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BIOGENESIS OF CHLOROPLAST MEMBRANES

X. CHANGES IN THE PHOTOSYNTHETIC SPECIFIC ACTIVITY AND THE RELATIONSHIP BETWEEN THE LIGHT HARVESTING SYSTEM AND PHOTOSYNTHETIC ELECTRON TRANSFER CHAIN DURING GREENING OF *CHLAMYDOMONAS REINHARDI* y-1 CELLS

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SUMMARY

Specific activities of photophosphorylation and light-dependent pH rise at different stages of the greening process, have been measured as a function of the illumination intensity.

During the early phase of greening, the specific activity of cyclic photophosphorylation increases rapidly. Transfer of greening cells to the dark causes a marked decrease in the specific activity after 2 h incubation. Such an effect is not obtained if the greening cells are transferred to the dark at a later stage of the greening process.

High illumination intensities (10^6 erg·cm^{-2}·sec^{-1}) are required for maximal activity during the early phase of the normal greening process or the initial phase of repair of inactive membranes formed in the presence of chloramphenicol. The requirement for high illumination decreases during the late phase of the greening process ($3 \cdot 10^5$ erg·cm^{-2}·sec^{-1}) or during repair of inactive membranes if additional chlorophyll is synthesized. Saturation could not be obtained at $5 \cdot 10^5$ erg·cm^{-2}·sec^{-1}, during repair of inactive membranes in absence of chlorophyll synthesis over a period of 6 h of incubation. Based on these and additional data, it is concluded that the photosynthetic membranes are built stepwise and that the quantitative and organizational relationships between the light harvesting and electron transfer systems can be modulated within wide limits.

INTRODUCTION

The greening of dark-grown *Chlamydomonas reinhardi* y-1 mutant cells has been used as an experimental system for the investigation of the biogenesis of chloroplast membranes[1–13]. Based on the analysis of changes in the content of specific lipids and proteins during the normal greening process[1–4] and during greening in the presence of protein synthesis inhibitors[5,7,8,11,12] it appears that the growth of photo-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulphate.

synthetic membranes in this mutant can occur stepwise, different components being added at different times or at different rates[5,8,12].

Similar results have been obtained also in other systems, including wild type and several *Chlamydomonas* mutants[14–18], *Euglena*[19–21] photosynthetic bacteria[22,23] and higher plants[24,25].

Thus, during their developments the photosynthetic membranes represent a dynamic system undergoing continuous changes in composition and structure, as seen by the delayed appearance of grana during the greening process[1,26,27].

The question arises as to what extent these changes might be expressed at the level of photosynthetic function. Changes in the requirement for saturative illumination intensity might reflect changes in the degree of organization of the photosynthetic membranes. In the present work, the illumination requirements for maximal photophosphorylation and light-dependent pH rise activities at different stages of the chloroplast membrane formation were measured.

It was found that the requirement for high illumination intensity was maximal at the early phase of the greening process when the photosynthetic specific activity rises to a maximum. As chlorophyll and photosynthetic membranes continue to accumulate, both the specific activity and the saturative illumination intensity decrease, and reach the normal value usually found in light-grown cells.

MATERIALS AND METHODS

Growth, harvesting and greening of dark-grown *Chlamydomonas reinhardi* y-1 cells were as described before[1,5,28].

Unless otherwise specified the photophosphorylation activity was measured with open-cell preparations obtained as described in the preceding paper[9]. The pH of the photophosphorylation reaction mixture was 8.2. The reaction was carried out in a Warburg apparatus. Illumination was provided by six 100-W Sylvania lamps placed under the shaking bath at 10 cm distance, and three 150-W Sylvania lamps placed above the incubation vessels at 30 cm distance. The different illumination intensities were achieved by shading the incubation vessels with an adequate number of metal wire grids.

The amount of esterified ^{32}P was measured according to the method of Avron[29] (*cf.* also ref. 9). Light-dependent pH rise activity was measured in whole cells, as described by Schuldiner and Ohad[2]. The light source was a slide projector equipped with a 500-W lamp, and the reduction of illumination intensity was achieved with metal wire grids, as described above. Chlorophyll content was measured according to the method of Arnon[30].

All the reagents used in this work were of analytical grade and were obtained as described in the preceding paper[9].

RESULTS

The changes in the photophosphorylation and light dependent pH rise activities during greening of dark-grown y-1 cells are shown in Figs. 1 and 2. It can be seen that the above activities are hardly detectable in dark-grown cells. Following a short period of illumination the photophosphorylation specific activity of Photosystem I

and light-dependent pH rise increase sharply, reaching a maximum after about 2–3 h of illumination and decline afterwards, while chlorophyll synthesis continues at a maximal rate. A similar behaviour can be noticed also for Photosystem II, but the change in the activity is less pronounced. It has been shown before that transfer of greening cells to the dark, at any time period of the greening process, results in an immediate cessation of chlorophyll synthesis, which can be resumed by reexposing the cells back to the light[1,10]. The increase in photosynthetic activity of greening cells requires continuous illumination.

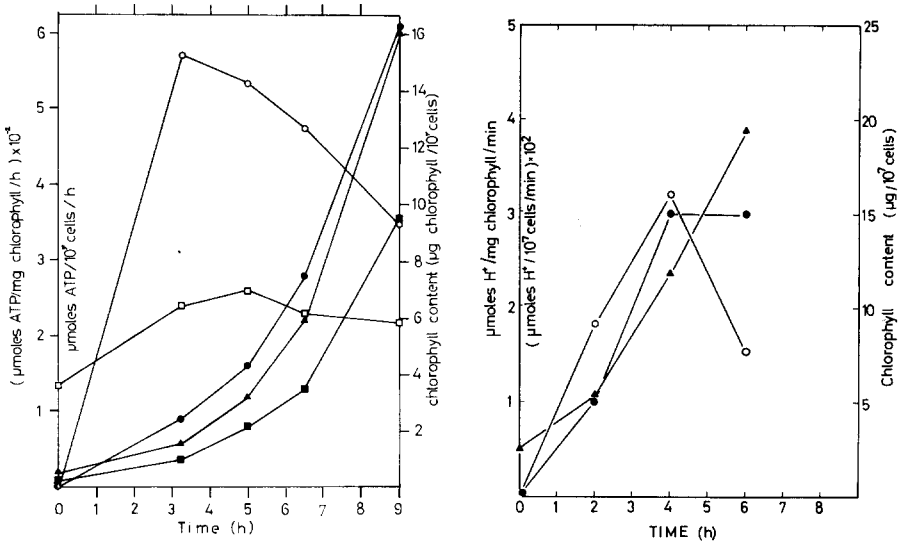

Fig. 1. Photophosphorylation activity during greening process. Photophosphorylation activity was measured with an open-cell preparation, as described in Materials and Methods. The assay system contained 30–60 μg chlorophyll. Photosystem II dependent photophosphorylation was measured in the presence of ferricyanide (2 mM) (■—■); the cyclic photophosphorylation of Photosystem I was measured with PMS ($5 \cdot 10^{-5}$ M) with addition of ascorbate (25 mM) as cofactors, in the presence of DCMU (10^{-5}M) (●—●); □—□, specific activity of Photosystem II; ○—○, specific activity of Photosystem I; ▲—▲, chlorophyll content.

Fig. 2. Light-dependent pH rise activity during greening process. The activity was measured with whole cells, in a system containing 30–60 μg chlorophyll. Measurements were carried out as described in Materials and Methods. ●—●, total activity; ○—○, specific activity; ▲—▲, chlorophyll content.

The activity seems to become more stable after illumination of the cells for several hours. Thus a decrease in the cyclic photophosphorylation specific activity is observed if cells greening for 2 h are transferred to the dark and further incubated for 4 h. The decrease in activity is less pronounced if the cells are transferred to the dark at a later stage of the greening process (Fig. 3A and 3B) and it is not observed when the light-dependent pH rise is measured under similar conditions (Fig. 4).

The changes in the illumination intensity required for photophosphorylation by both photosystems at different stages of the greening process are shown in Figs 5 and 6. It was found that during the early phase of the greening, before the activity per chlorophyll unit reaches the highest value high illumination intensities are required in order to saturate the above activities (Fig. 5, *cf.* also Fig. 1). The effect is

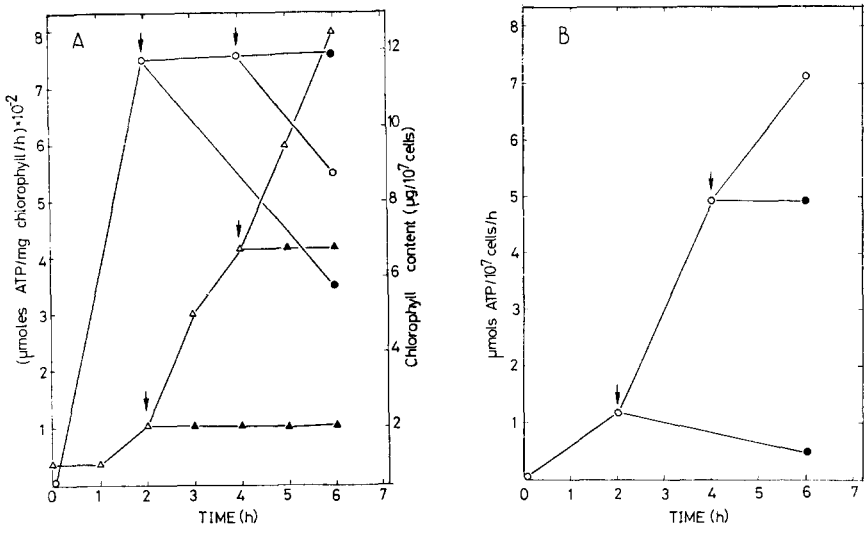

Fig. 3. Cyclic photophosphorylation activity of greening cells before and after transfer to the dark. A. Specific activity. B. Activity per cell. The activity was measured with an open-cell preparation, as described in Materials and Methods. The assay system contained 13–23 μg chlorophyll. Photophosphorylation was measured with PMS ($5 \cdot 10^{-5}$ M) with addition of ascorbate (25 mM) as cofactors, in the presence of DCMU (10^{-5} M) before (○—○) and after (●—●) transfer to the dark; chlorophyll content before (△—△) and after (▲—▲) transfer to the dark; arrows indicate time to the dark.

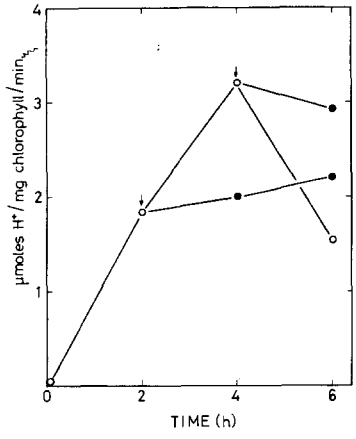

Fig. 4. Light-dependent pH rise activity of greening cells before and after transfer to the dark. The specific activity was measured with whole cells as described in Materials and Methods, in a system containing 30–60 μg chlorophyll, before (○—○) and after (●—●) transfer to the dark. Arrows indicate time of transfer to the dark.

more prominent for Photosystem I cyclic photophosphorylation, measured with phenazine methosulphate (PMS) and ascorbate in the presence of (3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). The maximal activity obtained in this case depends on the ascorbate concentration (Fig. 5). At high ascorbate concentration (10–25 mM), the activity during the early phase of the greening is not linear with the

illumination intensity, but becomes linear at ascorbate concentration below 10 mM (Fig. 7). However, the saturative illumination intensity is not affected by the ascorbate concentration (Fig. 5). The results of several experiments, in which the intensity of illumination required to obtain 50 % of the maximal photophosphorylation rate, achieved at saturative illumination, ($I_{S\frac{1}{2}}$), is plotted as a function of the greening time, are shown in Fig. 6. One can see that an exponential decrease in the requirement for illumination intensity is obtained for Photosystem I dependent cyclic photophosphorylation measured with PMS and ascorbate as cofactors in the presence of DCMU, as compared with a slight decrease for Photosystem II photophosphorylation with ferricyanide or Photosystem II dependent photophosphorylation by both photosystems with diquat as cofactor.

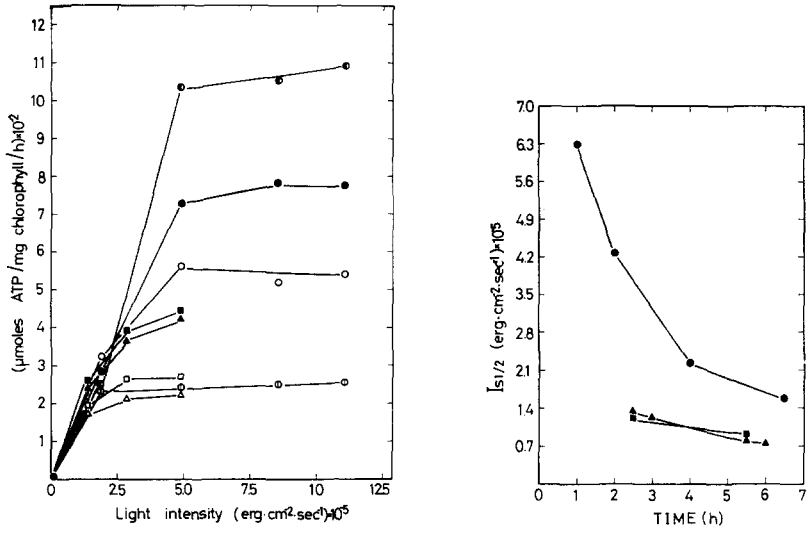

Fig. 5. Illumination intensity requirements of photophosphorylation activity during greening process. Same experimental conditions as in Fig. 1. The system contained 8–28 μg chlorophyll. The specific activity of Photosystem II was measured with ferricyanide (2 mM) after 2.5 h (▲—▲) and 5.5 h (△—△) of greening; specific activity of Photosystem I was measured with PMS ($5 \cdot 10^{-5}$ M) in the presence of DCMU (10^{-5} M) with addition of 25 mM ascorbate after 2 h (◐—◐) and 6.5 h (○—○) of greening, and with addition of 4 mM ascorbate after 2 h (●—●) and 6.5 h (①—①) of greening; the specific activity of Photosystem II *plus* Photosystem I was measured with diquat ($9 \cdot 10^{-6}$ M) as cofactor after 2.5 h (■—■) and 5.5 h (□—□) of greening. Different illumination intensities were obtained as described in Materials and Methods.

Fig. 6. Changes in $I_{S\frac{1}{2}}$ during greening process. Measurement conditions were identical to those described in Fig. 5. ▲—▲, Photosystem II photophosphorylation; ●—●, Photosystem I cyclic photophosphorylation; ■—■, Photosystem II *plus* Photosystem I photophosphorylation.

The requirement for high illumination intensity during the early phase of the greening process, is also evident for the light-dependent pH rise activity of whole cells (Fig. 8). As the greening proceeds, saturation can be obtained at low illumination intensities also for this system (Fig. 8). The response of the whole cells to illumination is not linear, the threshold being higher during the early phase of greening as compared with the later phase (see also Fig. 9).

It was shown before that inhibition of greening by chloramphenicol results in

the formation of non-photosynthetic chloroplast membranes[5,7,9]. The effect of partial inhibition of greening at low chloramphenicol concentration, on the saturative illumination requirement for light-dependent pH rise, is shown in Fig. 9. It appears that formation of membranes containing high amount of chlorophyll relative to the photosynthetic activity results in a lowering of the requirement for high illumination intensity in order to obtain saturation. Again, the response to the light is not linear, and the threshold intensity diminishes as a function of the greening time (Fig. 9).

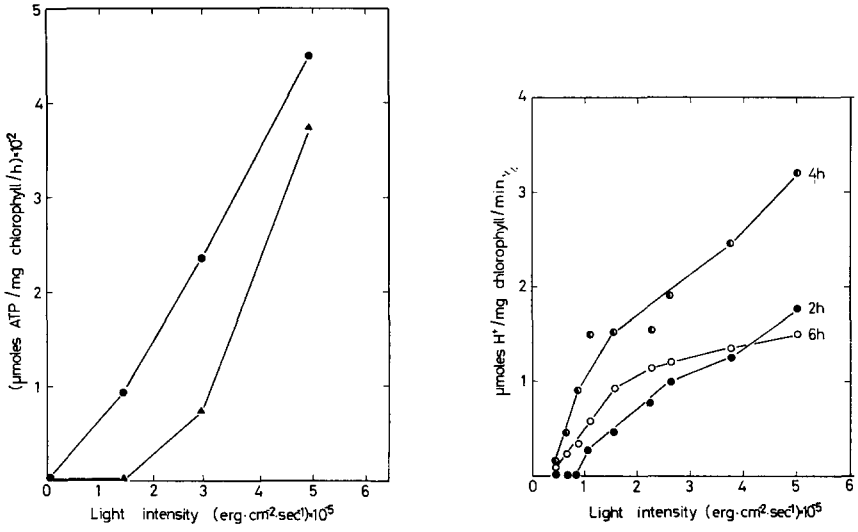

Fig. 7. The response of cyclic photophosphorylation activity to illumination intensity, as a function of ascorbate concentration. Same experimental conditions as in Fig. 1. The system contained 18 μg chlorophyll. The specific activity was measured with PMS ($5 \cdot 10^{-5}$ M) in the presence of DCMU (10^{-5} M), with addition of 10 mM ascorbate (▲—▲) or 4 mM ascorbate (●—●). The cells were incubated in the light for 3 h, and contained 2.6 μg chlorophyll per 10^7 cells. Different illumination intensities were obtained as described in Materials and Methods.

Fig. 8. Illumination intensity requirements for light dependent pH rise activity during greening process. Activity was measured with whole cells, in a system containing 30–60 μg chlorophyll. For details see Materials and Methods. The greening time was as indicated in the figure.

It was shown in the preceding paper[9] that photosynthetically inactive chloroplast membranes formed in the presence of chloramphenicol, can be reactivated under conditions which allow protein synthesis within the chloroplast (*cf.* also refs 5 and 7). The requirment for high illumination intensity for maximal photophosphorylation activity was found to persist during the repair of the inactive membranes, if no further chlorophyll synthesis occurred during the repair period (Fig. 10).

It should be noted that the response to the illumination was not linear in this experiment, in which photophosphorylation was measured with PMS and ascorbate (25 mM) in the presence of DCMU (see also Fig. 7). However, the threshold decreases, as the capacity of the preparation to photophosphorylate increases as a function of repair time.

When the repair of the inactive membrane, synthesized in the presence of chloramphenicol, occured under conditions which allowed an increase in the chloro-

phyll content, the value of $I_{s\,1/2}$ decreased during the repair period, in a manner resembling that of a normal greening process (Fig. 11; *cf*, also Fig. 6).

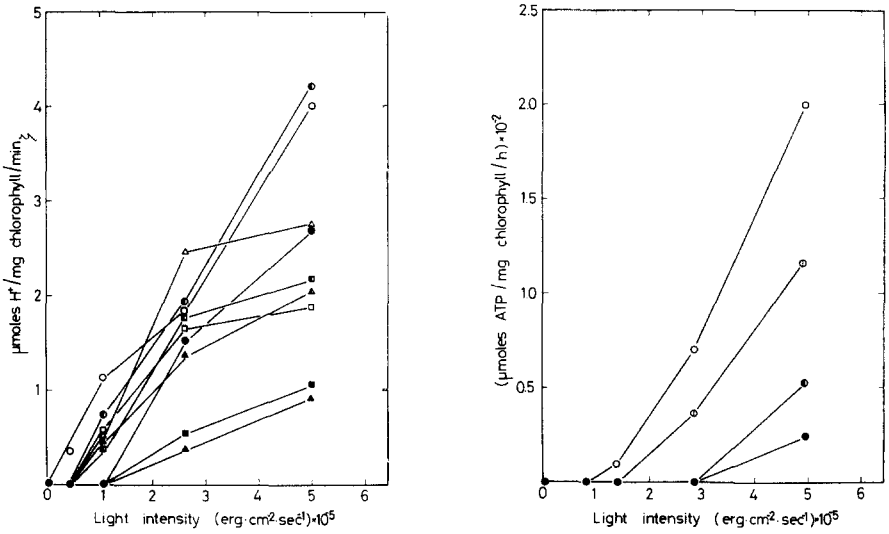

Fig. 9. Illumination intensity requirements for light-dependent pH rise activity of greening cells partially inhibited by addition of chloramphenicol. Activity was measured with whole cells, as described in Materials and Methods, in a system containing 30–60 μg chlorophyll. The specific activity was measured after 1 h (dark figures), 3 h (half closed figures) and 5 h (open figures) of greening, in a control system (circles), in a system greening in presence of 4 μg/ml chloramphenicol (triangles), and in a system greening in the presence of 7 μg/ml chloramphenicol (squares).

Fig. 10. Illumination intensity requirements for cyclic photophosphorylation activity, during repair of inactive membranes formed in the presence of chloramphenicol. The activity was measured with an open cell preparation, as described in Materials and Methods. The system contained 10 μg chlorophyll. The activity was measured with PMS ($5 \cdot 10^{-5}$ M) with addition of ascorbate (25 mM) as cofactors, in the presence of DCMU (10^{-5} M). At the time of transfer the chlorophyll content was 10 μg per 10^7 cells. Cells greening for 5 h in the presence of chloramphenicol (150 μg/ml) were washed free of chloramphenicol by centrifugation, resuspended in fresh growth medium containing cycloheximide (0.5 μg/ml), and further incubated in the light. Chlorophyll was not further synthesized during the repair process. The specific activity was measured after 1.5 h (●—●), 3 h (◐—◐), 4.5 h (ⓘ—ⓘ) and 6 h (○—○) of the repair process.

DISCUSSION

The results presented in this work demonstrate that the activity of the photosynthetic membranes in *Chlamydomonas reinhardi* y-1 cells varies during the greening process. This would indicate that throughout the process of their formation, the membranes grow stepwise and thus represent a dynamic system. The changes occuring in the photosynthetic membranes during their growth can be detected at two levels: (1) efficiency of photophosphorylation and light-dependent pH rise activities, expressed as rate per chlorophyll unit; (2) efficiency of photophosphorylation and light-dependent pH rise activities, as a function of the illumination intensity. The expression of the data in the above way rests on the assumption that the chlorophyll concentration per cell or assay system might be used as a marker for the content of photosynthetic membranes present in the greening cells. This assumption is derived from

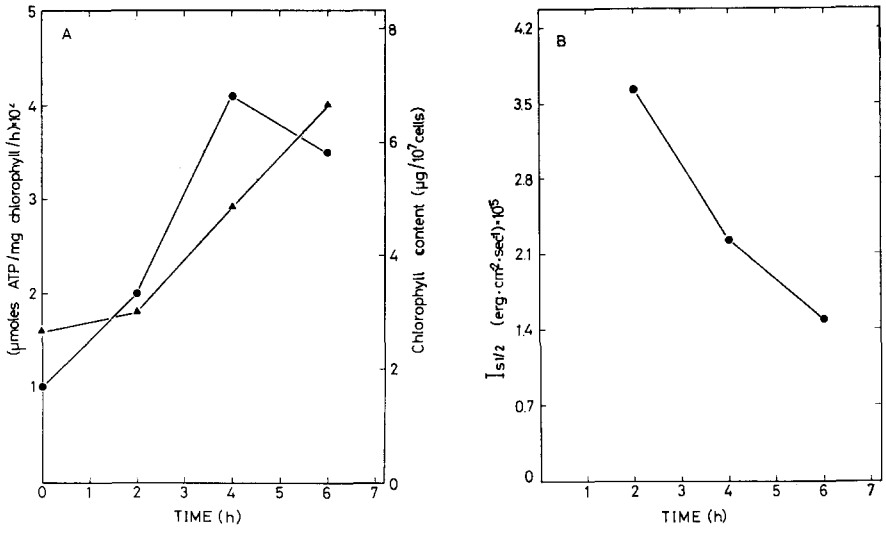

Fig. 11. The repair of the cyclic photophosphorylation activity in the presence of cycloheximide. A. Activity at different times of the repair process. B. Changes in $I_{s½}$ during the repair process. Same experimental conditions as in Fig. 10. The activity was measured with an open cell preparation, as described in Materials and Methods. The system contained 5–15 μg chlorophyll. The activity was measured with PMS ($5 \cdot 10^{-5}$ M) and ascorbate (4 mM) as cofactors, in the presence of DCMU (10^{-5} M). The cells were transferred to a medium containing 0.5 μg/ml cycloheximide after greening for 5 h, in the presence of chloramphenicol (150 μg/ml), when the chlorophyll content was 3 μg per 10^7 cells. Chlorophyll was further synthesized, and specific activity (●—●) and chlorophyll content (▲—▲) were measured at different times as indicated.

the following observations: (1) a correlation was found between the chlorophyll content and the amount of photosynthetic membranes profiles in cell sections seen in the electron microscope. This holds true for both processes, chlorophyll dilution during cell growth in the dark[28], and chlorophyll synthesis during greening[1,4]; (2) during the greening, membrane specific proteins (L-proteins)[5] are formed *de novo*, their synthesis being correlated to the amount of chlorophyll synthesized. It was also shown that a correlation can be found between the synthesis of chlorophyll and membrane specific lipids during greening of the y-1 cells[3]. These correlations hold during the linear phase of the normal greening process within the limits of the measurements accuracy for the different above mentioned parameters. However, departures from these correlations are evident under certain conditions: (1) during the initial or lag phase of the greening when only small amounts of chlorophyll are formed and become incorporated into the remnants of the photosynthetic membranes still present in the dark-grown cells; (2) during greening in the presence of chloramphenicol which inhibits synthesis of protein within the chloroplast[5,7,8,11]. In this case, lamellar proteins of cytoplasmic origin are formed, and chlorophyll and lipids synthesis are only partially blocked[11] resulting in the formation of membranes in which the relative content of the above-mentioned components will be drastically altered; (3) during repair of the altered membranes, under conditions permitting synthesis of proteins of chloroplastic origin with or without addition of chlorophyll[6]. Each of the above different situations seems to be characterized by a different range of activity of the photosynthetic apparatus.

Thus, the dramatic rise in photosynthetic activity during the initial lag phase,

might be regarded as a process of activation of the membrane remnants. It has been shown before that these vesicular remnants are relatively rich in components of the photosynthetic electron transfer chain such as cytochrome *f* [2,28] and ferredoxin[1,28]. One can assume that while other components might be present too, some components are present only in limiting amounts. The synthesis of these rate-limiting components following onset of illumination and their integration into the membranes, might explain the sudden activation of the photosynthetic system. Since the relative chlorophyll content of these membranes is low, the calculated specific activity per chlorophyll unit will have a high value. In addition, as indeed observed in this work, the low chlorophyll content per unit membrane area, will require a high illumination intensity in order to saturate the photosynthetic capacity or potential of the reactivated membranes. The transition in the degree of organization of the membranes during the reactivation process is gradual, as measured for Photosystem I activity, and seems to be partially reversible, at least during the early phase of the greening, as indicated by the decrease in the photosynthetic activity following transfer of the greening cells back to the dark (Figs 3A and 3B).

The reduction in the photosynthetic activity following transfer of greening cells to the dark, during the early phase of the process, was not evident when the light-dependent pH rise was measured*. In the whole cells proton uptake, ATP formation and $NADP^+$ reduction rely on electron supply by Photosystem II. It is thus possible that the stability of the light-dependent pH change, measured in whole cells, indicate a real difference between Photosystem II and I. Indeed, the variations in Photosystem I activity seem to be more drastic than the activity of Photosystem II. While chlorophyll continues to be synthesized together with other newly formed membrane components, and becomes integrated within the growing membrane, the ratio of the chlorophyll to the photosynthetic chain components increases and eventually reaches the value usually found in light-grown cells. This has the effect of reducing the specific activity per chlorophyll unit, and at the same time might permit a more efficient light utilization and decrease in the $I_{S\,1/2}$ value.

Thus, one can regard the early phase of the greening process, before the peak of activity is reached, as a phase in which the pigment system is limiting, while the later phase after the peak activity, as a phase in which the electron transfer chain and energy coupling are limiting.

The gradual change in the relative efficiency of the light absorbing system saturating the Photosystem I cyclic photophosphorylation is also demonstrated by the change in the relative PMS–ascorbate concentration required for a linear response to changes in the illumination intensity. Non-linear response to the illumination intensity, during greening of a yellow mutant of *Chlorella*[33], or in higher plants chloroplasts at high ascorbate concentration[32] was reported before. The fact that linearity of photophosphorylation with PMS–ascorbate during the early phase of the greening

* One should notice that these measurements were carried out with whole cells. The activity of whole *C. reinhardii* cells was regarded as the expression of the light-dependent proton uptake by the chloroplast at the level of the whole cell[2]. Recently, it was proposed that the light-dependent pH rise measured in this way might actually represent a CO_2 or HCO_3^- uptake due to photosynthetic CO_2 fixation[31,34]. Whether the light-dependent pH rise is due to proton uptake, CO_2 fixation or both, the appearance of this activity during the greening and its behavior towards light intensity, represents a change in the light-absorbing capacity activity of the electron transfer chain, and energy-coupled reactions.

can be achieved only at low ascorbate concentrations can be regarded as a result of the low activity of the light harvesting system, which cannot efficiently oxidize the Photosystem I so as to accept electrons from the more reduced PMS–ascorbate donor system.

An additional demonstration of the transit changes in the relative ratio of the light-absorbing system to the electron transfer system is provided by the results of the experiments in which chloramphenicol was used to block the chloroplast protein synthesis during greening. Here again, different sets of conditions could be obtained. At low concentration of chloramphenicol (10 μg/ml), the inhibition of protein synthesis required for activity of the photosynthetic membranes was partial, while chlorophyll synthesis was practically unaffected. In this case, membranes were obtained with a relative high ratio of light absorption capacity and low electron transfer capacity, and thus light saturation could be achieved at lower illumination intensity. On the other hand, use of high chloramphenicol concentrations which blocked completely the synthesis of chloroplast proteins and significantly inhibited chlorophyll accumulation, resulted in the formation of photosynthetically inactive membranes*. Such inactive membranes, however, can be repaired by incubation of the cells under conditions permitting synthesis of chloroplast proteins and their integration within the existing membranes, in absence or presence of additional chlorophyll synthesis. In the first case, the reactivated membranes required a high illumination intensity for maximal activity throughout the repair period (up to 6h), indicating that only small amounts of the light-absorbing chlorophyll present in the membranes can be utilized for electron transfer coupled with ATP formation. This small amount of active chlorophyll actually might have been present initially, and organised properly in the remnants of the membranes after cell division in the dark. This would imply that in the absence of chloroplast protein synthesis, in addition to the lack of proteins required for the formation of active membranes, the chlorophyll integrated in those faulty membranes is "misplaced" in such a way as to be unavailable for immediate utilization when the photosynthetic electron transfer activity has resumed.

When chlorophyll synthesis did occur during the repair, the requirement for high illumination intensity diminished drastically, as it did during the initial phase of the normal greening process, indicating that during the repair process the membranes can accept and utilize chlorophyll.

Whether the already present chlorophyll might become reorganized, and to what extent reutilized in the repair process, it remains to be studied.

The results obtained in this work were interpreted in terms of membrane modulation during growth. One can, however, regard the growing membranes as a mosaic system, in which regions of inactive membrane alternate with regions of normally active ones. In this case, the term "modulation" as used above will merely mean a change in the ratio between the two types of membrane regions. That this might not be the case is suggested by recent results indicating that the photosynthetic membranes of *C. reinhardi* y-1 cells grow as a homogenous system[11].

Changes in photosynthetic activity have been reported also for synchronous cultures of the wild type *C. reinhardi*[14] indicating that the membranes develop stepwise. Similar observations have also been reported for the photosynthetic mem-

* The relative contribution of membrane proteins of the cytoplasm and chloroplast to the growing membranes and its regulation in *C. reinhardi* y-1, are discussed in detail elsewhere[5,11,12].

branes of higher plants[24, 25]. Thus, one can conclude that the development of chloroplast membranes can occur stepwise, and withstand modulation of their composition and degree of organization, within certain limits.

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