Changes in Protein Synthesis Induced in Tomato by Chilling[1]

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PAM COOPER[2] AND DONALD R. ORT*
Department of Plant Biology and U.S. Department of Agriculture, Agricultural Research Service, University of Illinois, 289 Morrill Hall, 505 S. Goodwin Ave., Urbana, Illinois 61801

ABSTRACT

Impaired chloroplast function is responsible for nearly two-thirds of the inhibition of net photosynthesis caused by dark chilling in tomato (*Lycopersicon esculentum* Mill.). Yet the plant can eventually recover full photosynthetic capacity if it is rewarmed in darkness at high relative humidity. As a means of identifying potential sites of chilling injury in tomato, we monitored leaf protein synthesis in chilled plants during this rewarming recovery phase, since changes in the synthesis of certain proteins might be indicative of damaged processes in need of repair. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins pulse labeled with [^{35}S]methionine revealed discrete changes in the pattern of protein synthesis as a result of chilling. A protein of M_r = 27 kilodaltons (kD), abundantly synthesized by unchilled plants, declined to undetectable levels in chilled plants. Reillumination restored the synthesis of this protein in plants rewarmed for 8 hours. Peptide mapping analysis showed the 27 kD protein to be the major chlorophyll *a/b* binding protein of the photosystem II light-harvesting complex (LHCP-II). The identity of this protein was confirmed by its immunoprecipitation from leaf extracts by a monoclonal antibody specific for the major LHCP-II species. While chilling abolished the synthesis of the major LHCP-II species, it also induced the synthesis of an entirely new protein of M_r = 35 kD. The protein was synthesized on cytoplasmic ribosomes, and two-dimensional polyacrylamide gel electrophroesis showed it to exist as a single isoelectric species. This chilling-induced 35 kD protein is structurally distinct from the 27 kD LHCP-II and appears to be synthesized specifically in response to low temperature. While the 35 kD protein was found not to be associated with the chloroplast thylakoid membrane, chilling did cause selective changes in thylakoid membrane protein synthesis. The synthesis of two unidentified proteins, M_r = 14 and 41 kD, and the β-subunit of the chloroplast coupling factor were substantially reduced after chilling. These losses may provide clues as to the causes of the overall reduction in net photosynthesis caused by chilling.

Plant species evolutionarily adapted to warm habitats are quite susceptible to injury by low, above-freezing temperatures ($0 < T < 12$°C). For such plants, a relatively brief chilling exposure can have long-term, adverse effects on growth. An important element of this limitation on growth can lie in the susceptibility of photosynthesis to chilling damage.

Our studies have concentrated on tomato. In this species, net photosynthesis measured at 25°C and ambient CO_2 levels was observed to be inhibited by as much as 60% by 16 h of chilling in darkness (26). While increased stomatal resistance accounts for some of the inhibition, the major portion of the decrease was due to direct impairment of chloroplast activity (26). Yet plants damaged by chilling in darkness can eventually recover full photosynthetic capacity if they are rewarmed in darkness at high RH (24). Presumably, the damaged biochemical processes are repaired under these conditions.

Our research is directed toward identifying those elements that account for the susceptibility of chloroplast activity to chilling. Previous work has shown that dark chilling has vanishing little effect on water oxidation capacity (26), electron transfer reactions (20), or the regulation and activation of the photosynthetic carbon reduction cycle (31). Thus, although the effect of chilling on photosynthesis can be large, the underlying cause is subtle and is not revealed when individual processes are studied in isolation.

In the work presented here, we monitored leaf protein synthesis in tomato seedlings after chilling exposure. Chilling-induced changes in protein synthesis might reveal the resynthesis of damaged polypeptides vital to photosynthesis or the synthesis of acclimation proteins necessary for recovery or continuation of normal maintenance and development interrupted by chilling. We found that while most of leaf protein synthesis remained unchanged after chilling, there was a decline in synthesis of a 27 kD protein which we identified as the major Chl *a/b* binding protein of LHCP-II.[3] Chilling also induced the synthesis of a novel 35 kD polypeptide during the first 4 h after rewarming. Finally, chilling caused a reduction in the accumulated levels of three thylakoid membrane proteins, one of which was the β-subunit of the coupling factor.

MATERIALS AND METHODS

Treatment of Plant Material. Tomato plants (*Lycopersicon esculentum* Mill. cv Floramerica) were grown from seed under a 14 h, 29°C/10 h, 25°C light/dark regime and were fertilized twice weekly with half-strength Marvel 12-31-14 plant food (Plant Marvel Labs, Chicago, IL) supplemented with 5 mM KNO_3. Eighteen-d-old seedlings on which the third leaf was well expanded were used for experiments. Plants were chilled at 2 to 4°C for 16 h in darkness at 100% RH and allowed to rewarm in darkness at room temperature (22–24°C) and 100% RH. Control plants were maintained in darkness at room temperature and 100% RH for 16 h plus a length of time equivalent to the recovery period of the chilled plants. Plants to be used for thylakoid isolations were maintained in the growth chamber and used during the lighted portion of the diurnal cycle.

Labeling Conditions and Extraction of Proteins. Leaves incorporated label while still attached to the plant. A portion of the

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[2] Present address: Division of Biological Sciences, University of Missouri, Columbia, MO 65211.

[3] Abbreviations: LHCP-II: light-harvesting Chl *a/b* protein(s) of photosystem II; IgG, immunoglobulin G; SSU and LSU of Rubisco, small subunit and large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase.

oldest leaflet of the third leaf was abraded lightly with 400 grit carborundum. Fifty μCi [^{35}S]methionine (specific activity >800 Ci/mmol, Amersham) were applied in a 25 μL droplet to the abraded surface. The area was covered with a small square of Saran Wrap to prevent evaporation from the abraded surface. For protein synthesis inhibitor studies, the entire leaflet was abraded and then submerged in a solution of 1% Tween 20 containing the appropriate inhibitor. Excess inhibitor was rinsed away and the leaflet then labelled in the manner described above. Plants were manipulated under dim green light.

Following labeling, the leaflets were harvested and rinsed in ice cold, nonradioactive 1 mM L-methionine. The labeled portions were ground in Laemmli sample buffer (22) supplemented with 2 mM phenyl methylsulfonyl fluoride and 10 mM ascorbate. The homogenates were vortexed with polyvinylpolypyrrolidone (PVPP) and microfuged for 4 min to remove PVPP and insoluble cell debris. The supernatants were boiled for 2 min and stored at −80°C. Samples to be used for immunoprecipitations were treated similarly except proteins were extracted into 50 mM Tris-HCl (pH 7.5), 10 mM L-methionine, 5 mM EDTA, 4% SDS (w/v), 5 units/ml aprotinin (Sigma), and 10 mM ascorbate (grinding buffer).

Isolation of Chloroplast Thylakoid Membranes. Thylakoids were isolated from plants that had been labeled by painting 300 μCi of [^{35}S]methionine in 1 mL 1% Tween 20 on lightly abraded upper leaf surfaces. After 3 h under room light the abraded leaf surfaces were rinsed in cold methionine as described above. Labeled, rinsed leaves were de-veined and homogenized in a Virtis homogenizer at high speed for no more than 5 s in a grinding medium containing 50 mM Mes-KOH (pH 6.5), 0.3 M NaCl, 10 mM KCl, 2 mM EDTA, 1 mM EGTA, 0.2% (w/v) fatty acid free BSA, and 10 mM ascorbate. The homogenate was filtered through 16 layers of cheesecloth, and the filtrate was centrifuged for 2 min at 2400*g*. To promote the removal of adhering stromal proteins, the resulting pellet was resuspended with a soft paintbrush in a low osmotic strength medium containing 5 mM Mes-KOH (pH 6.5), 50 mM sorbitol, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM ascorbate. The sample was centrifuged for 15 s and then filtered through a Kimwipe to remove cells and large particles. The filtrate was then centrifuged for 4 min at 2500*g*. The pellet was resuspended in the same low osmoticum medium and recentrifuged for 4 min at 2500*g*. The final thylakoid pellet was resuspended in 0.5 mL of medium containing 5 mM Mes-KOH (pH 6.5), 0.4 M sorbitol, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM ascorbate. All manipulations were performed at 4°C. The labeled thylakoids were stored at −80°C until they were solubilized for polypeptide analysis.

Determination of Protein, Chl, and Incorporation of Radioactivity. Protein content of leaf extracts was determined by the method of Peterson (28). TCA precipitable radioactivity was determined as described in (23).

The Chl concentration was calculated according to equations derived elsewhere (13) using the specific absorption coefficients for Chl *a* and *b* published by Ziegler and Egle (37). Thylakoids were diluted into 80% acetone, and leaflets were homogenized in 80% acetone in a ground glass homogenizer. All samples were centrifuged to remove insoluble compounds prior to the Chl measurement.

Gel Electrophoresis of Proteins. Total leaf proteins were separated by SDS-PAGE using the buffer system of Laemmli (22). Two-dimensional gel electrophoresis was by the method of O'Farrell (27) as modified by Zurfluh and Guilfoyle (38). The SDS gels in both systems were 12% in acrylamide (w/v). Thylakoid membranes were solubilized in Laemmli sample buffer, boiled two min, and separated on SDS gels with a linear acrylamide gradient of 12 to 17.5% (w/v). Estimation of M_r was based on Bio-Rad low M_r standards run alongside samples. Proteins were stained with 0.025% Coomassie brilliant blue R in 25% isopropanol, 10% acetic acid, and gels were destained with 7.5% acetic acid. For detection of labeled proteins, Kodak XAR-5 X-ray film was exposed to dried gels. Gels intended for fluorography at −80°C were fixed overnight in 30% ethanol (v/v), 10% acetic acid (v/v) and impregnated with 2,5-diphenyloxazole (18) prior to drying.

Partial Proteolytic Cleavage of Proteins. *Staphylococcus aureus* V.8 protease (ICN ImmunoBiologicals, Lisle, IL) was used for limited digestion of proteins according to the method of Cleveland *et al.* (7). Gels on which thylakoid proteins were separated were stained for 30 min with Coomassie blue and destained briefly in 50% (v/v) methanol. The most abundant LHCP-II, which by our methods migrated at an apparent mol. wt. of 27 kD, was cut from the gel and equilibrated according to Cleveland's procedure. Bands cut from dried gels were rehydrated in 0.5% SDS (w/v) for 30 min prior to equilibration in Cleveland's buffer. Gel slices were loaded into slots of a second gel along with 0.5 μg V.8 protease. A 30 min digestion took place at the bottom of the stacking gel prior to electrophoretic separation of the proteolytic fragments on a 12 to 20% linear gradient polyacrylamide SDS gel containing 4 M urea. The peptide map was detected by fluorography.

Immunoblotting. Proteins were electrophoretically transferred from polyacrylamide gels to 0.2 μm nitrocellulose sheets (Schleicher and Schuell, Keene NH) using a Biorad Transblot cell operated at 40 V for 19 h. A portion of each gel was stained with Coomassie blue. The remainder of the gel was pre-equilibrated for 45 min in transfer buffer (12.5 mM Tris/96 mM glycine [pH 8.3], 20% methanol [v/v], 0.1% SDS [w/v]). Following transfer, one lane of the nitrocellulose filter was stained with 0.2% Coomassie blue R, 40% methanol, and 10% acetic acid and destained with 90% methanol, 2% acetic acid (5). The portion of the filter to be treated with antibodies was shaken overnight in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 3% fatty acid free BSA at 4°C to block remaining protein binding sites on the nitrocellulose filter.

Monoclonal antibodies MLH1 and MLH2 to LHCP-II proteins were the generous gifts of Dr. S. Darr. They were prepared from 50% ammonium sulfate precipitation of spent culture media. Precipitated MLH1 was solubilized and dialyzed against 20 mM Tris-HCl (pH 7.8) and 40 mM NaCl. MLH2 was resuspended and dialyzed against 20 mM Tris-HCl (pH 7.8), 375 mM NaCl, and 0.02% NaN_3 (w/v). The antibodies were supplied to us as lyophilized dialysates. The reaction of the filters with antibodies and the development procedure using alkaline phosphatase-conjugated goat anti-mouse IgG with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium was essentially that described by Darr *et al.* (11). The lyophilized antibodies were resuspended in 2 mL sterile deionized water. For detection of antigens, the stock solution of MLH1 was diluted 1:5000 and the solution of MLH2 was diluted 1:2500. Alkaline phosphatase-conjugated goat anti-mouse IgG (Boehringer-Mannheim, Indianapolis IN) was used as a 1:1000 dilution of the stock solution.

Detection of Radiolabeled Antigens by Immunoprecipitation. Indirect immunoprecipitations of radiolabeled antigens with the monoclonal antibodies were performed using a procedure based on that of Anderson and Blobel (1).

Equivalent amounts of radioactivity were taken from leaf extracts and diluted 1:1 in an Eppendorf tube with 'dilution buffer' (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 10 mM L-methionine, 5 mM EDTA, 5 units/ml aprotinin). Four volumes of 'Triton buffer' (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM L-methionine, 5 mM EDTA, 5 units/ml aprotinin, 2.5% [w/v] Triton X-100) were then added, which made the final detergent ratio 5 Triton:1 SDS by weight.

A volume of stock antibody solution was added to the samples. The volume added was proportional to the total volume of the sample so that the antibody concentration was identical in all the samples. Controls for each sample were diluted with a volume of 1:1:4 mixture of grinding/dilution/Triton buffers identical to the volume of antibody added. Samples were incubated overnight at 4°C to allow the antibody-antigen complexes to form.

When the primary antibody incubation was complete, Tachisorb M IgG immunoadsorbent (Calbiochem, La Jolla, CA), which is a suspension of heat-killed and fixed *Staphylococcus aureus* cells to which are conjugated goat anti-mouse IgGs, was added to samples and controls. The ratio of sample to Tachisorb was 1:1.2 by volume. The samples were further incubated for 1 h at room temperature with occasional vortexing to resuspend the immunoadsorbent.

The samples were microfuged for 20 min at 1500*g*. The immunoadsorbent pellets were washed and re-pelleted twice at 4C by microfuging for 1 min at 16,000*g*. The first wash was in 1 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 (w/v), 0.2% SDS (w/v), and the second wash was in 1 ml of the same buffer lacking detergents.

To release bound antigen, the final pellets were vortexed with 35 to 50 μL Laemmli sample buffer, boiled for 2 min, and microfuged at 16,000*g* for 3 min. The supernatants were removed and this process repeated. The supernatants were pooled and loaded onto a 12% polyacrylamide SDS gel. Radiolabeled immunoprecipitates were detected by fluorography.

RESULTS

Changes in Protein Synthesis Induced by Chilling. A time course experiment in which leaf protein synthesis was monitored after a 16 h chilling exposure showed that discrete changes in protein synthesis occurred as a result of the chilling treatment. It should be noted that the experimental procedure ensures only those proteins synthesized during the 30 min 'pulse' labeling interval are detected; previously accumulated proteins are unlabeled and thus not seen. The leaf synthesized a polypeptide of M_r = 35 kD in the first 0.5 h of rewarming (Fig. 1, lane B) that was absent in the control (lane A). Its synthesis reached a maximum after 2 to 3 h of rewarming, and thereafter declined to a low level (Fig. 1, lanes C–G). At the same time, there was a progressive loss of synthesis of a polypeptide of M_r = 27 kD in chilled leaves (Fig. 1, lanes B–G). Unchilled leaves normally synthesized relatively abundant levels of this polypeptide. (Fig. 1, lane A, and Fig. 2). Chilled plants rewarmed for 8 h in darkness completely lost the capacity to synthesize the 27 kD polypeptide (Fig. 1, lane G). However, when these plants are reilluminated the synthesis of this protein returned to the level of the unchilled control (Fig. 1, lane I).

The changes in protein synthesis described above were not the result of keeping the plants in darkness for prolonged periods. Unchilled control plants kept in darkness for the same total length of time as their chilled counterparts continued to synthesize the 27 kD protein for the length of the experiment (Fig. 2, lanes B–F). The 35 kD protein was not detectable in any of the controls.

Separation of proteins on two-dimensional gels showed the 35 kD protein exists as a single isoelectric species (Fig. 3B). The 27 kD protein synthesized by the control did not resolve well in these gels, running as a streak near the acidic end of the gel (Fig. 3A).

Identification of the 27 kD Polypeptide as LHCP-II. The abundance, size, and synthesis in response to light suggested the 27 kD protein whose synthesis is diminished by chilling might be the major Chl *a*/*b* binding protein of LHCP-II. We investigated this notion first by comparing the partial proteolytic digestion patterns of the 27 kD protein to that of authentic LHCP-II isolated from tomato thylakoid membranes (Fig. 4). The V.8 protease cleavage patterns of the 27 kD protein, whether isolated from leaves kept in darkness for 18 h (lane F) or maintained in the light (lane G), matched that of purified LHCP-II (lane E).

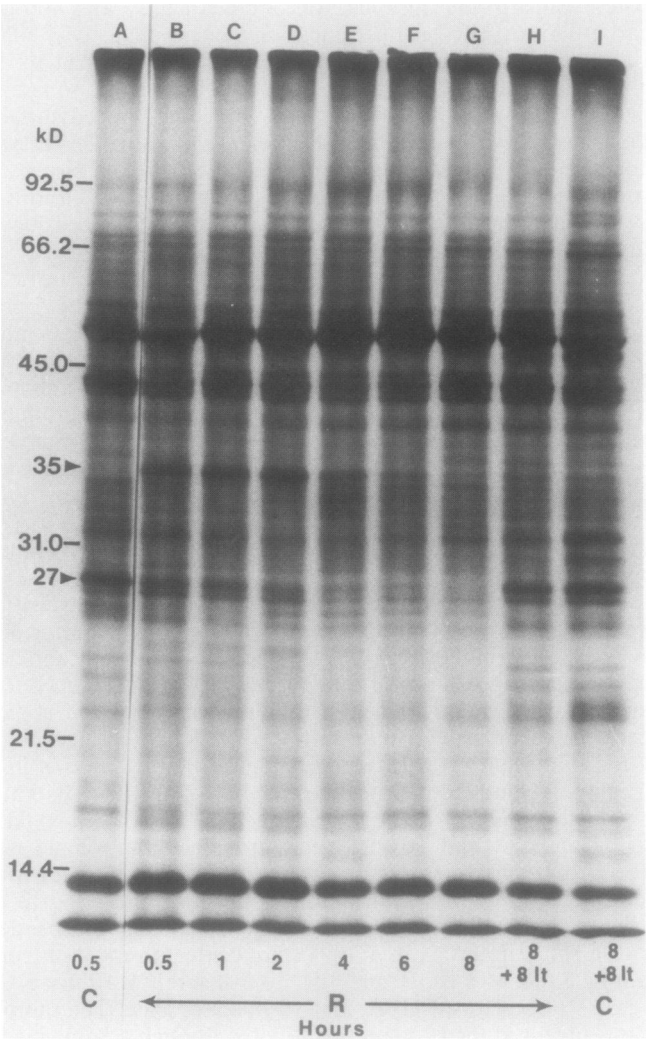

FIG. 1. Protein synthesis in tomato leaves after 16 h of chilling in darkness. Plants were rewarmed and incubated in darkness for increasing lengths of time. Leaves were pulse-labeled with [^{35}S]methionine during the final 30 min of treatment. Lanes: A, unchilled control, 16 + 0.5 h darkness; B, 0.5 h; C, 1 h; D, 2 h; E, 4 h; F, 6 h; G, 8 h rewarming after the 16 h of chilling; H, 16 h chilled +8 h rewarmed; I, unchilled control, 16 + 8 h darkness; H and I were returned to light for 8 h prior to labeling. Arrowheads indicate positions of 27 and 35 kD proteins whose synthesis is altered by chilling. Positions of M_r standards in kD are indicated at the left.

For further verification, we also tested for the antigenic relatedness of the 27 kD and LHCP-II by determining whether antibodies to LHCPs would precipitate radioactive 27 kD proteins from leaf extracts. It was first necessary to demonstrate that the monoclonal antibodies MLH1 and MLH2, which had been generated using pea LHCPs (11), would recognize tomato LHCPs in a specific manner. Western blot analysis of tomato thylakoid proteins showed that MLH1 and MLH2 specifically recognized two distinct LHCP-II (Fig. 5). MLH1 bound only the dominant 27 kD LHCP-II (Fig. 5, lane B), and MLH2 bound only the minor 30 kD LHCP-II component (Fig. 5, lane C) probably equivalent to CP-29 (11, 15, 16). A mixture of the two antibodies distinguished the two separate proteins (Fig. 5, lane D).

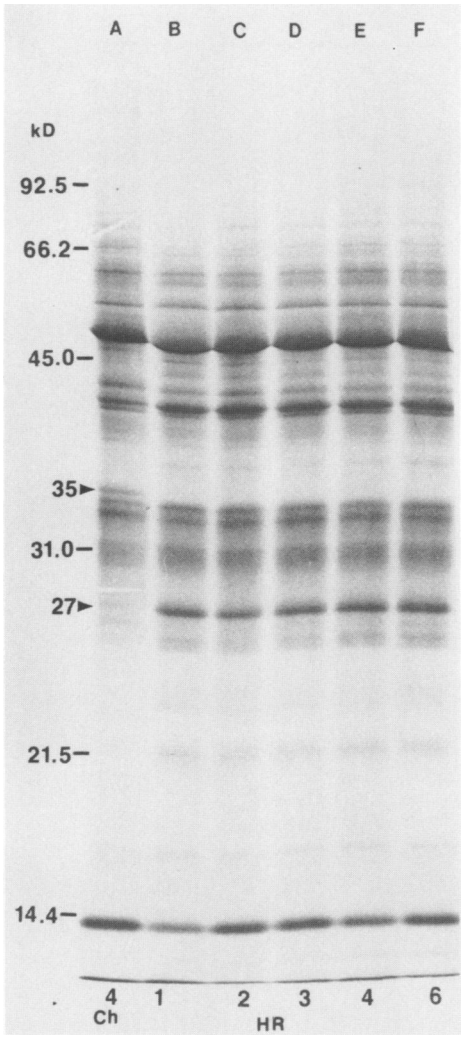

FIG. 2. Protein synthesis in unchilled tomato leaves when plants are maintained in continuous darkness. Isotope applied to leaves as in Figure 1. Lanes: B, 16 + 1 h; C, 16 + 2 h; D, 16 + 3 h; E, 16 + 4 h; F, 16 + 6 h. Lane A shows proteins synthesized in a 16 h dark-chilled plant rewarmed in darkness 4 h. Positions of M_r standards, 27 and 35 kD proteins are indicated at the left.

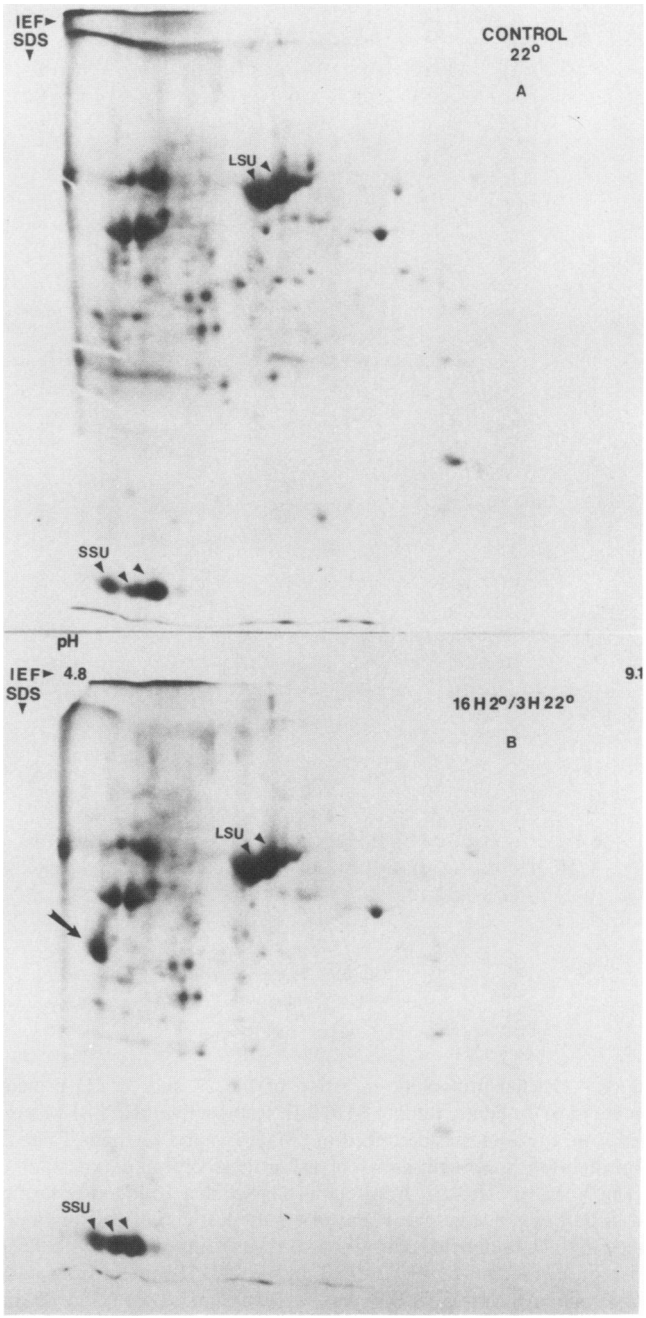

FIG. 3. Two-dimensional analysis of leaf proteins synthesized by A, unchilled plants maintained in darkness for 19 h, and B, plants chilled for 16 h and rewarmed in darkness for 3 h. Arrowheads indicate positions of Rubisco LSU and SSU. Arrow indicates the location of the chilling-induced 35 kD protein.

Having established that they would react specifically with tomato LHCPs, the antibodies were incubated with extract from labeled unchilled or chilled leaves (Fig. 6). Extracts were taken from plants chilled 16 h and rewarmed 2 or 4 h, or from control plants maintained in darkness for 18 or 20 h at room temperature. Leaves were labeled for the last hour of the treatments. The lanes of the gel in which total extracts were run show that chilled leaves (Fig. 6, lanes C and D) synthesized much less 27 kD protein than unchilled leaves (Fig. 6, lanes A and B). In chilled leaves rewarmed for 4 h, 27 kD protein synthesis was barely detectable (Fig. 6, lane D).

The antibody MLH1 precipitated radioactive 27 kD LHCP-II from all four extracts (Fig. 6, lanes E–H). The precipitated LHCP-II migrated in the gel to the same position as the 27 kD protein seen in total leaf extracts. The amount of LHCP-II precipitated corresponded to the level of 27 kD protein synthesis in each of the extracts. Some nonspecific binding of other abundantly synthesized proteins (*e.g.* Fig. 6, lane E) was a problem in samples in which large amounts of antibody were added in order to maintain a consistent sample-to-sample concentration of antibody. No protein was precipitated from leaf extracts incubated with buffer and Tachisorb only, and MLH2 did not precipitate detectable quantities of the 30 kD LHCP-II protein or any other protein from the extracts (not shown). The inability of MLH2 to precipitate any radioactive 30 kD LHCP-II probably resulted from very low levels of synthesis of the protein rather than nonreactivity of the antibody.

Although chilling significantly reduced the synthesis of the 27 kD LHCP-II, it had little if any effect on accumulated levels of the protein as judged from stained gels of thylakoid proteins (Fig. 8, lanes A–D) and from measurement of Chl a/b ratios. Leaves of lighted controls had an a/b ratio of 3.50 ± 0.06 ($n = 6$). Plants darkened for 16 h had a ratio of 3.59 ± 0.05 ($n = 6$) and plants

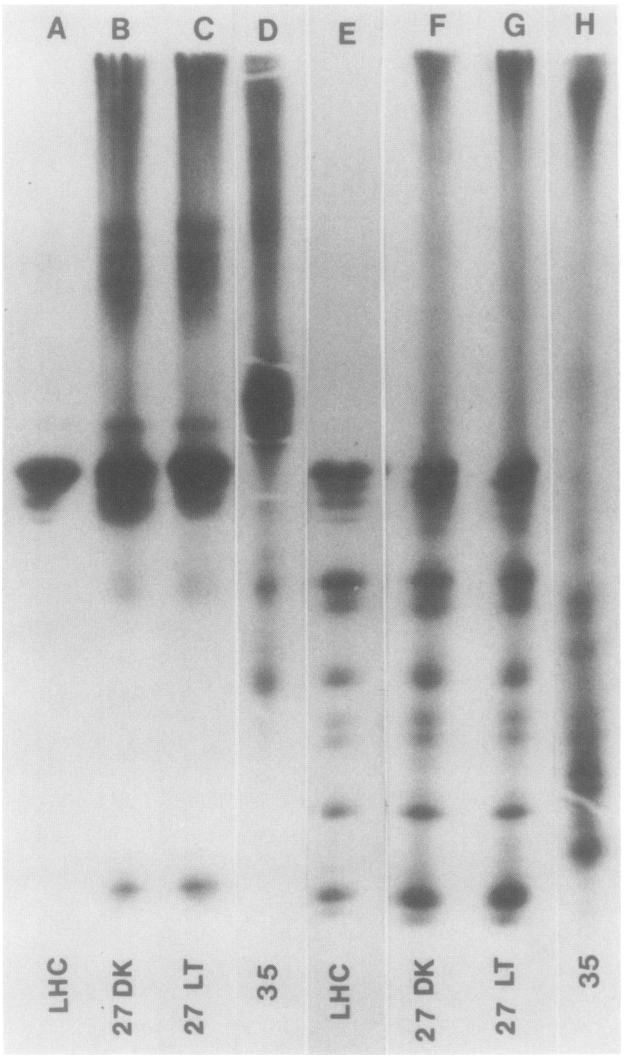

FIG. 4. Partial proteolytic digestion of the 27 and 35 kD proteins compared to authentic tomato LHCP-II. Radioactive LHCP-II was isolated from thylakoids as described in "Materials and Methods." The 27 kD protein was from light-grown plants or those kept in darkness for 18 h. The 35 kD protein was from plants dark-chilled 16 h and rewarmed 2 h in darkness. Leaves were labeled for the last 2 h of the treatment. Lanes: A to D, undigested controls; E to H, proteins digested with 0.5 μg V.8 protease. A and E, LHCP-II: B and F, 27 kD protein from dark-treated plants; C and G, 27 kD protein from light-grown plants; D and H, 35 kD protein from chilled plants.

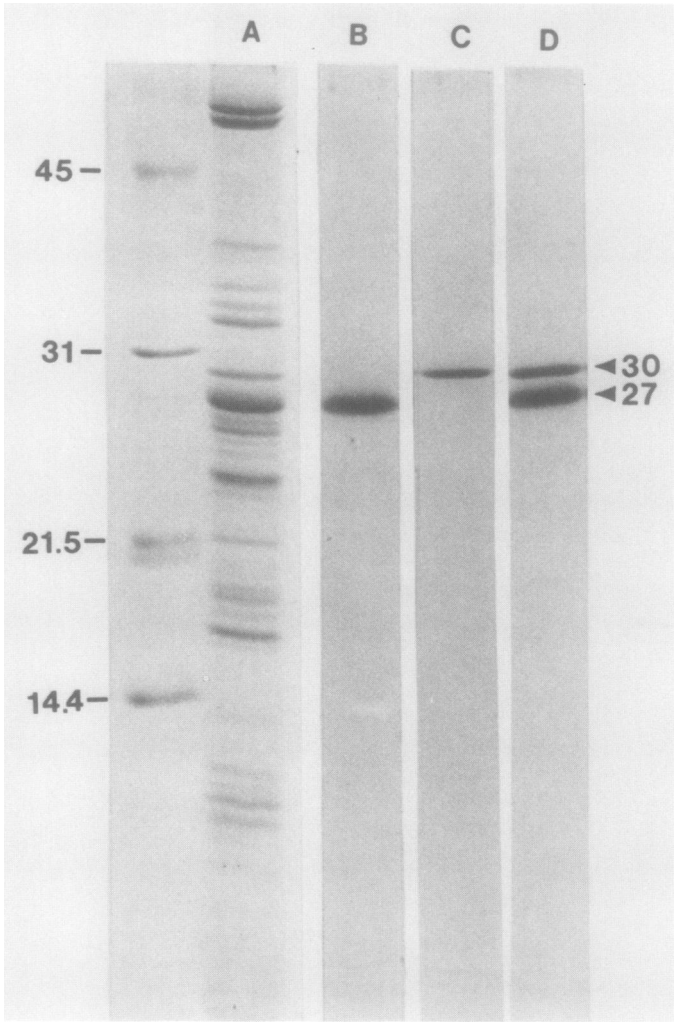

FIG. 5. Reaction of pea monoclonal antibodies MLH1 and MLH2 with tomato LHCP-II. Lane A, Coomassie blue stained tomato thylakoid proteins separated on a 12 to 17.5% linear gradient SDS polyacrylamide gel; B to D, blotted thylakoid proteins reacted with MLH1 (lane B), MLH2 (lane C), or a mixture of MLH1 and MLH2 (lane D). Standards of M_r in kD are indicated at the left. Positions of 27 and 30 kD LHCPs are indicated at the right.

chilled for 16 h in darkness had a ratio of 3.48 ± 0.09 (n = 6). These values did not change appreciably over the ensuing hours of incubation in darkness after rewarming.

Because the increase in synthesis of the 35 kD protein was nearly concomitant with the decline in synthesis of the 27 kD protein, we considered the possibility that a precursor-product relationship existed between these polypeptides. However, comparison of the V.8 protease digestion patterns of the 35 kD protein (Fig. 4, lane H) with the 27 kD protein (Fig. 4, lane F) showed that they have distinct amino acid sequences. Furthermore, MLH1 failed to precipitate a 35 kD protein from chilled leaf extracts (Fig. 6, lanes G and H).

Site of Synthesis of the 35 kD Polypeptide. The protein synthesis inhibitors cycloheximide and chloramphenicol were employed to determine whether the 35 kD protein was synthesized on cytoplasmic or organellar ribosomes. Plants were either chilled in the dark or maintained in the dark at room temperature for 16 h, then during the first 30 min after rewarming, a slightly abraded leaflet was submerged in 20 μg/ml cycloheximide or 200 μg/ml chloramphenicol dissolved in 1% (v/v) Tween 20. Controls were submerged in 1% Tween 20 alone. The leaves were rinsed and label was applied for 1 h prior to extraction of proteins.

Because the LSU of Rubisco is synthesized on chloroplast ribosomes and the SSU is synthesized on cytoplasmic ribosomes, the relative levels of Rubisco subunit synthesis served as internal controls for the efficacy and selectivity of the inhibitors. Cycloheximide-treated leaves synthesized the LSU but not the SSU (Fig. 7, lanes B and E). Chloramphenicol-treated leaves synthesized the SSU but not the LSU (Fig. 7, lanes C and F). Cycloheximide, but not chloramphenicol, inhibited the synthesis of the 35 kD polypeptide in chilled leaves (Fig. 7, lanes E and F). The same was true for the 27 kD polypeptide in unchilled leaves (Fig. 7, lanes B and C). Neither protein was detectable even when the film was overexposed (not shown).

Is the 35 kD Protein a Thylakoid Membrane Protein? Because we wished to determine whether the function of the chill-induced 35 kD protein was related to the photosynthetic process, we

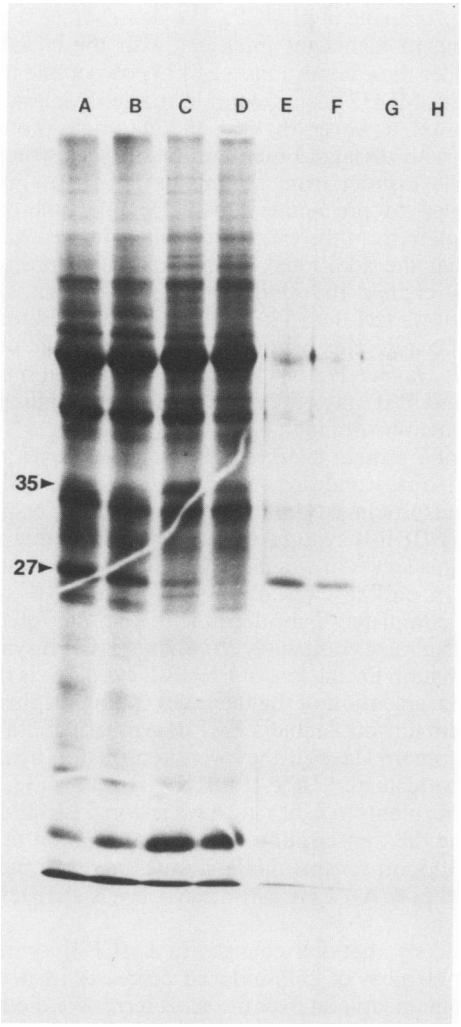

FIG. 6. Immunoprecipitation of radioactive 27 kD protein from leaf extracts by monoclonal antibody MLH1. Lanes: A to D, total unreacted leaf extracts; E to H, extracts incubated with MLH1 followed by Tachisorb. A, E, plant in darkness 18 h; B, F, plant in darkness 20 h; C, G, plant dark-chilled 16 h, rewarmed in darkness 2 h; D, H, plant dark-chilled 16 h, rewarmed in darkness 4 h. All samples in this fluorograph were run on the same gel. Lanes A to D contained 300,000 cpm/lane, and the film was exposed for 10 h. For lanes E to H, extracts contained 0.5×10^6 cpm/sample prior to reaction with antibodies and the film was exposed for 12 d.

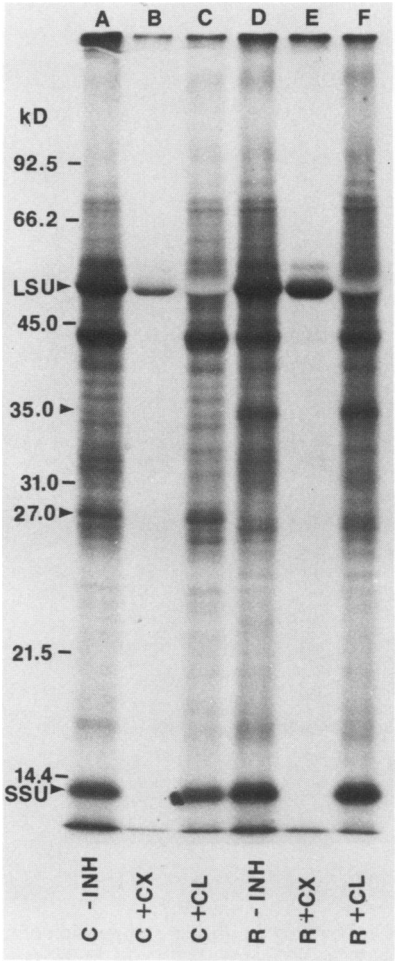

FIG. 7. Autoradiograph showing effects of cycloheximide and chloramphenicol on protein synthesis in chilled and unchilled leaves. Lanes: A to C, unchilled, D to F, chilled. A, D, 1% Tween 20 only; B, E, 20 μg/ml cycloheximide; C, F, 200 μg/mL chloramphenicol. Ten μg protein were loaded in each lane. Positions of LSU, SSU, 27, and 35 kD proteins indicated by arrowheads. Positions of M_r markers are indicated at the left.

isolated thylakoid membranes from chilled, [S^{35}]methionine labeled leaves to determine whether it was associated with this chloroplast subfraction.

The polypeptide composition of thylakoid membranes from chilled leaves was qualitatively identical to the thylakoid polypeptides from control leaves (Fig. 8). The 35 kD protein synthesized in chilled leaves was not associated with thylakoid membranes. However, several quantitative differences were apparent on both the stained gel and on its autoradiograph. The levels of newly synthesized β-subunit of the coupling factor and the dominant 27 kD LHCP-II were significantly reduced in thylakoids from chilled leaves (Fig. 8, lanes F and H) as compared to thylakoids from unchilled leaves (Fig. 8, lanes E and G). The same was true for two unidentified polypeptides of $M_r = 41$ and 14 kD. These differences were reflected in the levels of accumulated protein for the β-subunit and the unidentified polypeptides, but not for LHCP-II (Fig. 8, lanes A–D).

DISCUSSION

Tomato seedlings chilled at 2 to 4°C in darkness exhibited discrete changes in leaf protein synthesis during a subsequent dark period after rewarming. A 35 kD protein was synthesized within the first 30 min of rewarming. When [^{35}S]methionine was introduced into the leaf during the chilling exposure (*i.e.* at 2–4°C) exceedingly little radioactivity was incorporated and no synthesis of the 35 kD protein was detectable (data not shown). Thus, it is not clear whether the synthesis of this protein is induced at low temperature and continues after rewarming or if synthesis is initiated upon rewarming. It can be seen, however, that the rate of synthesis of the 35 kD protein increased slightly during the 3 to 4 h after rewarming. This behavior possibly suggests that the synthesis was initiated upon rewarming but a different sort of study will be required to specifically address this issue. Over the same period of time that the synthesis of the 35 kD protein was increasing, the synthesis of a 27 kD protein, abundantly synthesized in control leaves, exhibited a decline in chilled leaves.

Peptide mapping identified the 27 kD protein to be the dominant PSII Chl *a*/*b* binding protein. Identification of this protein as a LHCP-II was reinforced by the observation that the pea monoclonal antibody MLH1 (11) immunoprecipitated the ra-

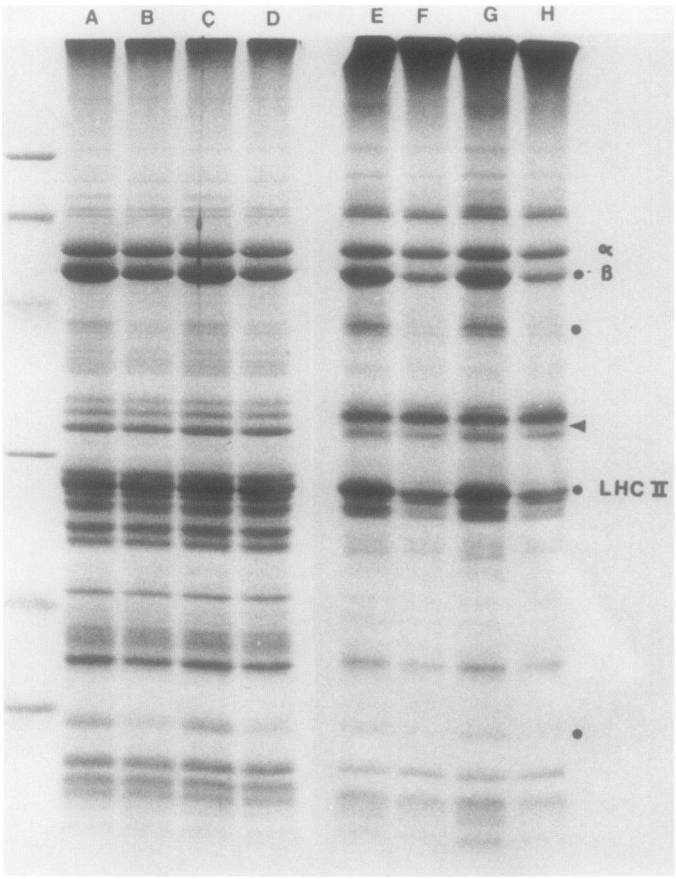

FIG. 8. Effect of chilling on protein composition of tomato thylakoid membranes. Leaves were labeled at 22°C for 3 h in darkness subsequent to 16 h in darkness at 22 or 2°C. Sample volumes equivalent to 20 μmol Chl were loaded in each lane. Lanes: A to D, Coomassie blue stained proteins; E to H, fluorograph of newly synthesized proteins present in membrane. Unchilled, lanes A, C, E, G; chilled, lanes B, D, F, H. Dots indicate proteins reduced in chilled leaves. α- and β-subunits of the coupling factor and the major LHCP-II indicated at right. Arrowhead denotes theoretical position of 35 kD protein calculated from M_r standard curve. M_r standards at far left, in kD from top to bottom: 92.5, 66.2, 45.0, 31.0, 21.5, 14.4.

dioactive 27 kD protein from tomato leaf extracts, and the amount of labeled, precipitated protein corresponded to the level of 27 kD synthesis in the leaf. By Western blot analysis we showed that MLH1 specifically recognized the dominant 27 kD LHCP-II of tomato, in agreement with the findings of Darr *et al.* (11), although these authors have sized the LHCP-II at 26 kD.

Multiple molecular forms of the LHCPs exist that vary somewhat in size but are similar in amino acid composition (17, 32). They are encoded by the nuclear genome and synthesized in the cytoplasm as soluble precursors (pLHCPs) that are 4 to 6 kD higher in molecular weight than the mature LHCPs as determined by SDS-PAGE (4, 32). The import of the pLHCPs into the chloroplast is a post-translational event (32). The first step is presumably recognition of the precursor by a putative receptor on the chloroplast envelope (3, 8, 10), followed by uptake and cleavage of the amino-terminal transit peptide (32) to form the mature LHCP. The cleavage step can occur after the insertion of pLHCP into the thylakoid membrane (6).

The appearance of the 35 kD protein concomitant with the decline in LHCP-II synthesis in the first hour after rewarming suggested to us that chilling might be interfering with the final uptake or processing of pLHCPs. Hayden *et al.* (15) have shown that chilling in high light interferes with the cleavage step in maize. Under these conditions a 31 kD polypeptide that is cross-reactive with MLH2 accumulates in maize thylakoids (16). Our results showed, however, that the 35 kD tomato protein was not associated with thylakoid membranes and was structurally and antigenically distinct from the major LHCP-II. Furthermore, MLH2 failed to precipitate any detectable quantities of the tomato protein from the extracts of chilled leaves. Although it is possible that the additional amino acids in a precursor could sufficiently change the ability of a monoclonal antibody to recognize it, we feel the 35 kD protein is not at all related to the LHCP-II because the peptide maps of the two proteins are distinct. As Hayden *et al.* (15) suggest, interruption of the processing of LHCP-II may only come about if the chilling treatment is in conjunction with high light.

Monitoring protein synthesis in unchilled leaves maintained under the same conditions of darkness as the chilled leaves showed that prolonged darkness alone was not responsible for the loss of LHCP-II synthesis. In this respect, tomato leaves are similar to pea leaves in which LHCP-II synthesis continues even after 2 d in darkness (2). By contrast, LHCP-II synthesis in *Lemna* is completely light-dependent; one day of darkness is sufficient to almost completely eliminate LHCP-II synthesis (34). One explanation for the loss of LHCP-II synthesis is that chilling causes the degradation of the messages coding for this protein or renders them untranslatable. LHCP-II synthesis could be restored in chilled tomato leaves if they were returned to light, and light is known to regulate LHCP-II mRNA synthesis (33, 34). Thus, returning the plants to light may have restored LCHP-II synthesis by inducing the transcription of a new population of functional mRNAs. Support for this model awaits the direct measurement of total and poly(A)$^+$ LHCP-II mRNA levels after rewarming of chilled leaves.

It is unlikely that the changes in LHCP-II synthesis are a significant element of chill-induced decreases in net photosynthesis of tomato, at least over the short term. We did not observe any effects of chilling on accumulated levels of the apoprotein or in Chl *a*/*b* ratios, and previous studies showed no effects on quantum yield or light saturation profile of photosynthesis (25). That is, the chill-induced reduction in LHCP-II synthesis had no significant effect on the photosystem II antenna size or on efficiency of energy transfer.

However, we did observe substantial decreases in the accumulated levels of three thylakoid polypeptides, including the β-subunit of the coupling factor. Santarius (30) has reported that low temperatures can release the coupling factor from isolated spinach thylakoid membranes. Although this may occur in tomato, we cannot disclude the possibility that chilling may alter the turnover rates of these proteins. Certainly the levels of newly synthesized proteins inserted into the membrane are reduced as a consequence of chilling treatment. Irrespective of the mechanism by which the levels of the proteins in the membrane decline, it is conceivable that such a loss can contribute in some way to an overall reduction in net photosynthesis. The decrease in the level of the β-subunit of the coupling factor is particularly intriguing. We are currently investigating the relaxation kinetics of the flash-induced electrochromic absorption change in attached leaves in order to determine whether the photophosphorylation competence of thylakoid membranes has been adversely affected by chilling (35).

The 35 kD protein is apparently unrelated to LHCP-II, and seems to be synthesized by tomato leaves as a direct response to chilling stress. Inhibitor studies showed the synthesis occurred in the cytoplasm, so the protein is almost certainly encoded by the nuclear genome. Once synthesized, the protein does not undergo

extensive post-translational modification, as two-dimensional gels showed it to exist as a single isoelectric species.

Plants are known to synthesize novel polypeptides in response to salt (12), anaerobic (21, 29), and high temperature (9) stress. Recently it has been shown that spinach (14) and maize (36) synthesize proteins subsequent to cold treatments, although these plants exhibit more extensive changes in the pattern of protein synthesis than we observed for tomato. The functional identities of most proteins induced by stress are unknown. In a few cases where the functions have been identified, as is the case for the maize anaerobic proteins (21, 29), the activity of the protein serves to correct or maintain those aspects of metabolism which have been perturbed by the inducing stress. This has led to the generally held concept that stress proteins function to homeostatically regulate metabolism.

A vast literature exists which supports the idea that organism's ability to tolerate stress is directly related to its capacity to synthesize stress proteins. However, our observations regarding the chilling response tomato has prompted us to consider an alternative possiblity. Tomato is a plant which is quite sensitive to chilling damage, yet it synthesizes a novel 35 kD polypeptide in response to low temperature treatment. Thus it may be a plant's sensitivity to stress rather than its tolerance that dictates the need and the capacity for synthesis of stress proteins. Kanabus *et al.* (19) put forth a similar idea after observing that cultured tobacco cells could synthesize more heat shock proteins during the portion of the growth phase in which they were most susceptible to killing by high temperatures. Keeping this alternative hypothesis in mind, we are continuing to investigate potential functions of the 35 kD protein, and what role, if any, it plays in the photosynthetic response of tomatoes to chilling.

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