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Biosynthetic Cause of *in Vivo* Acquired Thermotolerance of Photosynthetic Light Reactions and Metabolic Responses of Chloroplasts to Heat Stress

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ABSTRACT

Thermotolerance of photosynthetic light reactions *in vivo* is correlated with a decrease in the ratio of monogalactosyl diacylglycerol to digalactosyl diacylglycerol and an increased incorporation into thylakoid membranes of saturated digalactosyl diacylglycerol species. Although electron transport remains virtually intact in thermotolerant chloroplasts, thylakoid protein phosphorylation is strongly inhibited. The opposite is shown for thermosensitive chloroplasts *in vivo*. Heat stress causes reversible and irreversible inactivation of chloroplast protein synthesis in heat-adapted and nonadapted plants, respectively, but does not greatly affect formation of rapidly turned-over 32 kilodalton proteins of photosystem II. The formation on cytoplasmic ribosomes and import by chloroplasts of thylakoid and stroma proteins remain preserved, although decreased in rate, at supraoptimal temperatures. Thermotolerant chloroplasts accumulate heat shock proteins in the stroma among which 22 kilodalton polypeptides predominate. We suggest that interactions of heat shock proteins with the outer chloroplast envelope membrane might enhance formation of digalactosyl diacylglycerol species. Furthermore, a heat-induced recompartmentalization of the chloroplast matrix that ensures effective transport of ATP from thylakoid membranes towards those sites inside the chloroplast and the cytoplasm where photosynthetically indispensable components and heat shock proteins are being formed is proposed as a metabolic strategy of plant cells to survive and recover from heat stress.

Elucidation of the causal sequence of events leading to heat injury and thermotolerance is a key to our understanding of the plant's optimal temperature resistance. There is unequivocal evidence that, prior to the impairment of other cell functions, the photosynthetic apparatus of chloroplasts is irreversibly damaged if the environmental temperature exceeds the upper threshold of the temperature range to which plants are adapted by about 20°C within a few h (reviewed in Berry and Björkman [3]). Thermotolerance of photosynthetic processes considerably increases, parallel to the onset of synthesis and accumulation of heat shock proteins (for review see Nover *et al.* [16]), upon elevation of the temperature to supraoptimal levels (35–45°C) (3). At least the heat tolerance of photosynthetic light reactions, which are associated with the chloroplast thylakoid membranes, has been attributed to the heat-induced appearance of polar thylakoid lipids with more saturated fatty acids (3, 20). Such changes in the membrane lipid composition are thought to cause an upward shift of the temperature at which an irreversible inactivation of PSII (3) and inhibition of light-activated CO_2 fixation (31) occurs.

Measurements of the motion of labeled fatty acids have established that the more saturated lipid species considerably decrease the fluidity of thylakoid membranes at high temperatures, thus maintaining an ordered lateral movement of electron carriers between the photosystems, and give rise to a low passive ion permeability as a prerequisite to achieve a proton gradient sufficient to drive photophosphorylation (3, 19). Though direct proof is lacking, acquired heat tolerance of the most heat-sensitive light reactions is likely the result of both lipid-induced, more thermotolerant conformations of the membrane-anchored subunits of thylakoid protein complexes and adjustment in membrane lipid fluidity. Since the molecular mechanism inducing thermotolerance and the metabolic response to heat of chloroplasts *in vivo* remains to be elucidated, we have studied the effects of long-term heat stress on the biosynthesis and composition of chloroplast pigments, polar lipids, and proteins as well as thylakoid protein phosphorylation in heat-adapted and non-adapted plants. We found that a decreased ratio of MDGD[1] to DGDG and an increased content, in thylakoid membranes, of DGDG species with saturated fatty acids are thermotolerance-correlated traits that enable photosynthetic light reactions to proceed at supraoptimal temperatures. Furthermore, the results indicate a heat-induced recompartmentalization of the chloroplast matrix as a metabolic strategy for survival of and recovery from sublethal high temperatures. These changes cause an effective transport of ATP from thylakoid membranes towards those sites inside the organelle and the cytoplasm where indispensable photosynthetic components and heat shock proteins are being formed during heat shock.

MATERIALS AND METHODS

Plant Growth, Heat Adaptation, and Radiolabeling. Bean plants (*Phaseolus vulgaris* var Tcherenstarosagorsky) were grown in half-strength Knopp's medium under a 16 h light/dark cycle at 25°C. The plants were divided into two groups 5 d after germination. Control plants were grown further at 25°C whereas

[1] Abbreviations: MGDG, monogalactosyl diacylglycerol; DCPIP, dichlorophenol-indophenol; DPC, diphenylcarbazide; DBMIB, dibromothymoquinone; AQ, anthraquinone sulfonic acid; *had* and *nad* plants, heat-adapted and nonadapted plants; PMSF, phenylmethylsulfonylfluoride; DGDG, digalactosyl diacylglycerol; PC, phosphatidylcholine; RuBP, ribulose-1,5-bisphosphate; LHC complex, light-harvesting Chl *a*/*b*-protein complex.

the other group was heat-adapted during the light cycle the next days. Heat adaptation was performed in a thermostated piacryl plastic chamber by exposing the plants 5 h/d to an increased temperature followed by a 25°C recovery period of 19 h. The temperature during heat exposure at the 6th, 7th and 8th d of growth was 37, 45, and 47.5°C, respectively; 19 h after the last heat treatment 6 *had* plants and 6 control plants were gassed for 5 h with 259 MBq $^{14}CO_2$ (10.4 MBq/mg $Ba^{14}CO_3$) at 50°C. The light intensity was 670 $\mu E \cdot m^{-2} \cdot s^{-1}$ and air humidity 100%. The *had* plants before and after the 50°C stress differed from control plants by an about 50% reduction in size of the primary leaves. At this stage, Chl $a + b$ content per leaf area was 4 $\mu g/dm^2$ in control and 5 $\mu g/dm^2$ in *had* plants, whereas Chl a/b ratios of 2.89 ± 0.1 and 2.54 ± 0.12, respectively, were determined. None of the *had* plants was killed or lost turgescence during heat treatment while more than 80% of the *nad* plants were irreversibly damaged. Those *nad* plants that were expected to recover from 50°C stress due to a turgescent area which remained around the middle rib of the leaves, have been used for heat recovery experiments.

To investigate recovery from heat stress, 50°C-treated *had* plants and those *nad* plants that were expected to survive, were transferred to 25°C immediately following heat treatment and gassed with $^{14}CO_2$ for 5 h as indicated above.

To radiolabel lipids and phosphoproteins *in vivo*, plants were kept in distilled H_2O containing 37 mBq carrier-free ^{32}Pi per plant 18 h before and during the last treatment at 50 or 25°C.

Isolation of Chloroplasts, Thylakoid Membranes, and Stroma Proteins. Leaves, well cut, were vacuo-infiltrated with buffer (Tricine-NaOH [pH 7.8], 0.5 M sucrose, 1 mM diethyldithiocarbamate, 1 mM PMSF, 1 mM iodoacetamide, 0.2% (w/v) BSA) and afterwards homogenized in this medium. The buffer additionally contained 100 mM NaF_3 when ^{32}P-labeled leaves were used. Chloroplasts were prepared (14) and osmotically shocked by resuspending them in an excess of 0.5 mM Tricine-NaOH (pH 7.8), 0.1 mM PMSF, 0.1 mM iodoacetamide. Thylakoid membranes were sedimented at 10,000*g* for 10 min and washed again with the same buffer. The stroma supernatants were combined, centrifuged at 150,000*g* for 1 h, and the protein in the supernatant was precipitated with 1% (w/v) TCA.

Photochemical Assays. Intact chloroplasts were used. The PSII-dependent reduction of DCPIP by chloroplasts using either water or DPC as electron donors in the presence of 1 μM DBMIB was measured spectrophotometrically (17). Photoreduction of AQ as an electron acceptor, using either water or DPC (PSII to PSI electron transport) or DCPIP reduced with ascorbate (PSI-dependent) as an electron donor, was measured polarographically as O_2 uptake (28).

Analysis of Pigments, Polar Lipids, and Lipid Species. Thylakoid membranes were extracted twice with chloroform/methanol (2/1, v/v) or acetone and insoluble material was removed by centrifugation. Pigments were chromatographed on cellulose TLC plates impregnated with sunflower oil in a solvent system of methanol/acetone/water (60/20/4, v/v/v) (11). Polar lipids were separated in one or two dimensions by silica gel 60 TL chromatography (10). A solvent system of chloroform/methanol/water (65/25/4, v/v/v) and chloroform/methanol/acetone/acetic acid (100/20/40/10, v/v/v/v) was used in the first and second dimensions of chromatography, respectively. Lipids were located under UV light (260 nm) after spraying the plates with 0.2% anilinonaphthalene sulfonate in methanol. The TLC plates were checked for radioactivity by autoradiography using the x-ray sensitive film HS 11 (VEB Fotochemische Fabrik, Berlin, GDR) or the lipid zones were scraped off and radioactivity was measured by liquid scintillation counting (27). Alternatively, isolated lipids were separated into molecular species on $AgNO_3$-impregnated silica gel 60 TLC plates and characterized essentially as described previously (23). The plates were exposed to x-ray films which were scanned with a Zeiss-Schnellphotometer (VEB Carl Zeiss, Jena, GDR) to quantitate by planimetry radioactivity associated with the lipid zones. To determine the ratio of MGDG/DGDG in thylakoid membranes, lipid samples of inactive material were chromatographed on silica gel 60 TLC plates, the galactolipid-containing zones scraped off, and their galactose content was estimated with the phenol-sulfuric acid procedure (6).

Protein Analysis and Other Methods. Chloroplast stroma proteins and thylakoid membranes were dissolved in buffer (50 mM Na-borate [pH 8.0], 2% [w/v] SDS, 8 M urea, 1 mM PMSF, 5 mM DTT) and separated electrophoretically on 8 to 18% polyacrylamide gel slabs containing 0.1% SDS and 5 M urea, followed by Coomassie G250 staining and autoradiography of gels as described previously (27). A published method was used for measuring Chl concentrations (2).

RESULTS

Characterization of Thermotolerant Electron Transport. Partial reactions of the photosynthetic electron transport chain of isolated chloroplasts from control and heat-stressed (50°C, 5 h) *had* and *nad Phaseolus* plants were utilized to define the heat adaptive response of the photosynthetic apparatus. Table I shows that prolonged heat stress of *nad* plants completely inactivates PSII, including the O_2-evolving system, and severely inhibits PSI. In contrast, little or no effect on partial reactions had occurred in thermotolerant chloroplasts. An apparent inhibition, however, was established for the PSII-catalyzed reduction of DCPIP with DPC as an electron donor in the presence of DBMIB; the same reaction using water as a donor was slightly stimulated as compared to the control. This indicates that the electron donor site of PSII in thermotolerant chloroplasts is less accessible to the hydrophobic donor DPC. Furthermore, the comparably strong uncoupler-mediated stimulation of the PSI-dependent reduction of AQ (reactions 5, 6) suggests a much lower passive ion permeability in thylakoid membranes from heat-stressed *had* plants as compared to those from plants grown continuously at 25°C. We interpret these data to indicate that changes in membrane-bilayer organization and conformation of PSII within thylakoid membranes are prerequisites for thermotolerance and that these variations do not alter photosynthetic electron transport. It is worth mentioning in this respect that the rate of CO_2 fixation at 50°C of *had* plants exceeded the value of the *nad* plants kept at the same temperature about 20 times and was about 20% higher than in control plants kept 25°C (data not shown). The CO_2 fixation in *nad* plants diminished by more than 50% after reexposure to 25°C. This explains the weak radiolabeling of chloroplast components shown later.

Thylakoid Protein Phosphorylation under Heat Stress. Since a heat-induced change in the distribution of excitation energy between PSII and PSI has been suggested to be a primary event in achieving heat tolerance (32) and thylakoid protein phosphorylation was shown to regulate the light distribution between the photosystems (1), the phosphorylation of chloroplast thylakoid polypeptides was inspected in *had* and *nad* plants during and subsequent to prolonged exposure to 50°C. Thylakoid membranes labeled with ^{32}Pi *in vivo* were subjected to SDS/urea PAGE and the stained gels checked autoradiographically for radioactivity. Figure 1 shows that, independent of the source of membranes, six different polypeptides of 43, 33, 32, 26, 25.5, and 9 kD are predominantly phosphorylated in thylakoid membranes. Of these phosphoproteins, the 25.5 to 26 kD components represent subunits of the light-harvesting Chl a/b-protein complex II whereas the other are constituents of the PSII core particle (24, 26). Unexpectedly, thylakoid phosphoproteins in *had* plants were only weakly ^{32}P-labeled at 50°C and protein phosphoryla-

Table I. *Effect of Long-term Heat Stress (5 h, 50°C) of had and nad Phaseolus Plants on Photosynthetic Electron Transport Partial Reactions*

All reactions were carried out with chloroplasts suspended in 50 mM K-phosphate (pH 7.8). Reactions 1 and 2 require PSII only; reactions 3 and 4 require PSII and PSI; reactions 5 and 6 require PSI only. DCPIP = 200 μM; DPC = 10 mM; AQ = 200 μM; $DCPIP/_{asc}$ = 1 mM DCPIP reduced with 10 mM ascorbate; NH_4Cl = 4 mM; DBMIB = 1 μM.

Partial Reactions	Reaction rate, μmol mg^{-1} Chl h^{-1}				
	Control	*had*	% of Control	*nad*	% of Control
1. H_2O to DCPIP + DBMIB	47.18	54.00	114	0	0
2. DPC to DCPIP + DBMIB	39.17	15.75	40	0	0
3. H_2O to AQ + NH_4Cl	6.48	2.91	45	0	0
4. DPC to AQ + NH_4Cl	25.93	19.36	75	0	0
5. $DCPIP/_{asc}$ to AQ + NH_4Cl	252.91	242.10	96	54.47	22
6. $DCPIP/_{asc}$ to AQ	145.91	94.42	65	13.82	9

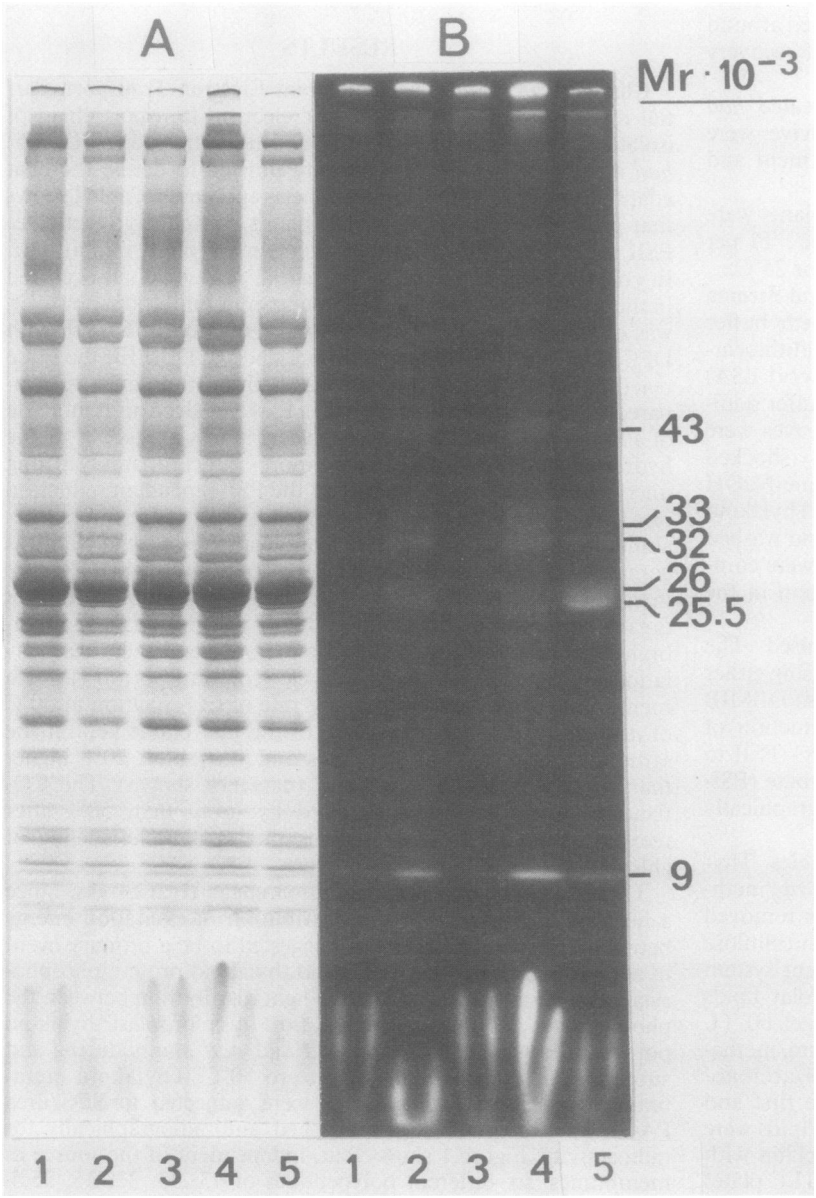

FIG. 1. Effect of heat stress on thylakoid protein phosphorylation of *had* and *nad* Phaseolus plants. *In vivo* ^{32}Pi labeled thylakoid membranes were isolated from control plants grown continuously at 25°C (lane 5) and from *had* (lanes 1, 3) and *nad* (lanes 2, 4) plants exposed 5 h to 50°C (lanes 1, 2) followed by reexposure to 25°C for 5 h (lanes 3, 4) and subjected to SDS/urea PAGE. Protein corresponding to 15 μg of Chl was applied to each lane. A, Coomassie blue-stained gel; B, autoradiogram.

tion declined slightly during the subsequent period of recovery from heat stress. In contrast, thylakoid protein phosphorylation in *nad* plants was markedly stimulated by prolonged 50°C-treatment as compared to the control, with the remarkable exception that a relatively lower amount of label was incorporated into LHC polypeptides. In contrast to the situation *in vitro* (15), the latter result demonstrates that the light-activated protein kinase(s) retains activity *in vivo* at elevated temperatures even when plants became turgescent and PSII was expected to be damaged.

Biosynthesis of Pigments and Polar Lipids under Heat Stress. Metabolic radiolabeling of plants with $^{14}CO_2$ was utilized to establish the effects of long-term heat stress and of a subsequent period of recovery from heat stress on the biosynthesis and composition of thylakoid pigments and polar lipids. Pigment samples were separated on cellulose TLC plates followed by autoradiography. An analysis revealed that, independent of the degree of heat tolerance of plants, high temperature irreversibly inhibit accumulation of Chl but do not significantly influence the biosynthesis and composition of the other pigments (Fig. 2). We noted, however, for heat-stressed *nad* plants the absence of violaxanthin as a colored pigment which is not recovered upon reexposure to 25°C for 5 h (not shown).

Heat tolerance of thylakoid membranes has previously been attributed mainly to changes in fatty acid composition of total polar lipids only (3), and for that reason we have separated and identified by silica gel 60-TL-chromatography the *in vivo* ^{14}C-labeled polar lipids. Autoradiograms of lipid patterns revealed a strong heat-stimulated accumulation of DGDG with respect to the major polar lipids MGDG and PC in thylakoid membranes of *had* but to a much smaller extent in *nad* plants during and subsequent to long-term heat exposure (Fig. 3A). To examine whether the rise in DGDG biosynthesis induced by heat treatment of plants changes the MGDG/DGDG ratio in thylakoid membranes, the galactose content of chromatographically separated galactolipids was determined. The analyses showed that the ratio of MGDG to DGDG decreased from 1.3 in control plants to 0.9 in heat-stressed *had* plants.

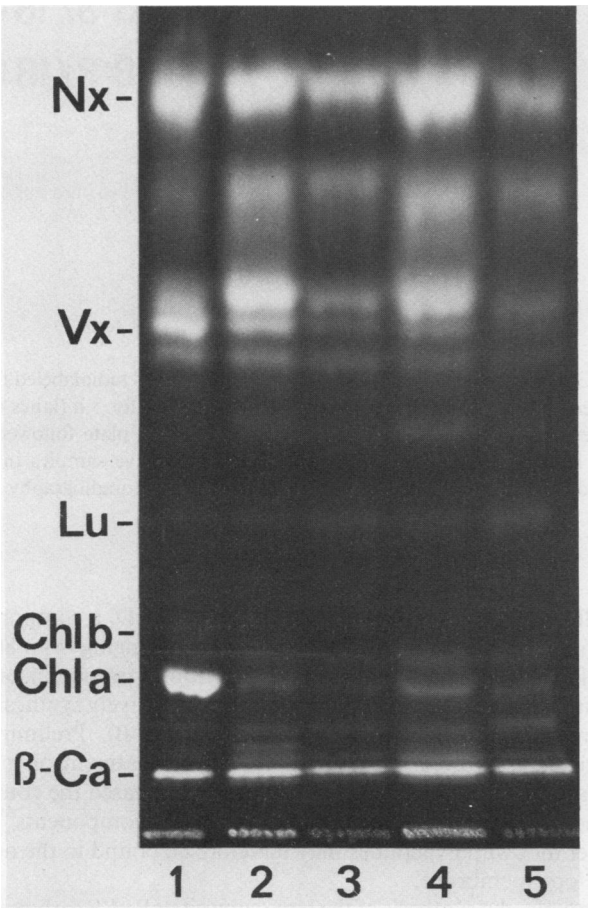

FIG. 2. Influence of heat stress on the *de novo* synthesis of thylakoid pigments. Pigments extracted from thylakoids of *had* (lanes 2, 4) and *nad* (lanes 3, 5) plants that were radiolabeled 5 h with $^{14}CO_2$ during exposure to 50°C (lanes 2, 3) or labeled subsequently, together with control plants (lane 1), at 25°C for the same time (lanes 4, 5) were chromatographed on cellulose-TLC plate followed by autoradiography. Sample containing 1 mg of Chl was applied to each lane. β-Ca, β-carotenoid; Lu, lutein; Vx, violaxanthin; Nx, neoxanthin.

The observation that an increased content of DGDG in thylakoids is a thermotolerance-correlated trait has led us to separate, by argentation-TL-chromatography, the DGDG, MGDG, and PC molecular species to identify those forms which become predominantly membrane-incorporated during prolonged heat stress. Figure 3B tentatively identifies DGDG with fatty acyl pairing 18:1/16:0, besides those species formed by control plants at 25°C, as being accumulated by heat induction in thylakoids of *had* but not *nad* plants. A quantitative evaluation of the ratio of newly synthesized DGDG species in control plants and heat-stressed *had* plants reveals that in the latter DGDG with 18:1/16:0 fatty acyls represents about 16% of the mass of DGDG molecular forms (Table II).

Reexposure of heat-stressed plants to normal temperatures (25°C) again stimulates incorporation of DGDG with 18:3/18:2, 18:3/18:1, and 18:3/16:0 fatty acyl pairings into thylakoids thus clearly indicating a fast-responding, temperature-controlled regulation of the synthesis of DGDG molecular forms. Since no thermo-dependent changes in the ratio of MGDG and PC molecular species have been observed (not shown), heat tolerance of thylakoid membranes *in vivo* is mainly achieved by an increased content of DGDG species with saturated fatty acyls and a decreased ratio of MGDG/DGDG. Essentially the same results have been obtained with wheat and barley.

Biosynthesis of Chloroplast Proteins under Heat Stress. To examine the action of high temperatures on the biosynthesis of chloroplast proteins, the *in vivo* ^{14}C-labeled thylakoid membrane and stroma proteins were separated on SDS/urea polyacrylamide gels and detected by staining and autoradiography (Figs. 4, 5). Although long-term heat stress did not visibly alter the polypeptide composition of thylakoid membranes (Fig. 4A), it strongly diminished the synthesis of several components with known function including the apoprotein of reaction center Chl *a*-protein of PSI, α, β and proteolipid subunits of ATP synthase, Cyt *f* and b_{559}, and the 47 kD component of PSII complex (Fig. 4B). These proteins are coded by chloroplast DNA and at least some of them were shown to be synthesized on membrane-bound ribosomes and inserted co-translationally into the thylakoids (7, 9). However, the rate of synthesis on thylakoid-bound ribosomes (11) of the smaller one of the two rapidly turned-over 32 kD polypeptides diminished during heat stress by only about 30% in *had* plants but more than 80% in *nad* plants as revealed by counting the radioactivity associated with bands (not shown). Notably, *had* and *nad* plants synthesized similar amounts of the larger 32 kD polypeptide at 50°C, although the rate of formation was only about 40% that of control plants. Comparably, much radiolabel appeared in the major 26 kD polypeptide of LHC complex and other thylakoid components of less than 25 kD. These proteins are formed on cytoplasmic ribosomes and then are actively imported into chloroplasts (7, 8). Reexposure of plants to 25°C significantly stimulated or even restored (as in the case of the smaller 32 kD polypeptide) the synthesis of thylakoid proteins formed inside the chloroplasts and in the cytoplasm of *had* but not in *nad* plants.

SDS/urea polyacrylamide gel electrophoresis has been used to analyze changes in the polypeptide composition of chloroplast stroma extracts from control and heat-stressed *nad* and *had* plants. No differences were revealed between the polypeptide patterns of control and heated *nad* plants (not shown). In contrast, the fraction of soluble chloroplast proteins of *had* plants contained, in addition to the normal set of stroma polypeptides, heat-accumulated components (Fig. 5A). These heat shock proteins can be subdivided into three groups. That is, a group of

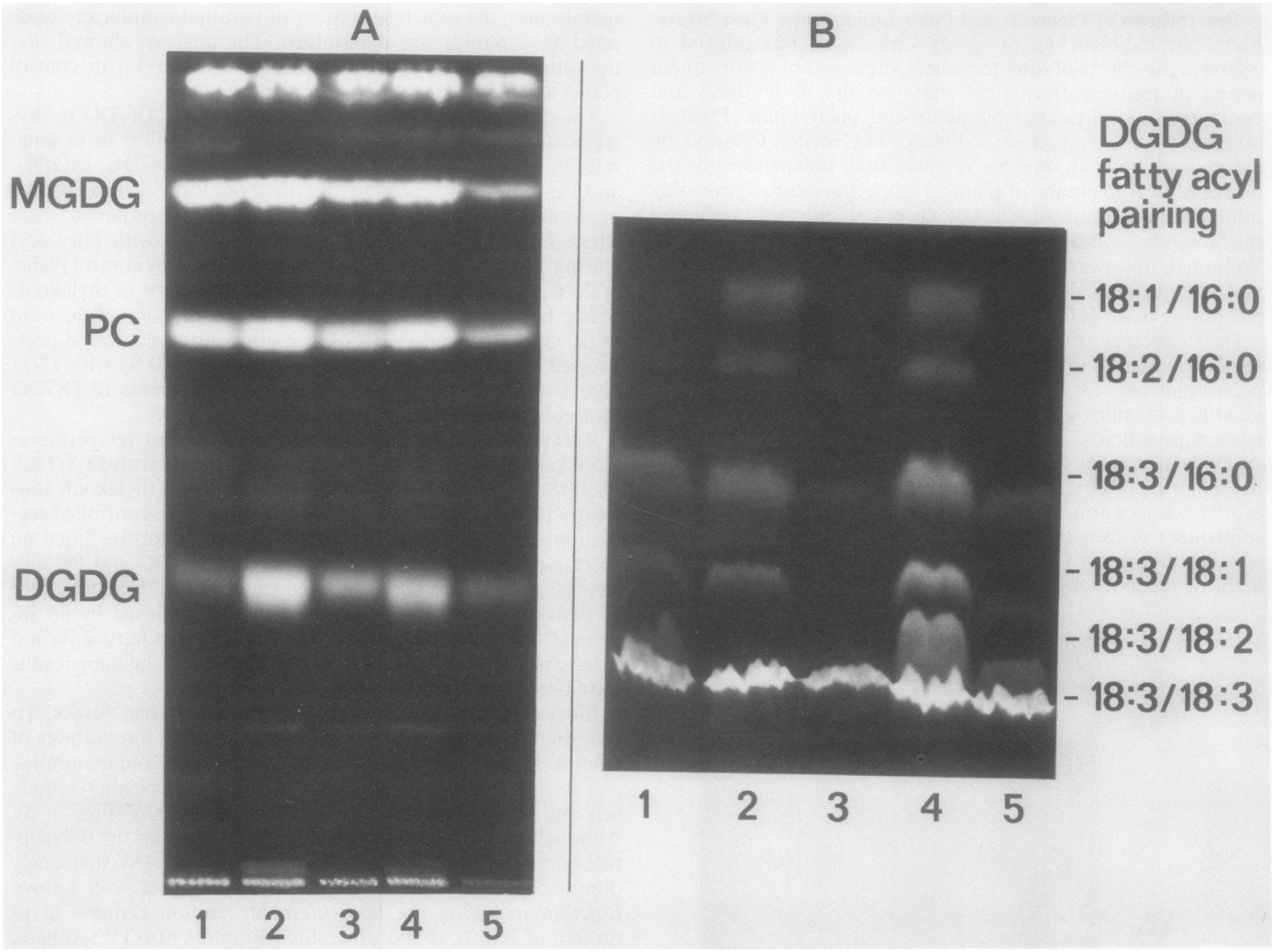

FIG. 3. Effect of heat stress on the synthesis of thylakoid lipids (A) and DGDG molecular species (B). A, Polar thylakoid lipids radiolabeled from $^{14}CO_2$ in *had* (lanes 2, 4) and *nad* (lanes 3, 5) plants when kept 5 h at 50°C (lanes 2, 3) or subsequent to heat treatment at 25°C for 5 h (lanes 4, 5) as well as in control plants (lane 1) were extracted of thylakoids and separated chromatographically on a silica gel 60-TLC plate followed by autoradiography. Sample corresponding to 1 mg of Chl was applied to each lane. B, DGDG was reextracted from respective samples in (A) chromatographed on a $AgNO_3$-impregnated silica gel 60-TLC plate and separated DGDG molecular species were detected by autoradiography.

Table II. *Ratio of Thylakoid DGDG Molecular Species Synthesized during and Subsequent to Heat Stress of had and nad Phaseolus Plants*

X-ray film with autoradiograms of ^{14}C-labeled DGDG molecular species shown in Figure 3B was scanned photometrically and radioactivity proportional to peaks quantisized by planimetry.

DGDG Species	^{14}C-Label in Species				
	5 h, 25°C	5 h, 50°C		5 h, 50°C → 5 h, 25°C	
	Control	*had*	*nad*	*had*	*nad*
		%			
18:1/16:0		16.6		10.8	
18:2/16:0	6.6	5.8		8.9	
18:3/16:0	30.8	23.4	18.3	20.7	21.0
18:3/18:1	9.4	10.5		13.9	6.0
18:3/18:2	9.7			20.2	16.2
18:3/18:3	43.4	44.3	81.7	25.9	56.7

smaller polypeptides in the range of about 22 kD, a component of 66 kD, and a group of four larger proteins ranging from 80 to 110 kD (Fig. 5). Autoradiographic detection of isotope incorporation confirmed these polypeptides were intensively synthesized during and subsequent to heat stress (Fig. 5B). Preliminary experiments showed, however, that trypsin treatment of intact chloroplasts from heat-stressed *had* plants decreased the content of the larger polypeptides but not of the 22 kD components. The higher mol wt polypeptides may therefore be bound to the outer envelope membrane.

Notably, the synthesis of the large subunit of RuBP carboxylase was completely impaired at 50°C and became only weakly reactivated upon reexposure of plants to 25°C. In contrast, although the rate of formation declined by about 70%, the cytoplasmic synthesis and import by chloroplasts of the small subunit of RuBP carboxylase proceeded during long-term heat stress. These results confirm previous findings on the subunit biosynthesis of this major plant protein during heat stress in cell cultures (30).

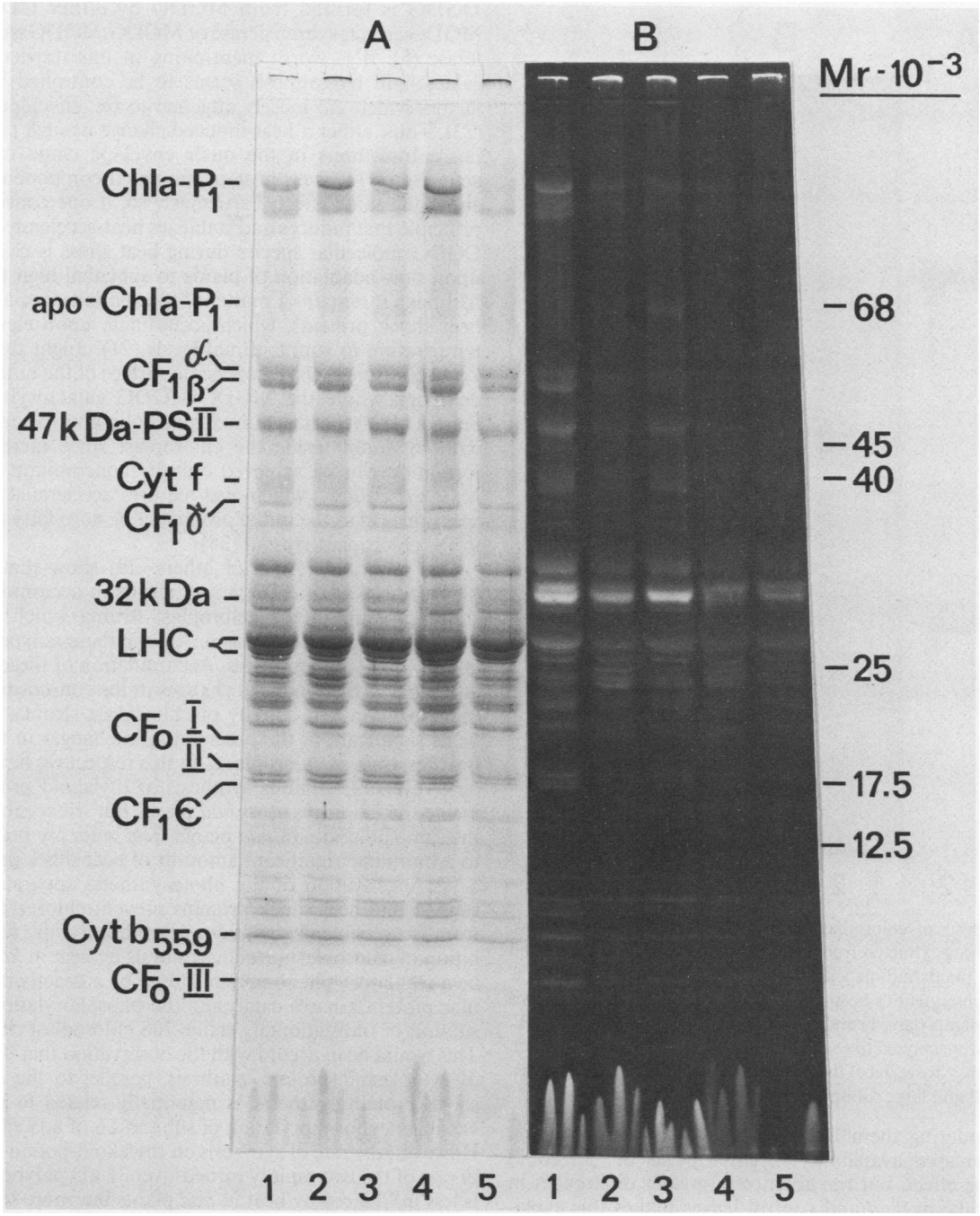

FIG. 4. Synthesis of thylakoid proteins in heat stressed *had* and *nad* plants. Thylakoids were isolated from *had* (lanes 2, 3) and *nad* (lanes 4, 5) plants which were $^{14}CO_2$ labeled 5 h at 50°C (lanes 2, 4) or radiolabeled subsequently to heat stress for 5 h at 25°C (lanes 3, 5) together with control plants (lane 1) were subjected to SDS/urea PAGE. A, Coomassie blue-stained ge; B, autoradiogram; CF_1, coupling factor 1; CF_o, membrane sector of ATP synthase; cyt., cytochrome; Chla-P_1, reaction center Chl *a*-protein of PSI.

DISCUSSION

This study provides evidence that an increased content of DGDG in thylakoid membranes correlates with heat tolerance of the photosynthetic apparatus of chloroplasts *in vivo*. As established for bean plants and confirmed for wheat and barley, it is tempting to suggest that both a decreased ratio of MGDG/DGDG and appearance of DGDG molecular species with saturated fatty acyls lead to a decreased fluidity of thylakoid membranes at supraoptimal temperatures (19), thereby decreasing the passive ion permeability of membranes and bringing about a conformation-induced rise in thermal stability of PSII units. These variations in lipid composition may function to keep photosynthetic electron transport and ATP synthesis virtually intact but somehow inhibit thylakoid protein phosphorylation which regulates the distribution of excitation energy between the two photosystems (1). Considering the fact that the light-activated protein kinase remains highly active in plants exposed to damaging high temperatures (Fig. 1), the weak thylakoid protein phosphorylation in thermotolerant chloroplasts *in vivo* is due either to an inhibition or severe activation of protein kinase and phosphoprotein phosphatase, respectively, or possibly by a changed conformation of the PSII-phosphoproteins within the

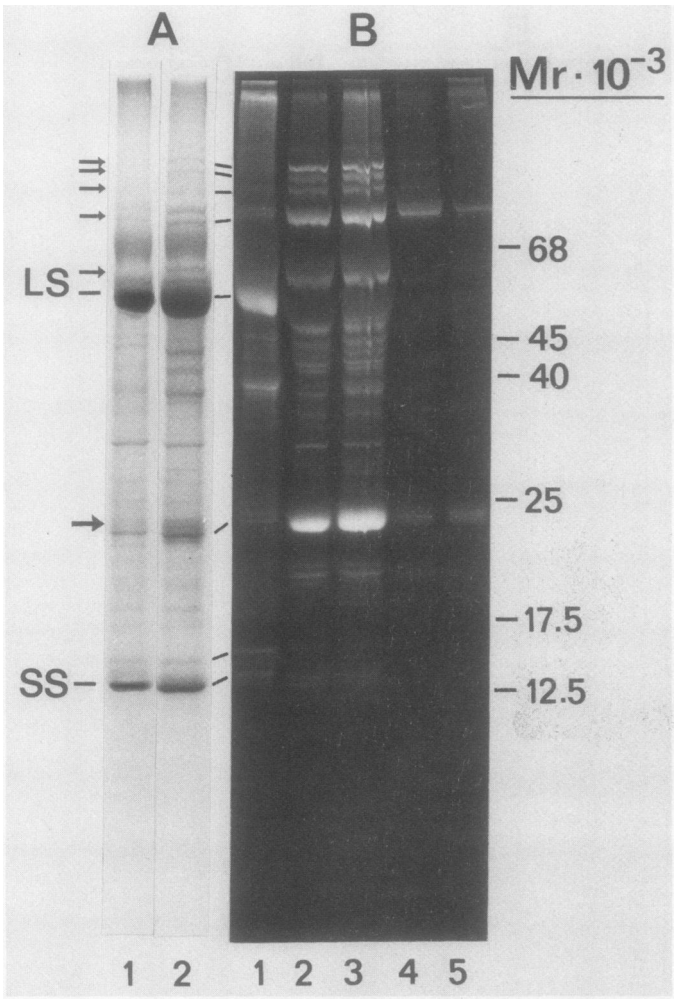

FIG. 5. Synthesis of chloroplast stroma proteins under heat stress. Stroma protein was extracted from chloroplasts of *had* (lanes 2, 3) and *nad* (lanes 4, 5) plants radiolabeled with $^{14}CO_2$ when kept 5 h at 50°C (lanes 2, 4) or subsequent to heat stress for the same time at 25°C (lanes 3, 5) as control plants (lane 1) and electrophoresed on SDS/urea PAGE. Protein (40 μg) were applied to each lane. A, Coomassie blue-stained gel; B, autoradiogram. Arrows label the positions of heat shock polypeptides. SS and LS, small and large subunit of RuBP carboxylase, respectively.

membrane rendering them less accessible to phosphorylation. Perhaps a decreased availability to chloroplasts of ^{32}Pi could account for the effect, but the absence of greater differences in phosphate uptake by *had* and control leaves makes this explanation unlikely. Hence, thylakoid protein phosphorylation is likely to be of less functional importance in thermotolerant chloroplasts but may contribute to a higher heat stability of PSII in nonthermotolerant thylakoids as has been assumed from fluorescence measurements (32). That protein phosphorylation might prevent, at least in part, heat-induced disconnection of the LHC complex from PSII core complex (3) is indicated by a significantly decreased and increased phosphorylation of LHC-polypeptides and phosphoproteins of PSII core complex, respectively, in heat-stressed *nad* plants. This explanation is supported by the finding that, at normal temperatures, phosphorylated LHC complexes migrate from PSII-enriched, appressed to nonappressed, PSII-deficient thylakoids (12).

We suggest acquired thermotolerance of chloroplasts can be attributed to activation of the DGDG-synthesizing enzyme(s). The heat-enhanced synthesis of DGDG is expected to take place in the outer chloroplast envelope membrane, *i.e.* the site where DGDG is formed from MGDG by either UDP-galactose: MGDG galactosyltransferase or MGDG:MGDG galactosyltransferase (5). It is worth mentioning in this respect that *in vivo* galactolipid biosynthesis seems to be controlled by regulatory factors which are loosely attached to the envelope membranes (23). Thus, either a heat-induced change of such factors or lipid phase transitions in the outer envelope could cause enzyme activation. However, heat-accumulated components may bring about the same effect. Particularly so, if one considers that the principle that induces and stabilizes heat-accelerated synthesis of DGDG molecular species during heat stress is clearly acquired upon slow adaptation of plants to sublethal high temperatures. Although it remains a matter of speculation, special cytoplasmic heat shock proteins, which accumulate upon elevation of the temperature to supraoptimal levels (21), might function as enzyme effectors at the cytoplasmic surface of the outer chloroplast envelope where the MGDG:MGDG galactosyltransferase is bound (4). A heat acquired metabolic control principle exists without doubt inside the chloroplast since increased DGDG formation can be achieved only by concomitant activation of MGDG synthesis which also requires accelerated formation of palmitic acid as the initial product of *de novo* fatty acid synthesis in the chloroplast stroma (25).

Our own and results of others (29) show that exposure of plants to elevated temperatures results in accumulation of heat shock proteins in the chloroplast stroma which are probably imported from the cytoplasm. Among these polypeptides one of about 22 kD predominates. Accumulation of these components and possible heat-induced changes in the composition, compartmentalization and activity of chloroplast stroma proteins may be as significant as the observed lipid changes in the metabolic heat response of chloroplasts. In this respect we have shown that heat reversibly inhibits synthesis of thylakoid proteins formed inside the chloroplasts in *had* plants but irreversibly inactivates the same processes in *nad* plants. The latter are probably unable to accumulate significant amounts of heat shock proteins due to a fast inactivation of the photosynthetic apparatus. One may suggest that heat shock proteins prevent chloroplast ribosomes from irreversible degradation. Alternatively, the reversible inhibition of chloroplast protein synthesis by heat in *had* plants may be a regulatory phenomenon caused by a deactivation of particular protein kinases catalysing the phosphorylation of the L16 subunit of translationally active 70S chloroplast ribosomes (18). This would be in accord with the observation that heat inhibition of cytoplasmic protein synthesis, parallel to the onset of heat shock protein synthesis, is potentially related to rapid, but reversible dephosphorylation of subunit S6 of 80S ribosomes (22). However, the rate of synthesis on thylakoid-bound ribosomes (9) of one of the two rapidly turned-over 32 kD polypeptides of PSII is less influenced by heat in *had* plants but more severely in *nad* plants. Consequently, continuous formation of 32 kD polypeptide mRNA and its translation at least by a fraction of bound ribosomes proceed at sublethal high temperatures. In contrast, the decline in synthesis on thylakoid-bound ribosomes of ATP synthase subunits, the 47 kD polypeptide of PSII, Cyt *f* and other thylakoid proteins seems to be caused by inhibition of message translation only. This conclusion is based on the observation that the majority of chloroplast DNA transcripts in pearl millet remains unchanged during heat shock (21).

The results reported here and by other authors (30) show that while the synthesis of proteins inside the chloroplast is strongly diminished, the formation of chloroplast proteins on 80S ribosomes, *e.g.* light-harvesting Chl *a*/*b*-binding proteins and the small subunit of RuBP carboxylase, and their ATP-dependent import by the organelle are preserved at high temperatures *in vivo*. This is an unexpected phenomenon if one considers that the outermost part of energy in a plant cell is being produced by

photosynthetic ADP phosphorylation and, furthermore, assumes that biosynthetic events in the chloroplast matrix would consume ATP before enzymic processes taking place in the cytoplasm can be supplied with energy. Obviously, the opposite seems to be the case because of the inhibition of Chl accumulation, chloroplast protein synthesis, and phosphorylation of thylakoid as well as stroma proteins. Therefore, we suppose that a heat-induced recompartmentalization of the chloroplast matrix causes the inhibition of various ATP-dependent stroma enzymes. We further propose that these changes ensure effective transport of ATP from thylakoid membranes towards those sites inside the organelle and the cytoplasm where components indispensable for normal functioning of the photosynthetic apparatus and particularly heat shock proteins are being synthesized. In this respect, maintenance of biosynthesis of the plastoquinone-binding, rapidly turned-over 32 kD thylakoid proteins of PSII appears to be an important result of the chloroplast heat shock response and acquired thermotolerance. This is understandable if the continuous replacement of this protein(s), representing the rapidly photodamaged secondary electron acceptor of PSII (13), is an absolute requirement to keep photosynthetic electron transport intact and thus to maintain ATP synthesis at supraoptimal temperatures.

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