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ORIGINAL ARTICLE

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Temperature-induced greening of *Chlorella vulgaris*. The role of the cellular energy balance and zeaxanthin-dependent nonphotochemical quenching

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Abstract When cells of the green alga *Chlorella vulgaris* Beij. are transferred from growth at 5 °C and an irradiance of 150 μmol photons m^{-2} s^{-1} to 27 °C and the same irradiance, they undergo what is normally considered a high-light to low-light phenotypic change. This involves a 3-fold increase in cellular chlorophyll content with a concomitant increase in light-harvesting complex polypeptide levels. This process appears to occur in response to the cellular capacity to utilize the products of photosynthesis, with the redox state of the plastoquinone pool sensing the cellular energy balance. The phenotypic adjustment can be enhanced or blocked using chemical inhibitors that modulate the redox state of the plastoquinone pool. The functional changes in the photosynthetic apparatus that occurred during the high-light to low-light acclimation were examined with special consideration paid to the paradox that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-treated cells, with non-functional photosystem II (PSII), accumulate light-harvesting polypeptides. At the structural and basic functional levels, the light-harvesting complex of the cells treated with DCMU was indistinguishable from that of the untreated, control cells. To examine how PSII was protected in the DCMU-treated cells, we measured the content of xanthophyll-cycle pigments. It appeared that a zeaxanthin-dependent nonphotochemical quenching process was involved in PSII protection during greening in the presence of DCMU. Metabolic inhibitors of mitochondrial respiration were used to examine how the change in cellular energy balance regulates the greening process. Apparently, the mitochondrion acts to supply energy to the chloroplast during greening, and inhibition of mitochondrial respiration diminishes chlorophyll accumulation apparently through an increase in the redox state of the plastoquinone pool.

Keywords Cellular energy balance · *Chlorella* Greening · Plastoquinone pool (redox state) Photosystem II · Nonphotochemical quenching

Abbreviations Chl: chlorophyll · Cyt: cytochrome · DBMIB: 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone · DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea · EPS: epoxidation state · LHC: light-harvesting complex · PQ: plastoquinone · PS: photosystem · q_N: nonphotochemical quenching of Chl *a* fluorescence · q_P: photochemical quenching of Chl *a* fluorescence · TPTC: triphenyl tin chloride.

Introduction

The growth of photosynthetic organisms at low temperature elicits many of the same responses as growth under high light (Huner et al. 1998). This is true in higher plants, green algae and cyanobacteria (Maxwell et al. 1994; Gray et al. 1997; Miskiewicz et al. 2000; Wilson and Huner 2000). However, temperature and light appear to have interactive effects on photosynthesis and its regulation (Huner et al. 1998; Wilson and Huner 2000). Thus, moderate temperature and high light can elicit the same high-light phenotypic response as low temperature and moderate light. At low temperature a low-light phenotype can still be observed but only at extremely low light levels (Gray et al. 1997; Wilson and Huner 2000). Growth of the green alga *Chlorella vulgaris* under moderate temperature and high light [27 °C and 1,200 μmol photons m^{-2} s^{-1} (27/1,200)] or under low temperature and moderate light (5/150) induces a high-light phenotype, characterized by a distinctive yellow

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pigmentation and high chlorophyll (Chl) *a:b* ratios. In contrast, growth at 27/50 or 5/50 resulted in a low-light phenotype, characterized by dark-green pigmentation and lower Chl *a:b* ratios (Maxwell et al. 1994; Wilson and Huner 2000). Thus, *C. vulgaris* adjusts the level of light-harvesting complex II (LHCII) polypeptides, Chl per cell, xanthophyll accumulation and the xanthophyll-cycle pigment epoxidation state (EPS) in response to different combinations of light intensity and temperature during steady-state growth (Maxwell et al. 1994; Wilson and Huner 2000). The plasticity of this phenotypic response can be observed when *C. vulgaris* cells are transferred from 5/150 to 27 °C and the same irradiance (27/150). The culture rapidly turns from yellow to green due to the accumulation of both Chl and LHCII polypeptides (Wilson and Huner 2000). This conversion from a high-light to a low-light phenotype was correlated with the oxidation of the plastoquinone (PQ) pool and diminishment of the trans-thylakoid pH gradient (Wilson and Huner 2000). The PQ-pool redox state and the thylakoid ΔpH are, in turn, a reflection of the cellular capacity to utilize the absorbed light energy and the products of photosynthesis for growth (Savitch et al. 1996; Wilson and Huner 2000; Paul and Foyer 2001).

The redox state of the PQ has been implicated as a mediator of a number of photosynthetic responses, including but not limited to the regulation of state-transitions, Chl biosynthesis, LHC polypeptide accumulation, rates of photosystem protein synthesis and the balance of photosystem stoichiometry (Allen et al. 1981, 1995; Escoubas et al. 1995; Maxwell et al. 1995; Melis et al. 1996; Pfannschmidt et al. 1999). There is a consensus that the PQ pool, localized between photosystems I and II (PSI and PSII, respectively) acts as a sensor of imbalances in electron transport (Allen 1995; Durnford and Falkowski 1997).

A high-light to low-light phenotypic change is also observed in many algal species upon treatment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a chemical which inhibits the capacity of PSII to reduce PQ, resulting in a highly oxidized PQ pool (Beale and Appleman 1971; Koenig 1990; Nauš and Melis 1992; Escoubas et al. 1995). In green algae, an oxidized PQ pool normally induces a change from State I to State II within a few minutes (Allen 1995), followed within hours by an increase in the accumulation of Chl and LHCII polypeptides (Nauš and Melis 1992; Escoubas et al. 1995; Maxwell et al. 1995). *Dunaliella salina*, a green alga that responds phenotypically to PQ redox state in a manner similar to *C. vulgaris* (Maxwell et al. 1995), also exhibits a concomitant change in the PSII:PSI ratio such that there is relatively more PSII following treatment with DCMU (Nauš and Melis 1992). This creates a paradox, whereby conditions that inhibit electron transport increase the light-harvesting capacity and hence the amount of light energy absorbed by the PSII antenna complexes.

The mechanism by which the greening of algal cells occurs in the presence of DCMU has never been addressed experimentally. It is hypothesized that *C. vulgaris* greens in the presence of DCMU because the redox sensor, PQ, remains in the oxidized state, which up-regulates LHCII polypeptide and Chl accumulation (Escoubas et al. 1995). However, the very characteristics that make Chl a good pigment for photosynthesis also make Chl a potent photosensitizing agent (Spikes and Bommer 1991). Excited-state Chl can spontaneously decay back to ground state with the possibility of an intersystem-crossing event generating triplet-state Chl (Spikes and Bommer 1991). Triplet-state Chl can then interact with O_2 to produce reactive oxygen species (ROS), which can damage cellular components (Spikes and Bommer 1991; Osmond et al. 1997). Not only will the PSII reaction centers of the DCMU-treated cells be unable to utilize the excitation energy from the antenna Chl (Melis 1996), the greening process continually increases the likelihood that more light will be absorbed. Because DCMU-treated cells accumulated more Chl, the cells should be under an increased level of PSII excitation pressure leading to greater photo-oxidative stress. Therefore, a mechanism must exist which acts to protect PSII and LHCII from over-excitation as the cells undergo temperature-dependent greening in the presence of DCMU. Two possible mechanisms that would protect PSII during greening in the presence of DCMU are nonphotochemical quenching either in the antenna (Niyogi et al. 1998; Niyogi 1999) or the reaction center itself (Bruce et al. 1997; Bukhov et al. 2001), or through the misassembly of the LHC polypeptides, such that they do not transfer excitation energy to PSII (Hippler et al. 2000).

Another important question regarding the greening of algal cells in the presence of DCMU is the supply of ATP and NADPH that is required for the production of Chl and the translation of chloroplast proteins. The activity of DCMU blocks linear electron transport (Trebst 1980). Therefore, ATP and NADPH levels should be rapidly diminished (Nicholls and Ferguson 1992; Cornic et al. 2000). Thus, it seems that the mitochondrion may play a significant role when algal cells green in the presence of DCMU, for the supply of ATP, reducing power, and the carbon skeletons required for Chl biosynthesis.

In this study we examined the accumulation of the photosynthetic electron transport chain components during the greening process that occurred when *C. vulgaris* cells were transferred from growth at 5/150 to 27/150. Because DCMU-treated cells somewhat paradoxically green under conditions where PSII electron transport was blocked, we investigated whether the greening process was the same at the structural/functional level of the photosynthetic apparatus in DCMU-treated cells as in untreated cells. To this end we used inhibitors of both photosynthetic and mitochondrial electron transport to examine where the cellular energy for greening is obtained and whether a photoprotective mechanism exists which allows DCMU-treated cells to avoid photo-oxidative damage.

Materials and methods

Culture conditions

Cells of *Chlorella vulgaris* Beij. (UTEX 265) were grown axenically in modified Bold's Basal Medium (5.89 mM $NaNO_3$) buffered with 5 mM Hepes:KOH (pH 7.2; Nichols and Bold 1965) and bubbled vigorously with air to keep the culture mixed and aerated, as described previously (Wilson and Huner 2000). To examine the response of cells to a temperature-induced change in PQ redox state, cells were initially grown at 5/150 to a Chl concentration of 2 μg Chl ml^{-1}. Subsequently, the culture tube was transferred to a temperature of 27 °C with the irradiance maintained at 150 μmol photons m^{-2} s^{-1}. Following the transfer, the cellular Chl content and Chl *a* fluorescence characteristics were monitored as a function of time. The effects of different metabolic inhibitors on the greening process were examined during the first 12 h following the transfer of the cells to 27 °C. The following inhibitors were dissolved in 95% (v/v) ethanol and employed at the concentration given: DCMU (1.0 μM), 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB; 10 μM), antimycin A (1 μM) and triphenyl tin chloride (TPTC; 1 μM). To ensure that the ethanol used to dissolve the inhibitors had no effect, control measurements were made in the presence of equivalent volumes of ethanol. Additionally, inhibitor experiments were always carried out in tandem with untreated control to allow direct comparisons to be made. The photon flux density was measured using a Li-Cor Model LI-189 radiometer equipped with a quantum sensor (Li-Cor, Lincoln, Neb., USA).

Pigment analysis

Total Chl and Chl *a:b* ratios were determined as described previously (Maxwell et al. 1994). Pigments were extracted with 90% (v/v) acetone and the equations of Jeffery and Humphrey (1975), were used to calculate Chl concentrations. The amounts of individual carotenoids and Chl *a* were measured by HPLC, as reported by Gray et al. (1996). Total xanthophyll-cycle pigment pool size was calculated as mmol of violaxanthin + antheraxanthin + zeaxanthin (V + A + Z) per mol of Chl *a* and the relative epoxidation state of the xanthophyll-cycle pigments was determined as (V + 0.5A)/(V + A + Z) (Thayer and Björkman 1990).

SDS–PAGE and immunoblotting

Cells were harvested during early- to mid-log phase by centrifugation at 1,500 *g* for 5 min. The thylakoid membrane fraction was isolated according to Wilson and Huner (2000). The protein concentration of the soluble and membrane fractions was determined using the Bio-Rad DC system following the instructions provided with the kit by the manufacturer, and samples containing 20 μg of protein were electrophoresed and immunoblotted as described previously (Wilson and Huner 2000). The nitrocellulose membranes were challenged with polyclonal antibodies raised against: LHCII from *Dunaliella salina*, the D1 protein of PSII from *Synechocystis* sp. PCC 6803, cytochrome *f* (Cyt *f*) of the Cyt b_6/f complex from spinach, psaA/B the PSI reaction center heterodimer of barley or against the Rubisco holocomplex of rye. The antibody complexes were visualized after incubation with a goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) using the ECL system (Amersham). The photographic films were digitally imaged and the intensity of the spots was estimated using Scion Image software (Scion Corp, Frederick, Md., USA).

Measurement of O_2 evolution

Measurements of O_2 evolution and cellular respiration were carried out according to Maxwell et al. (1994) using an aqueous phase Clark-type electrode and Hansetech O_2 electrode control box (Hansetech Instruments, King's Lynn, Norfolk, UK). The temperature of the cuvette was maintained at either 5 °C or 27 °C using a refrigerated water bath. Measurements were made on 2-ml culture samples at 27 °C and 150 μmol photons m^{-2} s^{-1} following the transfer of the cells from 5/150. Estimates of cellular respiration were made immediately following photosynthesis measurements and, therefore, represent the light-enhanced rate of respiration (Xue et al. 1996). To determine the optimum concentration of DCMU, DBMIB, antimycin A or TPTC, the chemicals were added to culture samples and O_2 evolution and consumption were measured, then the inhibitor was added and the measurements were repeated. During the shift experiments, samples were measured at each time point to ensure that O_2 evolution and consumption were inhibited in comparison to the non-treated control cultures.

Chlorophyll fluorescence measurements

Room-temperature Chl fluorescence measurements were made using a PAM fluorometer (PAM-103; Heinz Walz, Effeltrich, Germany), as described previously (Wilson and Huner 2000). While the fluorescence measurements were made, the temperature of the cuvette was maintained at either 5 °C or 27 °C using a refrigerated water bath. Fluorescence quenching parameters were calculated according to van Kooten and Snel (1990) [$q_P = (F_M' - F_S)/(F_M' - F_O')$ and $q_N = (F_M' - F_O')/(F_M - F_O)$]. Excitation pressure was determined as $1 - q_P$ [$1 - q_P = (F_S - F_O')/(F_M' - F_O')$], and was used to estimate the PQ-pool redox state (Tullberg et al. 2000).

Chlorophyll fluorescence measurements were also made at the temperature of liquid N_2 (77 K) to examine the relative fluorescence emission from PSI and PSII and their respective light-harvesting complexes. Isolated thylakoid membranes were diluted with Tricine buffer [50 mM Tricine:NaOH (pH 7.8), 10 mM NaCl, 5 mM MgCl] containing 50% (v/v) glycerol to a Chl concentration of 5 μg/ml. A 0.75-ml aliquot of each sample was placed in a 5-mm NMR tube (Alpha Scientific, Vineland, N.J., USA) and dark-adapted for 15 min at room temperature. The samples were then rapidly frozen in liquid N_2. At 77 K, the samples were excited at 436 nm and the corrected emission spectrum was collected from 650 to 800 nm. The emission spectrum of each sample was scanned 3 times and averaged. For presentation purposes, the spectra were normalized to the emission at 705 nm.

Separation of chlorophyll–protein complexes

Isolated thylakoid membranes were washed and solubilized as described by Huner et al. (1987). The samples were then loaded on an equal-Chl basis and separated electrophoretically at 4 °C in the dark (Waldron and Anderson 1979). The Chl–protein complexes were quantified as a percent of total Chl by scanning the gel lanes at 671 nm.

Results

Temperature induced greening of *C. vulgaris*

As described previously (Wilson and Huner 2000), when *C. vulgaris* cells were transferred from 5/150 to 27/150 the amount of Chl on a per-cell basis increased approximately 3-fold in the first 12 h (Fig. 1A, open circles). Concomitantly, the Chl *a:b* ratio declined to approximately 50% of the initial value (Fig. 1B, open circles), representing a change from 9.5 to 5.0. This change in Chl content resulted in the culture becoming visibly green, compared to the yellow-green color of the cells grown at 5/150. Thus, it appeared that the cells

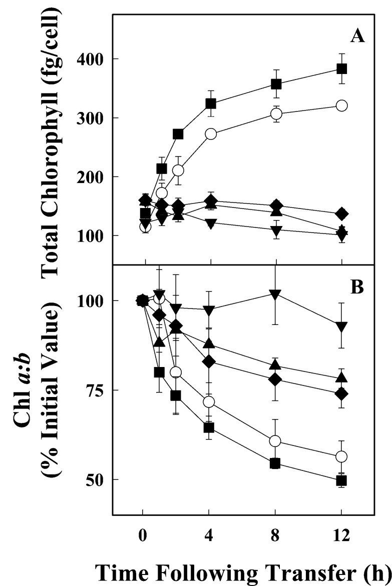

Fig. 1A, B The effect of metabolic inhibitors on changes in cellular Chl content (**A**) and Chl *a:b* ratio (**B**). Cells of *Chlorella vulgaris* were transferred from growth at 5/150 to 27/150 in the presence of the following: 1.0 µM DCMU (*black squares*), 10.0 µM DBMIB (*black triangles*), 1.0 µM antimycin A (*black diamonds*), or 1 µM TPTC (*black inverted triangles*). Control cells were treated with an equivalent volume of ethanol (*open circles*). All values represent means ± SE, $n = 6$ (when not visible, error bars are smaller than the symbol)

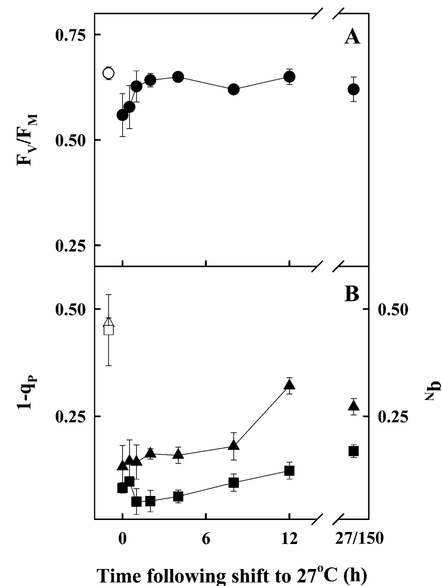

Fig. 2A, B Steady-state Chl fluorescence quenching parameters of *C. vulgaris* cultures. **A** Maximal photochemical efficiency of dark-adapted cells was measured as F_V/F_M. The *open circle* represents F_V/F_M of cells grown at 5/150 and measured at 5 °C. The *closed circles* reflect F_V/F_M measurements of cells following their shift to 27/150, measured at 27 °C, which can be compared to cells grown at 27/150. **B** The quenching parameters $1-q_p$ and q_N measured at 5/150 (*open triangle* and *square*, respectively) and at 27/150 (*closed triangles* and *squares*) following a shift to those growth conditions can also be compared to the quenching parameters of cells grown at 27/150. All values represent means ± SE, $n = 4$

underwent a typical high-light to low-light phenotypic acclimation during the 12 h following the shift from 5/150 to 27/150.

The functional aspects of this change in pigmentation were examined using Chl *a* fluorescence. Cells of *C. vulgaris* grown and acclimated at 5/150 had a relatively high PSII photochemical efficiency ($F_V/F_M =$ 0.658), indicating that the cells were not chronically photoinhibited (Fig. 2A, open circle). Furthermore, cells acclimated to growth at 5/150 exhibited exponential growth (data not shown). Following transfer to 27/150 (0 h) there was an initial decrease in F_V/F_M (Fig. 2A, closed circles). While F_V/F_M measures the dark-adapted efficiency of PSII, $1-q_P$ estimates the relative redox state of Q_A (Schreiber et al. 1994). When grown at 5/150, *C. vulgaris* exhibited a steady state $1-q_P$ of 0.467 (Fig. 2B, open triangle). When the cells grown at 5/150 were subsequently transferred to and measured at 27/150, $1-q_P$ decreased to 0.132, remained low during the first 8 h and then increased to 0.300 after 12 h, a level similar to that in cells grown at 27/150 (Fig. 2B, closed triangles). Thus, it appeared that PSII was maintained in a more oxidized state immediately following the shift to 27/150 than during growth at 5/150. Nonphotochemical quenching is a measure of the capacity to dissipate excess light energy under steady-state conditions (Niyogi 1999). The value of q_N primarily reflects changes in xanthophyll-cycle pigments and the trans-thylakoid ΔpH (Schreiber et al. 1994). When cells were grown at 5/150 the steady state q_N was 0.451 (Fig. 2B, open square); this decreased to 0.083 when cells were transferred to and measured at 27/150 (Fig. 2B, first closed square). During continued growth at 27 °C, q_N gradually recovered to 0.160, which was similar to the value observed in cells grown at 27/150 (Fig. 2B, closed squares). Thus, after 12 h at 27/150, the 5/150-grown cells appeared to be similar photosynthetically to the cells grown at 27/150.

When cells grown at 5/150 were measured at 5 °C, the net rate of O_2 evolution at 150 µmol photons m^{-2} s^{-1} was 0.200 µmol O_2 evolved (10^7 cells)$^{-1}$ h^{-1} (Fig. 3A, open circle) and the rate of cellular respiration was 0.170 µmol O_2 consumed (10^7 cells)$^{-1}$ h^{-1} (Fig. 3B, open circle). At the start of the shift to 27/150, a sample of the culture was warmed up to 27 °C for 4 min to examine the initial effect of the transfer to the new growth conditions. When the cells were measured at 27/150, the net rate of photosynthesis decreased to 0.120 µmol O_2 evolved (10^7 cells)$^{-1}$ h^{-1} (Fig. 3A, first closed circle), at the same time the rate of respiration increased dramatically to 0.530 µmol O_2 consumed (10^7 cells)$^{-1}$ h^{-1} (Fig. 3B, first closed circle), an increase of over 300%. The combination of the decrease in the rate of photosynthesis and increase in the rate of respiration upon the initial transfer of the cells to 27 °C resulted in the overall consumption of O_2 by the cells in the light. Therefore, immediately after the shift in temperature, the ability of the cells to use energy was much greater than their

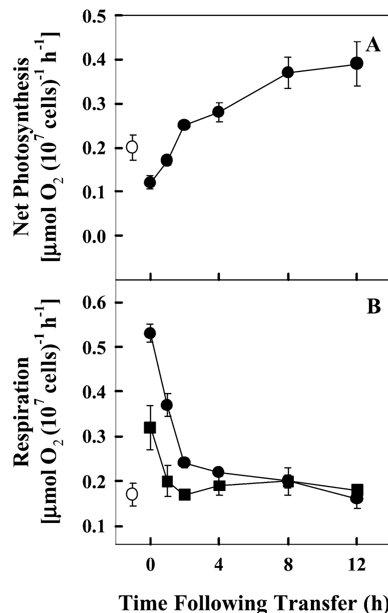

Fig. 3A, B Rates of net photosynthesis (**A**) and cellular respiration (**B**) measured on a per-cell basis at 5/150 prior to *C. vulgaris* cells being shifted (*open circles*), and following a shift to 27/150. The effect of adding antimycin A (*black squares*) is compared to control cells (*black circles*) during the shift to 27/150. Following the shift, all O_2-exchange measurements were made at 27/150. Rates of respiration were made immediately following the estimation of photosynthetic rates, and hence, represent light-enhanced respiration. All values represent means ± SE, $n=3$

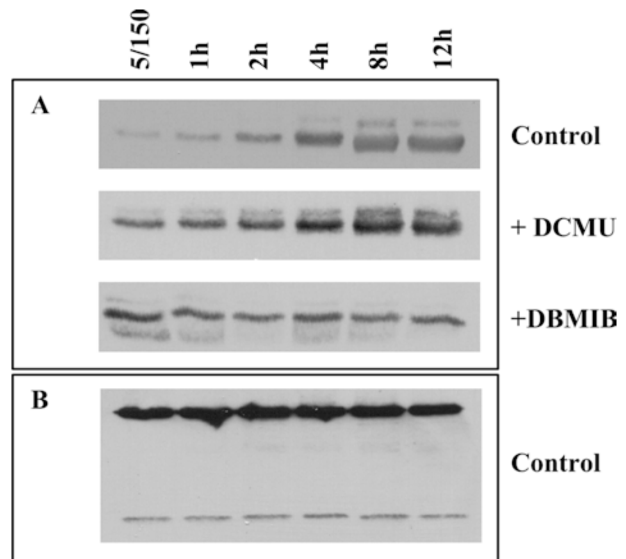

Fig. 4A, B Immunoblots illustrating changes in LHCII (**A**) and Rubisco (**B**) content of *C. vulgaris* cells grown at 5/150 and transferred to 27/150. To detect LHCII, thylakoid membrane fractions were loaded on an equal-protein basis (20 μg/lane) and challenged with antibodies raised against LHCII of *Dunaliella salina*. *Lane 5/150* represents aliquots of the same sample that are of different signal intensity due to differences in film exposure time. Thus, changes in LHCII content of each blot can be estimated by comparison to the 5/150 lane. To detect Rubisco, soluble protein fractions were loaded on an equal-protein basis (μg/lane) and challenged with antibodies raised against the Rubisco holocomplex of rye. Thus, the upper band represents the Rubisco large subunit and the lower band the small subunit

ability to produce it photosynthetically. As the cells began to acclimate to growth at 27/150 the net rate of photosynthesis increased on a per-cell basis (Fig. 3A). A maximum rate of 0.4 μmol O_2 evolved $(10^7\ \text{cells})^{-1}\ \text{h}^{-1}$ was reached after 12 h (Fig. 3A, closed circles) which corresponded with the increase in cellular Chl (Fig. 1A, open circles). After the initial burst in the rate of respiration, 0.530 μmol O_2 consumed $(10^7\ \text{cells})^{-1}\ \text{h}^{-1}$ immediately following transfer to 27 °C, respiration decreased to 0.120 μmol O_2 consumed $(10^7\ \text{cells})^{-1}\ \text{h}^{-1}$ during 12 h of growth at 27/150 (Fig. 3B, closed circles).

By examining the relative change in LHCII content, it was evident that the accumulation of LHCII correlated with the amount of Chl and the decline in the Chl *a:b* ratio, both in magnitude and in the kinetics of accumulation (Figs. 4A, 5A). In untreated cells, the increase in Chl and LHCII polypeptides occurred in conjunction with an increase in the other photosynthetic electron transport complexes, as estimated by immunoblots of specific component proteins (Fig. 5, open circles). The D1 protein of PSII, the Cyt *f* protein of the cytochrome b_6/f complex and the psaA/B heterodimer of the PSI complex accumulated in a similar manner as a percent of the total membrane protein (Figs. 5, open circles, and 6). However, shifting *C. vulgaris* cells from 5/150 to 27/150 did not induce a noticeable change in the level of Rubisco (Fig. 4B). Therefore, not all chloroplast-localized proteins increased in abundance during the temperature-induced greening process.

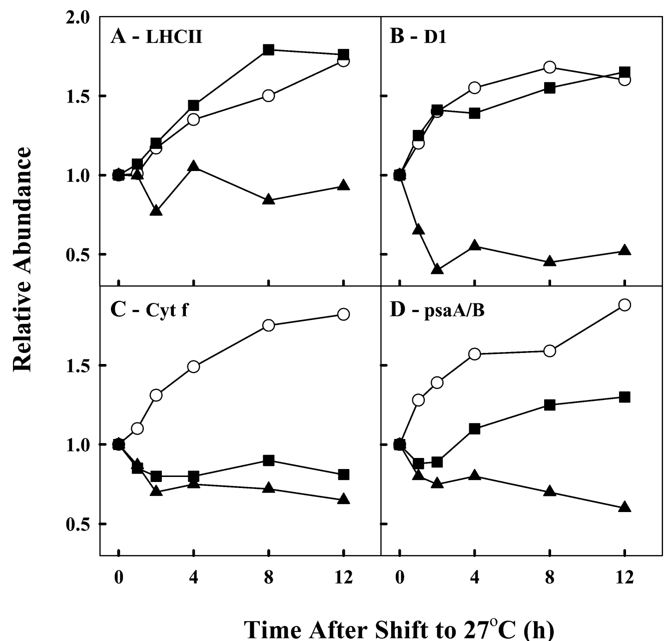

Fig. 5A–D Densitometric estimation of the relative abundance of the main thylakoid protein complexes following the shift of *C. vulgaris* cells from 5/150 to 27/150. Immunoblot films were digitally imaged and the relative density of the spots determined to estimate the abundance of LHCII, PSII (D1), the cytochrome b_6/f complex (Cyt *f*), and PSI (psaA/B). Each point is the average to two independent immunoblots

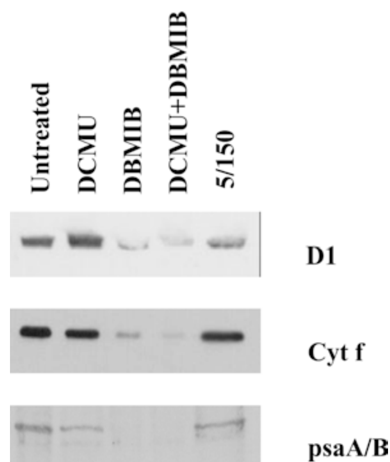

Fig. 6 Immunoblots illustrating the relative amounts of the main thylakoid protein complexes PSII (D1), Cyt *b*/*f* (Cyt *f*) and PSI (psaA/B). The amounts of these protein complexes were compared before (5/150) and following transfer of *C. vulgaris* cells to 27/150 for 12 h. The amount of each component was compared following transfer in the presence of 1.0 μM DCMU, 10.0 μM DBMIB, 1.0 μM DCMU + 10 μM DBMIB, or an equivalent volume of ethanol (*Untreated*), to the amount present in cells grown at 5/150. Thylakoid membrane fractions were loaded on an equal-protein basis (20 μg/lane) and challenged with antibodies raised against the protein indicated

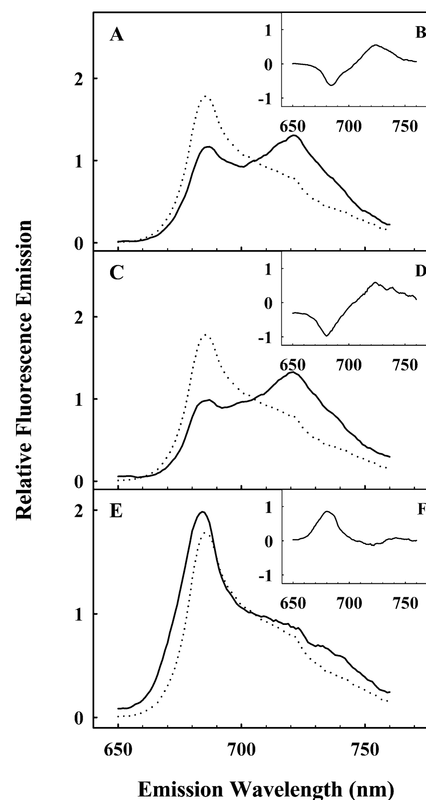

Fig. 7A–F Low-temperature Chl fluorescence emission spectra taken following the transfer of *C. vulgaris* cells from 5/150 to 27/150. Thylakoid membranes were isolated from either untreated cells (**A, B**) or from cells treated with 1.0 μM DCMU (**C, D**) or 10.0 μM DBMIB (**E, F**). The *dotted lines* are spectra from cells grown at 5/150. *Solid lines* are from cells transferred for 12 h. The samples had equal chlorophyll content (5 μg/ml) and the spectra are normalized at 705 nm. Difference spectra (**B, D, F**) were determined by subtracting the normalized spectrum of the cells grown at 5/150 from the spectrum obtained following 12 h at 27/150

Table 1 The distribution of Chl within the pigment–protein complexes of the thylakoid membrane of *Chlorella vulgaris*, as estimated by mildly denaturing SDS–PAGE. Thylakoidmembrane samples were electrophoresed as described in the Materials and methods section, and gel lanes were scanned at 671 nm to determine the percent of the total Chl present in each fraction

Algal culture treatment	PSI	PSII	LHC	Free Chl	Oligomer: monomer
Untreated (no inhibitor)	23±1.9%	19±1.7%	51±7.2%	7 ± 4.7%	0.65
+ DCMU	20±2.3%	20±2.5%	55±9.3%	5 ± 3.6%	0.65

The relative amount of LHCII polypeptides does not provide information on their functional capability or the supramolecular organization of the antenna complexes and reaction centers. Changes in the energy distribution between PSI and PSII were examined using 77 K Chl fluorescence following the shift from 5/150 to 27/150. There appeared to be a change in the excitation energy distribution from a situation where PSII fluorescence dominated to one where there was a more equal fluorescence emission from PSI and PSII over the 12 h following transfer to 27/150 (Fig. 7A). Calculation of a difference spectrum allowed the change in excitation energy distribution to be visualized more clearly (Fig. 7B).

The organization of the light-harvesting complex was also examined using mildly denaturing SDS–PAGE (Table 1). The percent of total Chl that was associated with dissociable LHC (the major LHC proteins which are not tightly bound to PSI or PSII) was 51%, while the ratio of oligomeric to monomeric LHC was 0.65 (Table 1). The percent of total chlorophyll associated with PSI and its tightly associated LHC was 23%, and PSII and its core antenna accounted for 19% of the total Chl (Table 1). The remaining Chl (7%) was present as free pigment that had become dissociated from the various pigment–protein complexes as a result of the extraction and solubilization procedure.

As the cellular Chl concentration increased in response to the shift from 5/150 to 27/150, the amount of zeaxanthin present in the cells decreased from 75.0 to 15.0 mmol (mol Chl *a*)$^{-1}$ (Table 2). As a result, the EPS of the xanthophyll-cycle pigments increased from 0.46 to 0.64 (Table 2). Thus, based on the theory that antheraxanthin and zeaxanthin content, and hence the EPS, is regulated by the trans-thylakoid ΔpH (Gilmore and Yamamoto 1993; Gilmore 1997) it appeared that the ΔpH gradient diminished following the shift to 27 °C (Table 2).

Table 2 The effect of metabolic inhibitors on zeaxanthin content and nonphotochemical quenching in*C. vulgaris* cells following temperature-dependent greening. The measurements of q_N were carried out at 27 °C at 150 μmol photons m^{-2} s^{-1}. The levels of the individualxanthophyll cycle pigments were determined by HPLC, as described in the Materials and methods section and are presented in units of mmol (mol Chl *a*)$^{-1}$

Algal culture treatment	EPS	Zeaxanthin	q_N
5/150-grown cells	0.46 ± 0.06	75.0 ± 3.6	0.451 ± 0.083
Untreated (no inhibitor)	0.64 ± 0.01	15.0 ± 1.3	0.129 ± 0.041
+ DCMU	0.60 ± 0.01	22.1 ± 3.2	0
+ DBMIB	0.14 ± 0.06	59.5 ± 7.9	0.731 ± 0.051
+ DTT	0.85 ± 0.02	9.4 ± 1.3	0.059 ± 0.015
+ DCMU + DTT	0.81 ± 0.01	15.3 ± 0.2	0

Table 3 The effect of metabolic inhibitors on the redox state of Q_A, and the rates of photosynthesis and respiration of *C. vulgaris* cells. The measurements were carried out at 27 °C and 150 μmol photons m^{-2} s^{-1}. Respiration measurements were made in the dark immediately following the photosynthesis measurements and, hence, represent light-enhanced respiration. Net photosynthesis and respiration are presented as % control (no inhibitor)

Algal culture treatment	$1-q_P$	Net photosynthesis	Respiration
Untreated (no inhibitor)	0.135	100	100
DCMU	1.00	12 ± 2.5	92 ± 3.8
DBMIB	1.00	10 ± 5.0	91 ± 6.2
DCMU + DBMIB	1.00	5 ± 4.0	83 ± 6.9
Antimycin A	0.375	105 ± 10.0	35 ± 5.9
TPTC	1.00	23 ± 5.7	26 ± 3.5

The Effects of DCMU on temperature-induced greening of *C. vulgaris*

When *C. vulgaris* cells grown at 5/150 were treated with 1.0 μM DCMU prior to transfer to 27/150, they greened in a manner that was visually indistinguishable from the untreated, control cells. The cells actually accumulated greater amounts of Chl/cell (Fig. 1A, closed squares), and correspondingly, the Chl *a:b* ratio was slightly lower than observed in the control cells (Fig. 1B, closed squares). Thus, DCMU did not inhibit, and may have enhanced the phenotypic adjustment of the cells following the transfer from 5/150 to 27/150. Because DCMU binds to the Q_B site of PSII, blocking electron transport out of PSII, Chl *a* fluorescence was always maximal, indicating that PSII was highly reduced. However, the PQ pool should become oxidized as DCMU blocks its reduction by PSII (Trebst 1980). Thus, room-temperature Chl fluorescence could not be used to ascertain the functional acclimation of the cells. Chlorophyll fluorescence and O_2 measurements were used to indicate that the 1.0 μM DCMU treatment was sufficient to almost completely inhibit photosynthesis (Table 3).

The changes observed in Chl accumulation and the Chl *a:b* ratio were also reflected in the accumulation of LHCII polypeptides (Figs. 4A, 5A). The increase in the amount of LHCII per unit membrane protein was slightly greater when compared to cells shifted from 5/150 to 27/150 in the absence of inhibitors, and the kinetics of accumulation were almost identical (Figs. 4A, 5A). The accumulation of the D1 protein followed a nearly identical pattern in DCMU-treated and untreated cells (Figs. 5B, 6). However, Cyt *f* did not accumulate and there was only a limited increase in the abundance of the PSI heterodimer (Figs. 5, 6). As a result, while LHCII and PSII accumulation was very similar, the stoichiometry of the photosynthetic electron transport complexes, on a per-membrane-protein basis, was very different following greening in the presence of DCMU. As observed in untreated cells the accumulation of Rubisco appeared unaltered by the shift in growth temperature in the presence of DCMU (data not shown).

At the functional and supramolecular level, the similarity in the Chl and LHC accumulation continued to be evident based on 77 K Chl fluorescence and the relative distribution of Chl in the various Chl–protein complexes. The 77 K Chl fluorescence emission spectra of the cells following the shift from 5/150 to 27/150 in the presence of DCMU indicated, as observed in control cells, a more balanced distribution of excitation energy capture between the two photosystems (Fig. 7C, D). At the level of the Chl–protein complexes, dissociated LHC accounted for 55% of the total Chl and the ratio of oligomeric:monomeric was 0.65 (Table 1). Similarly, the percent of total Chl associated with PSI and PSII was 23% and 20%, respectively, again almost exactly what was found for untreated cells shifted from 5/150 to 27/150 (Table 1).

The relaxation of the xanthophyll-cycle-dependent energy dissipation process also occurred in the DCMU-treated cells. The zeaxanthin content decreased to 22.1 mmol (mol Chl *a*)$^{-1}$, which, while lower than 5/150-grown cells, was slightly higher than that observed in the untreated cells (Table 2). Thus, the EPS also remained slightly lower in the DCMU-treated cells (0.60) (Table 2), suggesting a greater ΔpH than in control cells (Gilmore and Yamamoto 1993; Gilmore 1997).

The Effect of DBMIB on temperature-induced greening of *C. vulgaris*

Contrary to the effects of DCMU, DBMIB inhibited the temperature-dependent greening of *C. vulgaris* cells grown at 5/150 during a 12-h shift to 27/150. At the end of 12 h the cells remained visibly yellow-green. There was no increase in the cellular Chl content (Fig. 1A, closed triangles), and only a small decrease in the Chl *a:b* ratio (Fig. 1B, closed triangles). Treatment with DBMIB blocks the transfer of electrons from PQ to the Cyt b_6/f complex, which causes the PQ pool to become highly reduced (Trebst 1980). Therefore, like DCMU, DBMIB minimizes photochemical quenching, and as a result of the build up of electrons in the PQ pool, PSII remains reduced, and hence, $1-q_P$ was nearly 1.00

(Table 3). This was also true when cells were treated with a combination of DBMIB and DCMU. The inhibition of electron transport by DBMIB or DBMIB + DCMU resulted in the near cessation of photosynthetic O_2 evolution with only minor effects on cellular respiration (Table 3).

Predictably the accumulation of LHCII polypeptides mirrored the level of cellular Chl and no increase was observed (Figs. 4A, 5A). This was also true in the DCMU + DBMIB-treated cells, indicating that the effect of DCMU can be overridden by the simultaneous addition of DBMIB (data not shown). The addition of DBMIB not only blocked the accumulation of Chl and LHCII polypeptides, it also stopped the increased accumulation of the other major thylakoid membrane protein complexes (Figs. 5, 6). The level of PSII, estimated by D1 decreased rapidly in DBMIB-treated cells (Fig. 5). The Cyt b_6/f complex, estimated by Cyt *f* content, and PSI estimated by psaA/B content, also decreased from 5/150 levels when cells were shifted in the presence of either DBMIB or DCMU + DBMIB although not as rapidly as D1 (Fig. 5). The overall stoichiometry of the complexes appeared to remain approximately the same despite the treatment as all three complexes decreased to approximately 50% of the 5/150 level (Figs. 5, 6).

It was not possible to analyze the distribution of Chl into the LHC and reaction-center complexes in the DBMIB-treated cells, as the extremely low Chl:protein ratio in the thylakoid membranes made the partial solubilization of the Chl–protein complexes impossible. However, by examining the cells using 77 K Chl fluorescence, it appeared that the cells did not re-organize the light-harvesting complexes (Fig. 7E). Additionally, the peak representing LHCII and PSII in the 685-nm region shifted towards shorter wavelengths. When a difference spectrum was generated by subtracting the emission spectrum prior to shifting from that after 12 h at 27/150 in the presence of DBMIB, a peak at 680 nm was evident (Fig. 7F). Fluorescence emission at this wavelength is normally associated with isolated LHC proteins (Butler 1978). The addition of DBMIB also inhibited the relaxation of the xanthophyll-cycle pigment EPS. The level of zeaxanthin remained high [59.5 mmol (mol Chl *a*)$^{-1}$], and the EPS actually decreased from 0.46 to 0.14 (Table 2), suggesting that the ΔpH remained high (Gilmore and Yamamoto 1993; Gilmore 1997).

The effect of inhibitors of mitochondrial respiration on temperature-dependent greening of *C. vulgaris*

The gross rate of O_2 evolution was negative during the first 4 h following transfer from 5/150 to 27/150 in untreated cells, and for the entire 12 h when the cells were treated with DCMU. Therefore, the cellular energy required for Chl biosynthesis and the translation of chloroplast proteins may have come from the mitochondria. To investigate this, cells were transferred from 5/150 to 27/150 in the presence of antimycin A, an inhibitor of mitochondrial electron transport. Treatment with 1 μM antimycin A had no effect on net photosynthetic O_2 evolution, but decreased cellular respiration to 35% of that measured in control cells (Table 3), and greatly diminished the rapid burst of O_2 consumption by the cell upon the initial transfer from 5/150 to 27/150 (Fig. 3B, black squares). In terms of greening, antimycin A inhibited Chl accumulation as the amount of Chl per cell did not increase (Fig. 1A, black diamonds), and the Chl *a:b* ratio stayed higher than in control cells (Fig. 1B, black diamonds). The addition of antimycin A also caused $1-q_P$ to remain higher than in control cells (0.375 compared to 0.135 in control cells) (Table 3), suggesting that PSII was more reduced as a result of inhibition of mitochondrial electron transport. The increased reduction of PSII should reflect a correspondingly more reduced PQ pool as antimycin A had no apparent effect on O_2 evolution (Table 3; Tullberg et al. 2000).

Treatment of the cells with TPTC had an even stronger effect on Chl accumulation (Fig. 1A, black inverted triangles) and Chl *a:b* ratio (Fig. 1B, black inverted triangles). TPTC inhibits both chloroplastic and mitochondrial ATPases and, hence, the cell should have no source of ATP (Gould 1976). The net result was an almost complete inhibition of photosynthesis and respiration as determined by both O_2 exchange and Chl fluorescence measurements (Table 3). Correspondingly, no greening occurred.

The role of zeaxanthin-dependent nonphotochemical quenching during greening in the presence of DCMU

Following the shift from 5/150 to 27/150 in the presence of DCMU, PSII should be under conditions of 'high-light' stress, but the PQ pool is kept oxidized because PSII was unable to transfer electrons to the PQ pool. Zeaxanthin and the trans-thylakoid ΔpH are thought to play critical roles in the quenching of excess light-energy and, thus, protecting the photosynthetic apparatus from photo-oxidative damage (Gilmore 1997; Niyogi 1999). In cells shifted from 5/150 to 27/150, the level of zeaxanthin decreased and the EPS increased correspondingly (Table 2). When the cells were treated with DCMU, the level of zeaxanthin also decreased, but not to as great an extent as in the untreated cells. Similarly, the EPS increased but not as much as observed in untreated cells (Table 2). Dithiothreitol (DTT) is an inhibitor of violaxanthin de-epoxidase, the ΔpH dependant enzyme that converts violaxanthin to zeaxanthin (Gilmore 1997). When 5/150-grown cells were shifted to 27/150 for 12 h in the presence of DTT, they greened normally. The Chl/cell was 305 fg Chl/cell, and the Chl *a:b* ratio was 5.5 (55% of the initial value). Because DTT acts as an inhibitor of zeaxanthin formation, the level of zeaxanthin decreased to 9.4 mmol (mol Chl *a*)$^{-1}$

only 60% of that found in the untreated cells, and the EPS was 0.85 dramatically higher than the untreated cells (Table 2). This resulted in a concomitant decrease in q_N (Table 2). When cells were transferred to 27/150 for 12 h in the presence of DCMU and DTT the greening of the cells was partially blocked, as indicated by the fact that the amount of Chl per cell was 194 fg Chl/cell compared to 383 fg Chl/cell in cells treated with DCMU alone. The Chl *a:b* ratio also remained higher than in cells treated with only DCMU (69% of the initial value compared to 50%). While the actual concentration of zeaxanthin was similar to that observed in control cells [15.3 mmol (mol Chl $a)^{-1}$], the EPS was much higher than in untreated cells (Table 2), indicating very little zeaxanthin was present as a function of the size of the xanthophyll-cycle pigment pool.

Discussion

We previously demonstrated that the temperature-dependent accumulation of Chl and LHCII polypeptides in *C. vulgaris* was regulated by the redox state of the PQ pool and the trans-thylakoid ΔpH, in a process which appeared dependent upon the cellular capacity to utilize the products of photosynthesis (Wilson and Huner 2000). During the acclimation process, the PQ redox state was expected to rapidly become more oxidized before establishing a level similar to that observed in cells grown at 27/150. Additionally, it was evident, as described previously, that in a somewhat paradoxical manner, treatment with DCMU did not inhibit Chl and LHCII accumulation (Beale and Appleman 1971; Koenig 1990; Nauš and Melis 1992). The results presented in this study extend this premise by examining the accumulation of Chl and LHC at the level of both structural and functional organization during the temperature-induced greening process and following treatment with DCMU or DBMIB. The greening of *C. vulgaris* cells in response to a temperature-dependent oxidation of the PQ pool was very similar to the response observed when *C. pyrenoidosa* and *D. tertiolecta* cells were transferred from high light to low light (Fujita et al. 1989; Sukenik et al. 1990). There was a rapid increase in Chl per cell, LHCII polypeptide accumulation, and a corresponding increase in the amount of the photosynthetic electron transport complexes, as a percent membrane protein (Figs. 5, 6). This rapid increase in the photosynthetic components indicated a coordination of nuclear and chloroplastic protein synthesis, which is required for the stable accumulation of these complexes (Wollman et al. 1999).

When cells were treated with DCMU there was a greater accumulation of Chl and LHCII polypeptides on a per-cell basis than in untreated cells. The amount of Chl associated with both photosystems and the relative amount of Chl incorporated into the LHCII polypeptides was also very similar as determined using mildly denaturing SDS–PAGE (Table 1). At the protein level there appeared to be a coordinated increase in the photosynthetic electron transport complexes in the untreated cells and a similar coordinated decrease in DBMIB-treated cells (Figs. 5, 6). In DCMU-treated cells, the kinetics of LHCII and PSII accumulation matched those of untreated cells. However, while the accumulation of PSI in DCMU-treated cells was delayed and diminished, the cytochrome b_6/f complex decreased in abundance in a manner similar to that of DBMIB-treated cells (Figs. 5, 6). It is known that translation of the psbA (D1) transcript is regulated by a thioredoxin-mediated mechanism (Danon and Mayfield 1994), and there are a large number of nuclear-encoded factors involved in the assembly of PSI (Rochaix 2002). Thus, a simple regulation of these complexes may not occur. However, we suggest that the PQ-pool redox state, acting as a sensor of the cellular energy balance, regulates LHCII, PSII and PSI abundance. It is also likely that the cytochrome b_6/f complex is regulated in the same fashion, but due to secondary effects it does not accumulate in the absence of linear electron transport.

It was interesting to note the lack of a noticeable change in the accumulation of Rubisco (Fig. 4B), which, while not part of the electron transport chain, does play a critical role in photosynthesis, utilizing ATP and NADPH to reduce CO_2. Thus, not all components of the photosynthetic apparatus are sensitive to the redox state of the PQ pool. The lack of change in Rubisco accumulation at the protein level was somewhat contradictory to the observations of Savitch et al. (1996), who demonstrated a 50% greater abundance in Rubisco in 5/150-grown *C. vulgaris* cells compared to those grown at 27/150. There are two possible explanations for this apparent contradiction. First, in this study Rubisco levels were estimated by immunoblotting on the basis of an equal protein level rather than enzymatically on a per-cell basis. Second, Savitch et al (1996) examined cells grown under steady-state conditions; perhaps if Rubisco levels were examined over a longer period following the shift to 27/150 a difference could be observed. Thus, the rapid, temperature-dependent accumulation of photosynthetic components appears to be specific for thylakoid membrane proteins. Our results appear to extend the model of Sukenik et al. (1990), which proposes that the photosynthetic protein components of the thylakoid membranes accumulate first, followed by lipid and structural components upon a shift from high to low light conditions. Therefore, temperature-dependent greening appears to mimic light-dependent greening in *C. vulgaris*.

When *C. vulgaris* cells were transferred from 5/150 to 27/150 in the presence of DCMU, PSII was under very high excitation pressure (Table 3, Wilson and Huner 2000). It was not clear how the DCMU-treated cells were able to avoid photo-oxidative damage. Normally, when photosynthetic organisms are exposed to short-term high excitation pressure conditions two protective mechanisms are utilized. The first is the rapid induction of xanthophyll-cycle-dependent nonphotochemical

quenching (Niyogi 1999; Gilmore 1997); the second is an increase in the rate of degradation of the D1 protein of PSII (Barber and Andersson 1992; Aro et al. 1993). Xanthophyll-cycle-dependent nonphotochemical quenching requires a high trans-thylakoid ΔpH (Gilmore 1997); however, because DCMU blocks electron transport out of PSII, the ΔpH should remain low. In addition, DCMU interacts with PSII by binding to the D1 protein, protecting the D1 protein from directed proteolysis (Jansen et al. 1993). It was seen that D1 levels increased in DCMU-treated cells rather than decreasing (Figs. 5, 6). Therefore, when cells were treated with DCMU, to block electron transport out of PSII, they should have been under a high-light stress, but Chl and LHCII accumulated to greater levels. Thus, a mechanism must exist to protect the cell from high-light-associated oxidative damage.

We suggest that zeaxanthin-dependent nonphotochemical quenching of excitation energy protects PSII during greening in the presence of DCMU. Our primary evidence that zeaxanthin played a role in the protection of PSII and LHCII is that DCMU-treated cells contained more zeaxanthin on a per-Chl basis than the untreated cells, and the xanthophyll-cycle pigment pool EPS was lower, indicating that the ratio of zeaxanthin to violaxanthin and antheraxanthin was greater. In addition, when DTT was added in combination with DCMU, greening was partially inhibited relative to greening in the presence of only DCMU or DTT alone. We suggest that the decrease in zeaxanthin and q_N caused by the addition of DTT resulted in the decreased protection of PSII and LHCII when electron transport was inhibited by DCMU. The mechanism of zeaxanthin-dependent nonphotochemical quenching is thought to occur by nonradiative decay of the zeaxanthin excited state to ground state, and the accumulation of zeaxanthin is triggered by the trans-thylakoid ΔpH (Gilmore 1997). Under conditions where PSII is inactive, it has been suggested that cyclic electron transport around PSI may be able to maintain a significant ΔpH to allow xanthophyll-cycle-dependent nonphotochemical quenching to occur (Ivanov et al. 2001). Alternatively, chlororespiration may supply electrons to the photosynthetic electron transport chain via extra-chloroplastic sources, such as the mitochondria (Hoefnagel et al. 1998; Casano et al. 2000). While DCMU-treated cells had less zeaxanthin on a per-Chl basis than cells grown under high $1-q_P$, relatively small differences in zeaxanthin could be very important in protecting PSII during greening in the presence of DCMU, especially as the irradiance was relatively low at 150 μmol m^{-2} s^{-1}. Zeaxanthin has not been shown to be present in the PSII reaction center core, however, it is found in the inner antenna pigment protein complexes (Gilmore 1997). Additionally, small proteins (Cbr, Elips, Hlips, PsbS) that are structurally similar to LHC polypeptides have been shown to be up-regulated by high light and high excitation pressure conditions (Braun et al. 1996; Li et al. 2000) and appear to be important in the nonphotochemical quenching process (Braun et al. 1996; Li et al. 2000). A second piece of evidence in favor of a quenching mechanism was the relatively small LHC-PSII peak observed in the 77 K fluorescence emission spectra of the cells greened in the presence of DCMU (Fig. 7). It has been shown previously that quenching of PSII excitation can occur at 77 K and that independent PSII-associated quenchers could diminish the fluorescence emission of the 685 nm peak (Kyle et al. 1983). Thus, if sufficient quenching could be maintained, a limited number of excitons would be transported to PSII.

Another mechanism which could act to protect PSII is the improper assembly or insertion of the LHCII polypeptides, such that they do not transfer excitation energy to PSII (Hippler et al. 2000). However, when the 77 K Chl fluorescence was examined 12 h after the shift to 27/150, the DCMU-treated cells appeared very similar to the untreated cells, while the DBMIB-treated cells appeared to have a population of unbound LHC based on the increased emission at 680 nm. Additionally, based on the mildly denaturing SDS–PAGE, the ratio of oligomeric to monomeric LHC, as well as the percent of total Chl associated with each reaction center was nearly identical to that observed in control cell cultures. Therefore, it does not appear that LHC dissociation from the reaction centers occurred in DCMU-treated cells as a protection mechanism.

The acclimation process in response to temperature is more complicated than adjustment to light intensity (Machalek et al. 1996; Savitch et al. 1996). When early- to mid-log phase *C. vulgaris* cells that had been grown at 5/150, were transferred to 27 °C, the rate of cellular respiration initially increased more than 3-fold (Fig. 2B; Maxwell et al. 1994). Thus, the short-term cellular energy demand was greater at 27 °C than at 5 °C, which is consistent with our previous hypothesis that acclimation to PQ redox state occurs ultimately in response to the balance between the amount of light being absorbed and the utilization of that energy by the cell (Wilson and Huner 2000). This would be especially true in cells treated with DCMU, as no net photosynthetic production of NADPH would occur. Although limited amounts of ATP could be derived from starch breakdown (Ball 1998), it would seem unlikely that the ATP, reducing equivalents and carbon skeletons required for the rapid greening to occur could be produced without oxidative phosphorylation. This was supported by the rapid and large increase in O_2 consumption by the cells immediately following transfer to 27 °C. The results of mitochondrial inhibitor treatments suggested even more strongly that the total cellular capacity to utilize photosynthate regulates photoacclimation. Both antimycin A and TPTC block the cellular ability to use the energy produced by photosynthesis, and inhibited the acclimation process. Although the use of these inhibitors did not help in the further elucidation of the sensing/signaling mechanisms involved in initiating and regulating the acclimation process, they support the possibility that

both the PQ redox state and the trans-thylakoid ΔpH act as potential sensing mechanisms for the cell to identify cellular energy imbalances. Chlorophyll fluorescence measurements indicated that the addition of either antimycin A or TPTC caused an increase in $1-q_P$ (Table 3) suggesting a more reduced PQ pool (Tullberg et al. 2000). Therefore, it appeared that mitochondrial electron transport and the reducing power produced in the mitochondrion was coupled to photosynthetic electron transport. This provided an important link in establishing that changes in whole-cell energy balance could be sensed by the chloroplast through the PQ redox state and/or the trans-thylakoid ΔpH.

In summary, we have demonstrated that when *C. vulgaris* cells are transferred to 27/150 from growth at 5/150 not only is there an increased accumulation of LHCII proteins but also of the other main photosynthetic electron transport chain complexes. Based on inhibitor studies, it appeared that the PQ-pool redox state regulated the accumulation of Chl, LHCII, PSII and PSI. Strikingly, the cells treated with DCMU during the transfer from 5/150 to 27/150 were indistinguishable from untreated cells, at the level of Chl and LHCII accumulation and distribution, despite the fact that PSII was not functioning and should have been under a high-light stress condition. Thus, it appears likely that PSII and LHCII are protected from potential photo-oxidative damage through a zeaxanthin-dependent nonphotochemical quenching mechanism. This allowed greening to occur under conditions that should normally result in the destruction of Chl–protein complexes and cellular damage. Finally, in keeping with our previous hypothesis, it appeared that the photosynthetic adjustment *C. vulgaris* cells undergo in response to a change in growth temperature, occurred in order to counteract changes in, and re-establish a balance between, photosynthetic energy production and cellular energy consumption. This interconnection appears to occur via feedback loops that link the mitochondrion to the chloroplast, likely via metabolic intermediates, to alter the redox state of the PQ pool and thereby regulate changes in LHCII accumulation.

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