the inactivation of electron transport during aging. Siegenthaler (26) has shown that aging is correlated with liberation of unsaturated fatty acids from the membrane which in turn induced inhibition of both photosystem activities. Although it was shown by Hoshina *et al.* (11) that lysolecithins are liberated along with free fatty acids and are powerful detergents that disintegrate chloroplasts into small particles, no specific site of inactivation is known in the membrane which causes PSII or PSI inhibition.

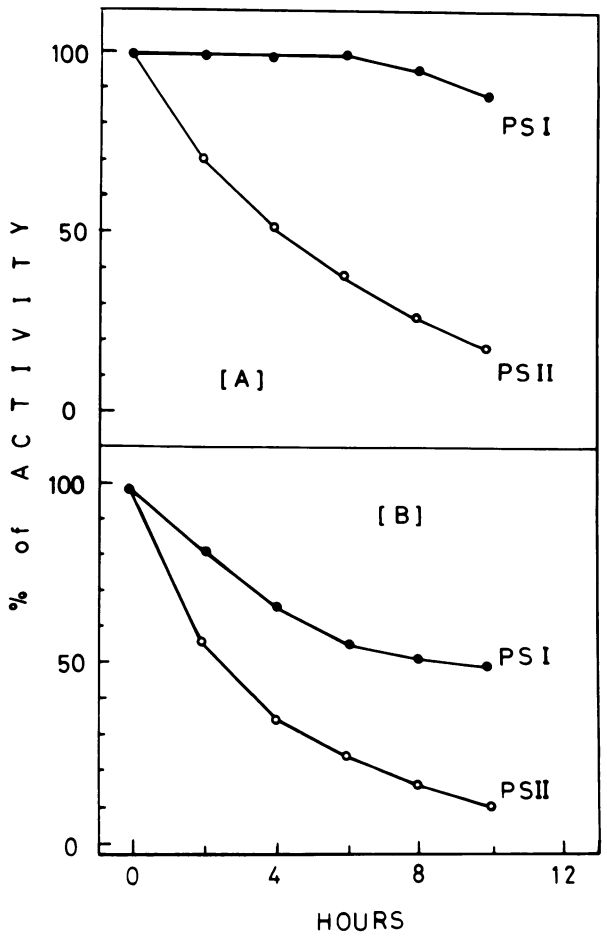

FIG. 1. Stability of PSII activities under saturating red light. Fresh (A) and frozen (B) chloroplasts (0.5 mg Chl/ml). Activities were measured as O_2 uptake or evolution. PSI (DCPIP red → MV). PSII (H_2O → PD_{ox}).

Interestingly, Hoshina *et al.* (11) found that the free fatty acid levels did not increase under light and also that the supposed lipid peroxidation is not the cause of photoinhibition of photosystem activities.

SDS-PAGE[3] has been demonstrated to be an excellent tool in the study of the chloroplast membrane composition and identification of photosystem reaction centre components in some organisms (20). Despite studies with mutants and enriched PSI and PSII fractions in higher plants, which showed correlation between

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[2] Part of this work was presented at the 5th International Congress of Photosynthesis at Halkidiki, Greece, September 1980 (Abstract p. 496).

[3] Abbreviations: PAGE, polyacrylamide gel electrophoresis; DCPIP, 2,6-dichlorophenolindophenol; FeCy, potassium ferricyanide; PD, *p*-phenylenediamine; MV, methyl viologen; DPC, sym-diphenylcarbazide; kD, kilodalton; TMPD, *N,N,N',N'*, tetramethyl *p*-phenylenediamine; EPR, electron paramagnetic resonance; CPI, chlorophyll-protein complex I; CF_1, coupling factor.

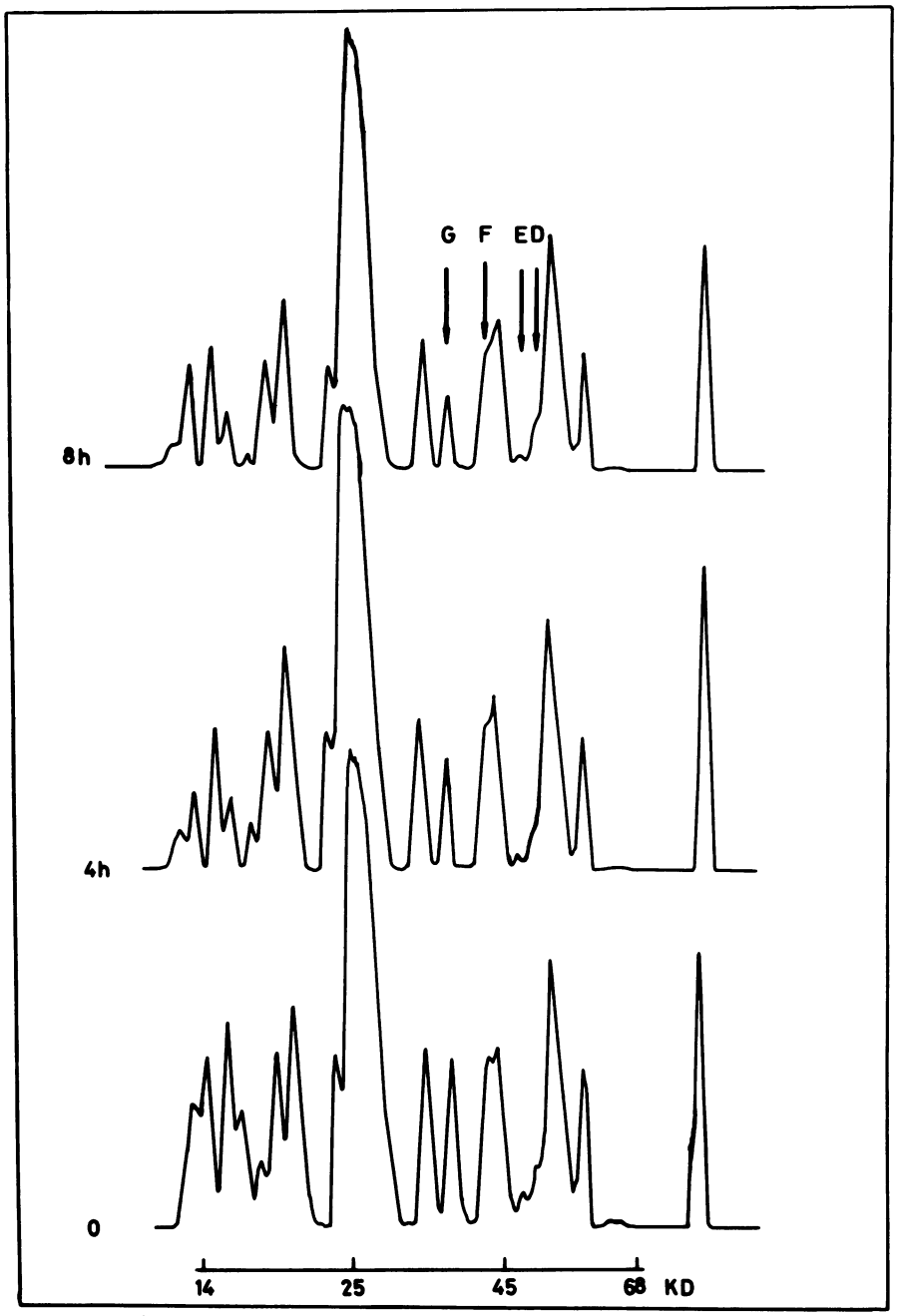

FIG. 2. Polypeptide pattern of fresh chloroplast membranes in SDS-PAGE after exposure to saturating red light for 0, 4, and 8 h at 4°C.

a Chl protein complex (CPI) and PSI reaction center (13, 25), there are only few indications of the components of the PSII reaction center (18, 24).

Our work shows that the loss of PSII and PSI activities is correlated with the disappearance of specific polypeptides as visualized by SDS-PAGE, and also that the decay of PSI occurs simultaneously with a decrease in the amount of CPI.

MATERIALS AND METHODS

Chloroplast Isolation. Spinach leaves (*Spinacia oleracea*) grown in our greenhouse or bought in the market were exposed for 30 min in an ice bath to white light (2.5×10^5 erg cm^{-2} s^{-1}) prior to extraction (23) to yield type A (9) intact chloroplasts. The isolated chloroplasts were resuspended in a small volume (approximately 1 ml) of 2 mM EDTA, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 0.33 M sorbitol, 50 mM Hepes buffer (pH 7.5). When indicated, BSA (fatty acid free) was also added (30 mg/mg of Chl). Chl estimates were performed by the method of Arnon (2).

Photosystem Activities. These were measured under saturating red light (1.2×10^6 erg cm^{-2} s^{-1}) as O_2 uptake or evolution in an O_2 electrode (Rank Brothers, Cambridge, England), at 20°C. Chloroplasts (100 μg Chl) were broken by suspension in 2 ml of water for 30 min, then 2 ml of twice-concentrated medium was added in order to give a final concentration (4 ml) of 0.33 M sorbitol, 5 mM $MgCl_2$, 2 mM EDTA and 50 mM Hepes (pH 7.5).

PSII activity was assayed with 0.75 mM PD oxidized with 2.5 mM FeCy. PSI activity was measured with DCMU (5 μM), MV (50 μM), NaN_3 (2 mM) using DCPIP (50 μM) reduced with ascorbate (2 mM) as electron donor. NH_4Cl (5 mM) was added to both reaction mixtures.

Electron transport from DPC to DCPIP and H_2O to DCPIP were assayed at 580 nm in a Shimadzu—MPS 50L (Kyoto, Japan)

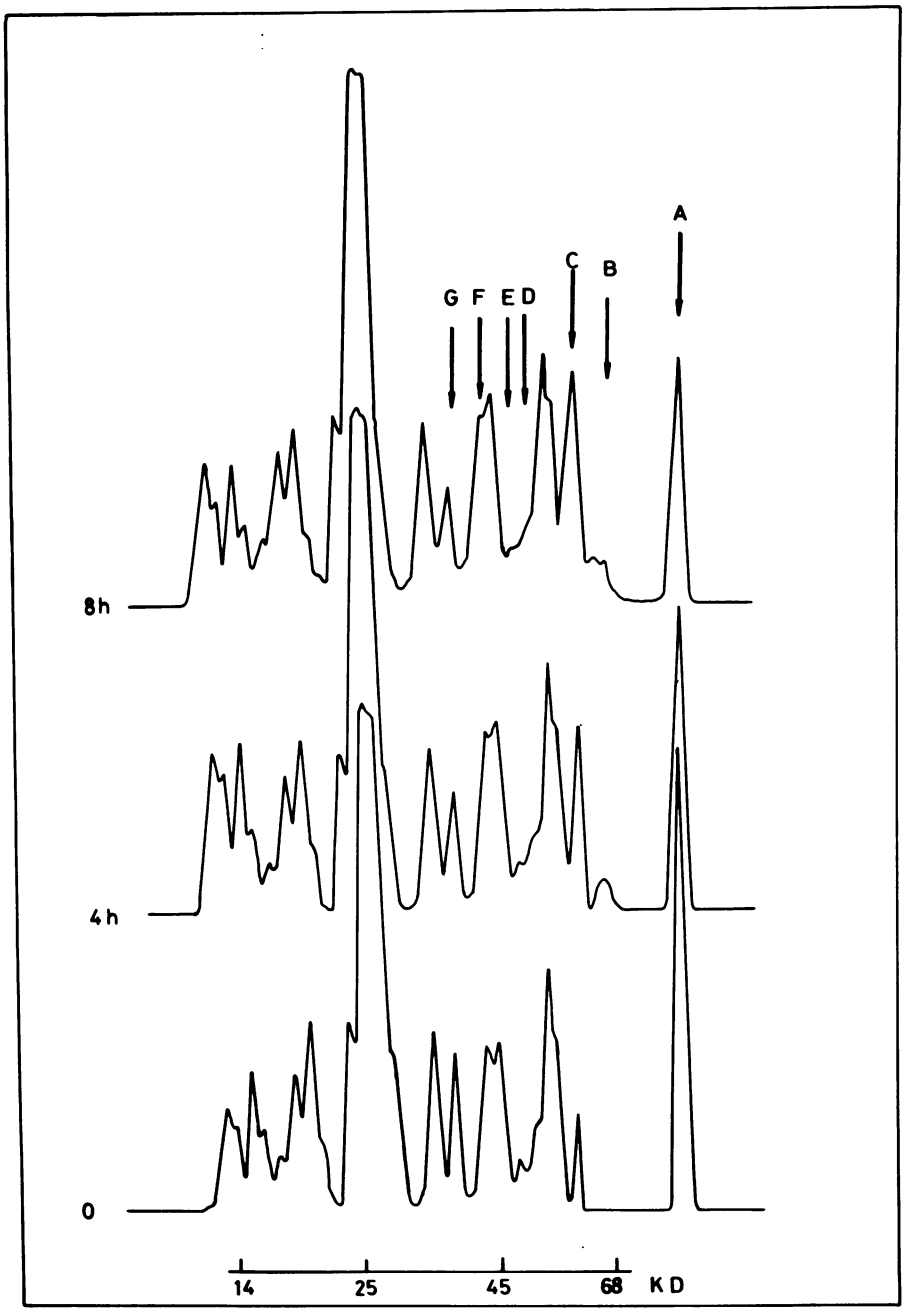

FIG. 3. Polypeptide pattern of frozen chloroplast membranes in SDS-PAGE after exposure to saturating red light for 0, 4, and 8 h at 4°C.

dual beam spectrophotometer modified for lateral illumination. Light from a 300 w slide projector was passed through a Barr and Stroud red filter (RG-645, cut off at 645 nm) and then conducted by a 1 m light guide (Barr and Stroud LG-5) to the cuvette (light intensity at the cuvette surface was 2×10^5 erg cm^{-2} s^{-1}). The photomultiplier was protected by a blue filter (Corning Glass 9780). Final concentrations in the cell were DCPIP (20 μM), DPC (300 μM), NH_4Cl (1 mM), Hepes (pH 7.0) (50 mM), $MgCl_2$ (5 mM), and EDTA (2 mM), plus 2 μg Chl/ml.

SDS-Gel Electrophoresis. This was performed basically as in Chua and Bennoun (5) with the acrylamide gradient used being 7% to 15% (Figs. 7 and 8) or 6% to 12% (Figs. 2–6). Forty μl of chloroplast membranes (1 mg Chl/ml) were washed twice with 1 ml water followed by centrifugation for 5 min at 9,000*g*. The pellet was redissolved in 20 μl 0.1 M DTT with 0.1 M Na_2CO_3 for 20 min then 20 μl of 4% (w/v) SDS, 12% (w/v) glycerol and 0.05% (w/v) bromophenol blue was added and the mixture allowed to stand for 20 min at 20°C.

Electrophoresis was run in a slab gel apparatus (Raven Scientific, Suffolk, England) for 12 to 14 h at constant voltage (50 v) and at room temperature. Mol wt standards used were from Sigma, Poole, England (Dalton VII marker kit). Gels were stained for 1 h in a solution containing 0.25% (w/v) Coomassie blue G-250, 50% (v/v) methanol, and 10% (v/v) acetic acid, and were destained in methanol:acetic acid:H_2O (4:1:5). Scanning was done in a Joyce-Loebl Microdensitometer, Model MK III (Gateshead, England).

Chloroplast Aging. This was performed in the dark or under broad red light (620–1,200 nm) at 4°C (±1°C). The light source was a 150 w tungsten floodlight lamp (Cryselco, London, England) passed through a Cinemoid filter No. 5A (Rand-Strand Electric, London, England). Light measurements were made with a YSI Kettering Radiometer model 65. Chloroplasts (0.5 mg Chl/ml of the suspension medium) were kept in an ice bath with continuous

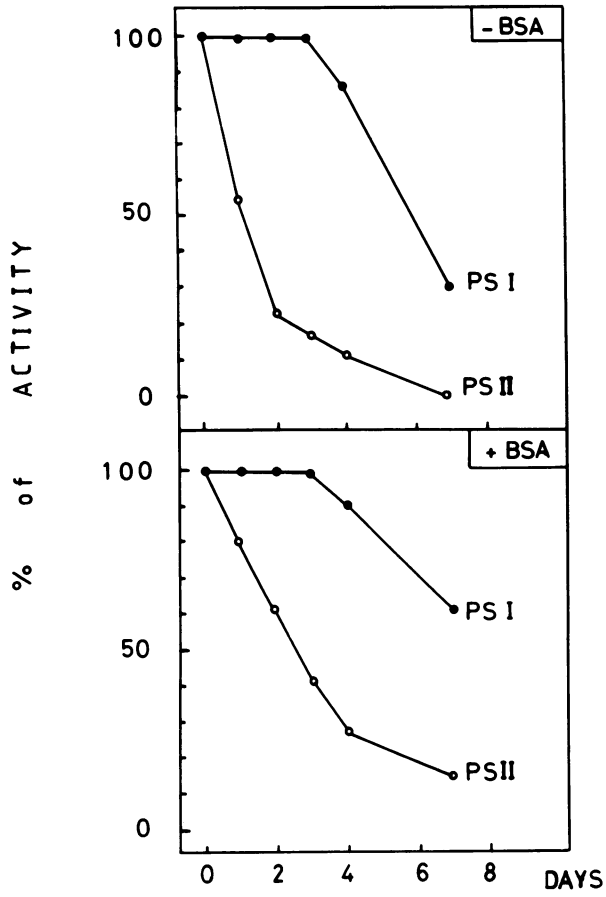

FIG. 4. Storage stability of chloroplast membranes. Chloroplasts (0.5 mg Chl/ml) were kept in the dark at 4°C for the time indicated, with (+) or without (−) BSA (30 mg/mg Chl).

stirring for the light experiments. At the time indicated samples were withdrawn for measurement of electron transport activities and SDS-PAGE analysis.

Protein Isolation. Coupling factor (ATPase) was obtained by EDTA treatment of isolated spinach chloroplasts according to Lien and Racker (17). Cyt *f* was prepared as described by Singh and Wasserman (27) and purified PSI particles were prepared by Triton extraction after Evans *et al.* (6).

RESULTS

Photoinhibition Experiments. Using saturating red light (1×10^6 ergs cm^{-2} s^{-1}) and varying the time of illumination we obtained the PSI and PSII activity decay curves shown in Figure 1A. It can be seen that PSI activity was very stable during the first 7 to 8 h of illumination, with a small decrease (12%) at the end of this experiment (10 h of illumination), whereas PSII activity showed a characteristic decay with a half-life of approx. 4 h. This response varies slightly with chloroplast preparations, reaching only 20% of the initial activity after 10 h of illumination. If MV is used as the electron acceptor (PSII + PSI) instead of PD/Fe Cy (PSII only), the same pattern of PSII decay was observed. We confirmed that the rate of decay of PSII activity under red light is dependent on the light intensity with a maximum decay rate at 8.10^5 ergs cm^{-2} s^{-1} under our experimental conditions (data not shown).

Between the point at which water acts as an electron donor to PSII and the point at which oxidized PD accepts electrons, there are at least two sites where photoinhibition may occur *viz.* the water splitting enzyme itself and the PSII reaction center. As there is some indication (14) that the first site is more sensitive to photoinhibition, we measured the reaction DPC to DCPIP and compared it with the H_2O to DCPIP reaction. Table I shows these two reactions before and after 7 h of illumination, together with the H_2O to PD reaction. It can be seen that the decay of activity (% inhibition) of the three reactions are identical, which indicates that the site of photoinhibition is probably common to the three reactions. Furthermore, the shape of the decay curves are the same as in Figure 1A (data not shown).

Table I. *Photoinhibition of Electron Transport*

Chloroplasts were illuminated with saturating red light for 7 h at 4°C. Activities were measured as described in "Materials and Methods." Electron transport rates are μmol O_2 evolved (or absorbed) or μmol DCPIP reduced mg^{-1} Chl h^{-1}.

Assay	Electron Transport Rates		
	Control	Photo inhibited	Inhibition
			%
PSII			
$H_2O \rightarrow$ DCPIP	180	33	82
$H_2O \rightarrow PD_{ox}$	310	56	82
DPC → DCPIP	208	47	80
PSI + PSII			
H_2O \ MV	240	56	77
DPC → MV	224	56	78
PSI			
$DCPIP_{red} \rightarrow$ MV	304	304	0
$PD_{red} \rightarrow$ MV	370	320	15

Using different chloroplast concentrations (0.3–1.0 mg Chl/ml), and varying the volume of the illuminated suspension between 4 and 8 ml caused no differences in the decay pattern which led us to infer that there were no 'shade effects' limiting light absorption.

The polypeptide patterns derived from the photoinhibition experiments of Figure 1A are shown in Figure 2. It is of interest that concomitant with the loss of PSII activity there was a disappearance of polypeptides with apparent mol wt of 36 kD (G) and two small bands at 48 kD (E) and 50 kD (D). It can also be seen that there was a small decrease of band F (40 kD); however, this decrease was not consistently observed when PSII activity decayed.

Freezing the chloroplasts at −22°C for 2 d and then repeating the above experiment led to a decrease of PSI stability (Fig. 1B) and a further slight decrease of PSII stability. Figure 3 shows the polypeptide pattern of Figure 1B after SDS-PAGE. The observed difference compared to the unfrozen membranes shown in Figure 2 is the progressive loss of CPI (band A), an increase of polypeptide band C (60 kD) and the increase in a diffuse band B (65 kD).

Comparing the photoinhibition of the fresh chloroplasts (Fig. 1A) with the frozen material (Fig. 1B) and the respective polypeptide patterns (Figs. 2 and 3), we deduced that the CPI decrease and the 60 kD polypeptide (band C) increase are correlated with PSI activity, while the decay of the other polypeptides, bands D, E, F and G, seem related to the loss of PSII activity.

The protective effect of BSA was observed in the illumination experiments. In the presence of BSA, the half-life of the PSII activity (as measured by O_2 evolution using oxidized PD as artificial electron acceptor) was increased more than 2-fold (data not shown).

Dark Decay Experiments. A typical experiment with chloroplasts aged in the dark at 4°C is shown in Figure 4. Despite the fact that the decay was somewhat variable, depending on the source of chloroplasts and the time of the year, certain conclusions can be drawn: the decay of PSII activity starts after a few h (4–6

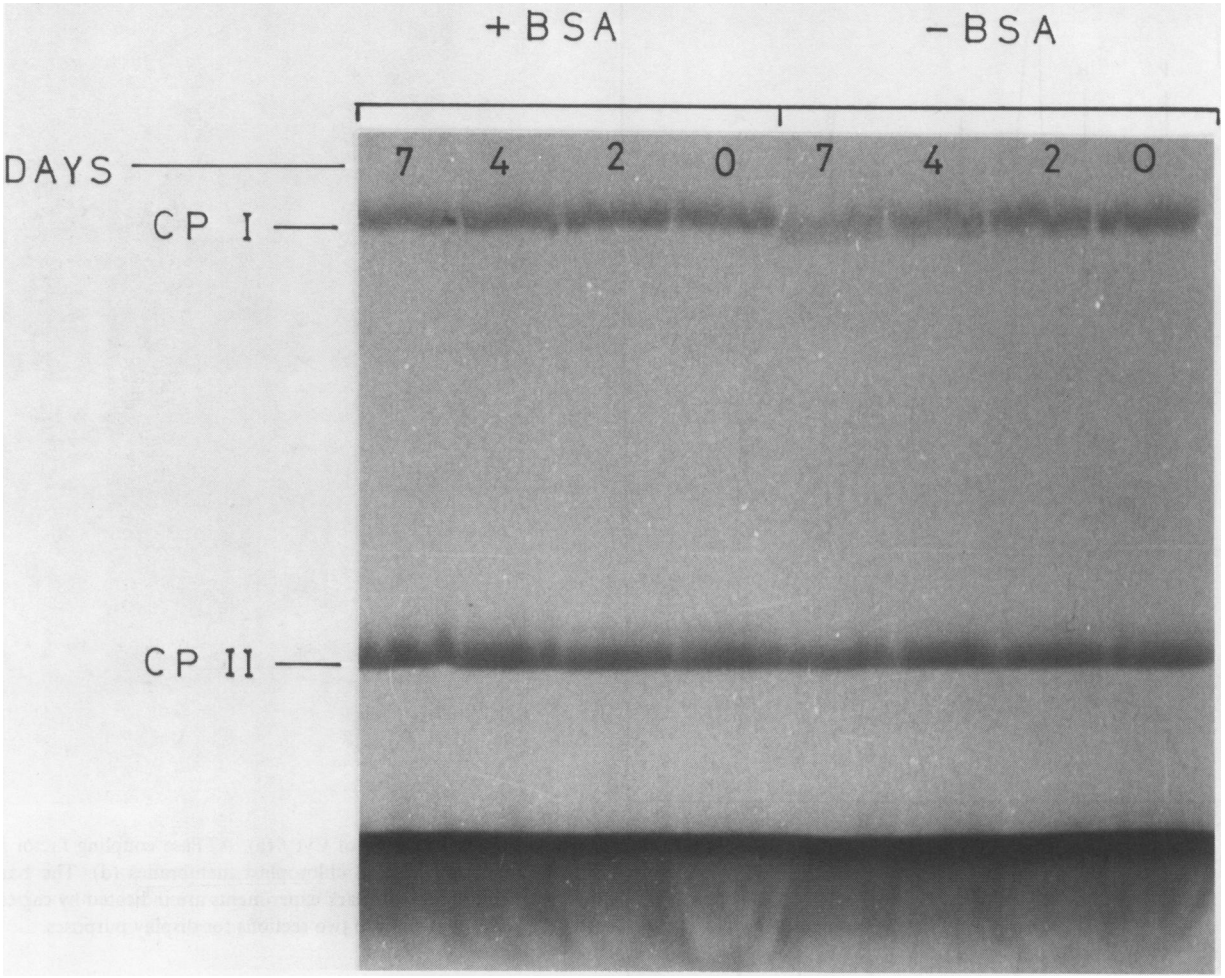

FIG. 5. Chl-protein complexes of dark-aged chloroplast membranes after SDS-PAGE. Samples were kept in the dark at 4°C for the time indicated and stored in liquid N_2 until use.

h) and shows a half life of about 30 h; PSI activity was very stable over the first 3 to 4 days (half-life of about 6 d). The use of BSA (30 mg/mg Chl) increased the stability of PSII (as has been shown by others [15, 29]). More remarkable, however, was the improvement in the stability of PSI; 60% activity remained after 7 d against only 30% activity remaining in the samples without BSA (Fig. 4).

Examination of the Chl-protein complexes showed that the stability of CPI was much improved by the use of BSA, and there was no modification in the amount of CPII (Fig. 5). Furthermore, the visible absorption spectra of both isolated CPI and CPII did not change during dark or light experiments in the presence of BSA (data not shown).

Figure 6 shows that the decay of PSI activity is correlated with the loss of the 100 kD polypeptide (band A) which is associated with CPI (Fig. 5). This correlation is substantiated by examining the polypeptide patterns of Figures 2 and 3 where PSI activity only decays when band A (100 kD) starts to disappear.

It is interesting to note that two other polypeptide bands (H-22 kD, I-13 kD) decay in the dark. Also, there was a diffuse band appearing between 60 and 100 kD (bands A and C) and as the chloroplasts aged this band disappeared and apparently shifted to a mol wt of about 65 kD (band B). This effect is more clearly shown in the gel which has chloroplast membranes aged without BSA than with BSA. These observations are of unknown significance at this stage.

Comparative Electrophoresis of Isolated Proteins. Purified Cyt *f*, CF_1, and Triton PSI particles were prepared and their electrophoretic pattern compared with isolated thylakoid membranes when solubilized by SDS.

Figure 7 compares the Cyt *f* subunit (32 kD), the five subunits of CF_1 (59, 52, 38, 20, 13 kD) and the polypeptides of the Triton PSI particle (95, 60, 22, 18, and 16 kD) with the normal electrophoretic pattern of the washed fresh thylakoid membranes. It can be seen that the PSI particle only shows a Chl-protein complex near band A (95 kD), a 60 kD polypeptide at band C, a small amount of 22 kD polypeptide (band H), and two close bands believed to be the iron-sulfur proteins (16–18 kD). These particles are able to catalyze light-induced O_2 uptake with reduced TMPD (300 μM) but showed only slight activity with reduced DCPIP (this was expected due to the lack of Cyt *f*). EPR spectra made in our laboratory by Dr. S. Chamorovsky show the presence of P-700 and the membrane bound iron-sulfur proteins (data not shown in this report). Differential spectroscopy showed that the Chl:P-700 ratio was 45:1.

Cyt *f* was characterized by low temperature absorption spectra and also by staining the SDS gel with tetramethylbenzidine (28); this stain indicates the presence of heme and was at the same position as the 32 kD band stained by Coomassie Blue. Unfortunately, the stain for heme-proteins easily disappears on the gel and the band was not intense enough to allow good photography.

Figure 8 shows that purified PSI particles when heated for 1 min at 60°C lose their CPI and show a simultaneous increase in the 60 kD band. The observation that Triton PSI particles show

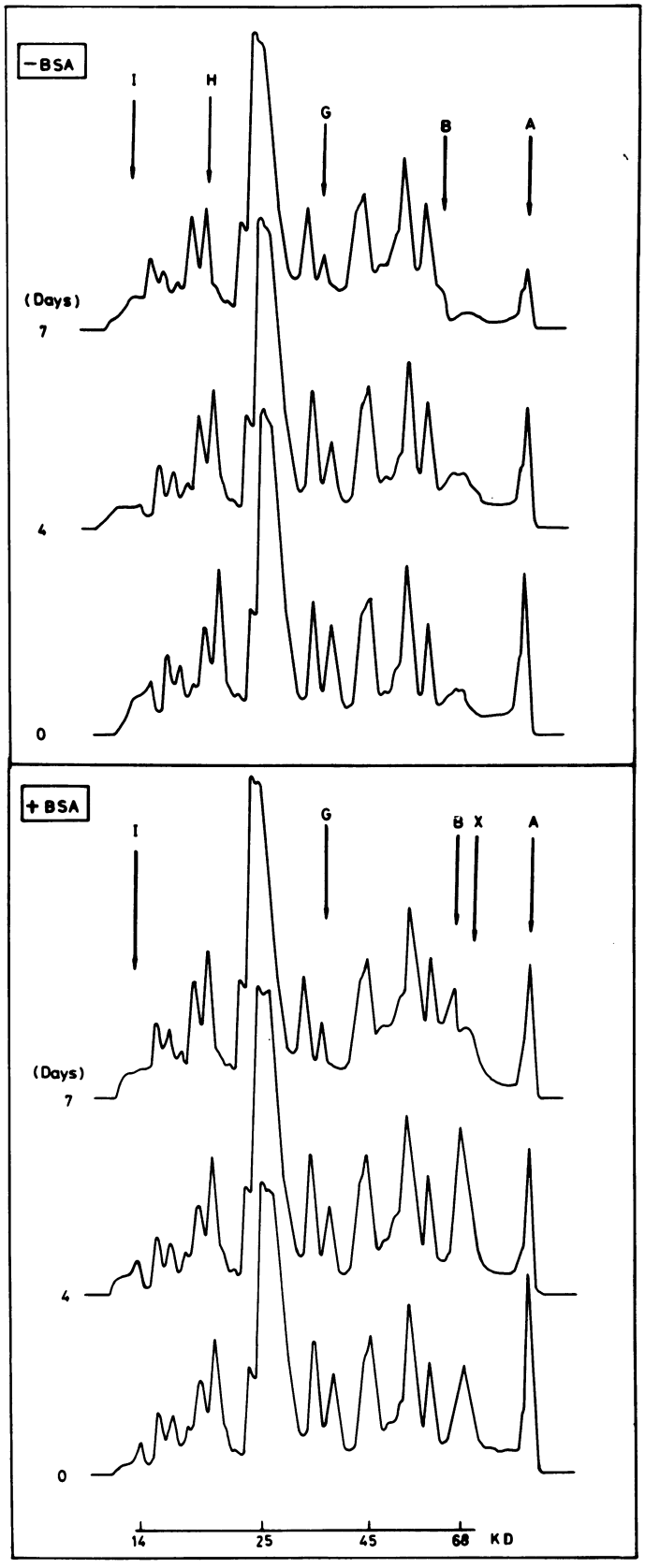

FIG. 6. Polypeptide pattern of dark-aged chloroplast membranes with or without BSA (30 mg/mg Chl) after 0, 4, and 7 d at 4°C.

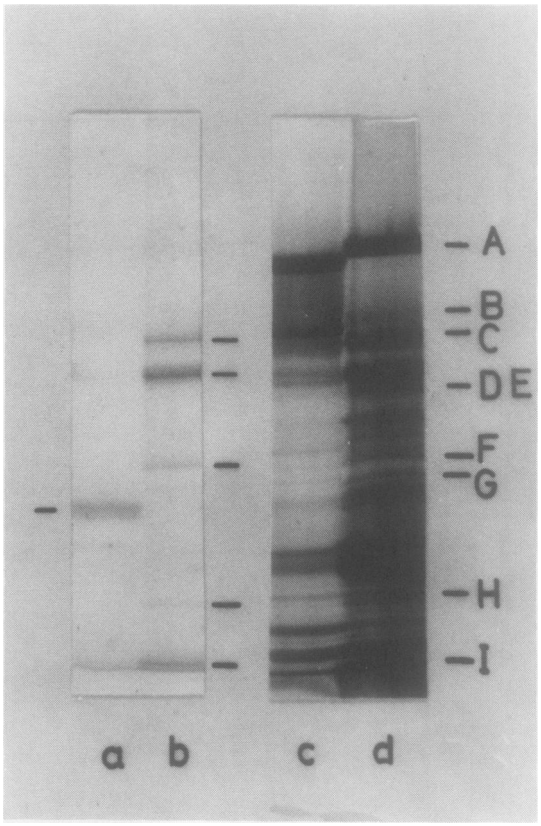

FIG. 7. PAGE in SDS of Cyt *f* (a), ATPase coupling factor subunits (b), PSI particles (c), and chloroplast membranes (d). The bands that disappear during light or dark experiments are indicated by capital letters (A–I). The gel was cut into two sections for display purposes.

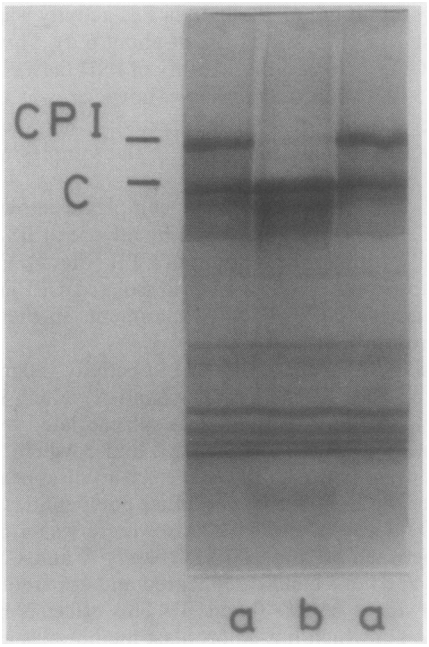

FIG. 8. Polyacrylamide gel electrophoresis in SDS of PSI particles. Samples were incubated at 20°C (a) or 60°C (b) for 1 min before electrophoresis.

a slightly lower apparent mol wt (95 kD) CPI complex than the one normally found in chloroplast membranes (100 kD) has recently also been shown by Lagoutte *et al.* (16).

DISCUSSION

We show in this paper that light causes a decay of PSI and PSII activities, the latter being much more sensitive to light inhibition. Light-inactivation of photosynthetic activities of isolated chloroplasts has been known for a long time. Early findings (14) indicated a photoinhibition which was believed to be due to lipid peroxidation (10). It was found by Hoshina *et al.* (11) that inhibition of the photosystems can also be caused by some fatty acids (*e.g.* linolenic) during dark aging; however, peroxidation of these lipids did not induce an inhibition of photochemical activities. Golbeck *et al.* (7) have shown that when linolenic acid was added to chloroplast suspensions, there is a loss of plastocyanin which causes a decay of PSI activity. They also showed that the decay of PSII was associated with the inhibition of the water splitting system and an interaction of linolenic acid with the reaction center of PSII. However, the importance of this finding in the aging of chloroplasts is still in question as recently Percival *et al.* (22) showed that there is no direct correlation between photosynthetic O_2 evolution and the release of free fatty acids during aging (the level of free linolenic acid in aged chloroplasts was 10 times lower than the level necessary for the inhibition of O_2 evolution reaction).

Observations on the loss of PSII activity in our light-aging experiments indicate that the component affected by light in the electron transport chain is close to or is part of the PSII reaction center. Critchley (4) examined photoinhibition using intact leaves and found evidence from fluorescence studies that there is a specific destruction of the PSII reaction center.

Our results with SDS gel electrophoresis suggest that the loss of polypeptides with mol wt of 36, 48, and 50 kD is related to the decay of PSII activity and probably to the reaction center of PSII itself. CPI disappearance seems to be correlated with the decay of PSI activity which indicates that the problem is directly related to disintegration of the Chl-protein complex believed to carry the reaction center of PSI (19). Our results agree with the idea that the 60 kD polypeptide comes from the CPI complex (1, 16).

It is known now that purified P700 (the primary electron donor of PSI reaction center) contains Chl *a*, carotene, and a protein. Electrophoresis of this purified P700 complex in SDS produces a single Chl-protein band around 100 kD (for a review see [20]). This Chl-protein called CPI in SDS-PAGE experiments, is the same protein which is seen to disappear in our gel during aging of the chloroplasts.

There is less available data for the PSII reaction center. We do however have some indication about the main components of PSII reaction centers. Satoh (24) has identified a 43 kD and a 27 kD polypeptide in a purified PSII reaction center complex from spinach chloroplasts; using *Chlamydomonas* mutants, Chua *et al.* (5) have observed that the lack of PSII was correlated with the absence of 40 kD and 50 kD polypeptides. New Chl-protein complexes have been shown in SDS electrophoresis using mild extraction conditions; among them are Chl_a-P_3 of Machold (18) which has a polypeptide of mol wt 41 kD, the 30 kD Chl-protein band of Wessels and Borchert (30), and the CPa (39 kD) band of Anderson (1)—all three have been correlated with the reaction center of PSII (or part of it). The most interesting aspect of the CPa band is that reelectrophoresis shows a 50 kD and 47 kD band and some minor components at 43 kD and 41 kD.

Comparison of our light and dark aging experiments shows that the disappearance of the 48 kD and 50 kD polypeptides is related to photoinhibition, whereas the 36 kD band is common to both experiments. This may indicate, if one assumes that these bands are directly related to the PSII reaction center, that the 48 kD and 50 kD polypeptides are more closely related to the light transduction at PSII than the 36 kD polypeptide. Another view could be that 2 of the 3 bands which disappear are coupling factor subunits (38 kD, 52 kD); but the fact that the other ATPase subunits are seen to be associated with the membrane and show different mobilities to the 36 kD, 48 kD, and 50 kD bands rules this out. Of course, one could also surmise that these three membrane polypeptides are not part of PSII itself but control the flow of electrons through PSII.

In summary, in this work it is shown that light inactivation is correlated with the loss of polypeptides with apparent mol wt of 36 kD, 48 kD and 50 kD when only PSII activities are lost. The loss of PSI activity is associated with the degradation of the Chl-protein CPI and the simultaneous increase in a polypeptide with apparent mol wt of 60 kD.

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