Biochimica et Biophysica Acta, 503 (1978) 316—332

BBA 47524

FUNCTIONAL AND STRUCTURAL ORGANIZATION OF CHLOROPHYLL IN THE DEVELOPING PHOTOSYNTHETIC MEMBRANES OF *EUGLENA GRACILIS* Z.

I. FORMATION OF SYSTEM II PHOTOSYNTHETIC UNITS DURING GREENING UNDER OPTIMAL LIGHT INTENSITY

G. DUBERTRET and M. LEFORT-TRAN
With the technical assistance of F. AMBARD-BRETTEVILLE

Laboratoire de Cytophysiologie de la Photosynthèse, C.N.R.S., 91190 Gif-sur-Yvette (France)

(Received October 31st, 1977)
(Revised manuscript received January 28th, 1978)

Summary

The relationships between light-harvesting chlorophyll and reaction centers in Photosystem II were analyzed during the chloroplast development of dark-grown, non-dividing *Euglena gracilis* Z. Comparative measurements included light saturation of photosynthesis, oxygen evolution under flashing-light and fluorescence induction. The results obtained can be summarized as follows: (1) Photosystem II photocenters are formed in parallel with chlorophyll synthesis, but after a longer lag phase. (2) As a consequence, the chlorophyll : reaction center ratio (Emerson's type photosynthetic unit) decreases during greening. (3) This decrease is accompanied by considerable changes in the energy transfer and trapping properties of Photosystem II. Most of the initially synthesized chlorophylls are inactive in the transfer of excitations to active photochemical centers and are shared among newly formed Photosystem II photocenters; as a consequence, the number of chlorophylls functionally connected to each Photosystem II photocenter decreases and cooperativity between these centers appears. Results are discussed in terms of chlorophyll organization in developing photosynthetic membranes with reference to the lake or puddle models of photosynthetic unit organization.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Introduction

The photosynthetic apparatus can be easily modified by acting either on internal controls (by mutation or the use of antibiotics and antimetabolites) or on external stimuli. This offers a unique opportunity for correlating functions, structural organization and the molecular composition of photosynthetic membranes. In such experimental systems, the development of these membranes during the light-induced differentiation of proplastids or etioplasts into chloroplasts has been extensively studied, since it constitutes a dynamic system which also enables the biogenesis of such membranes to be analyzed (for review, see refs. 1 and 2).

Although not well understood when observed at the overall level of the chloroplast and the photosynthetic reaction, such correlations should also be present at the level of clusters of molecules, partial photosynthetic reactions and the molecular architecture of chloroplast membrane. In this context, the relationships between light-harvesting and photochemical reactions associated with the photosynthetic unit concept, as proposed by Gaffron and Wohl [3] from the experiments of Emerson and Arnold [4] have been analyzed in vivo by several authors using normal [5,6] or mutant organisms [7,8], or during the differentiation process [9—13]. An unresolved point is whether such units, in which about 2400 chlorophyll molecules cooperate in harvesting the required light quanta for fixation of one CO_2 and for evolution of one O_2 molecule correspond to organized structural entities. As a first step, the constancy of the chlorophyll : reaction center ratio in different organisms and under different conditions had to be verified. Unit size was generally evaluated on whole cells either by measuring oxygen evolution under a regime of repetitive saturating short light flashes [5—7] or by analyzing light saturation curves of photosynthesis [8—12].

Unit size appeared to be approximately constant in most of the algae analyzed [6] but seemed to be rather variable in higher plants, depending on the leaves analyzed [5]. However, mutations can change unit size by reducing the chlorophyll concentration of cells without altering that of their reaction centers [8]. With the exception of some organisms which seem to retain a constant unit size [12], most experiments demonstrate an increase in the size of the photosynthetic unit during chloroplast differentiation [9—11] resulting from a step-wise insertion of reaction centers and chlorophyll into the photosythetic membranes.

These methods of analysis, although very useful in quantitative determinations, are limited to evaluations of overall chlorophyll : reaction center ratios. This is particularly true during the transitory phases of greening where it is not known if the two Photosystems are present in a functional state and in equivalent amounts. While broadened to cover the more specific level of Systems I and II reactions [14—17], the use of these methods should be combined with still other methods that provide more precise information on the distribution of chlorophyll between the two Photosystems and on the functional and structural interrelationships between the light-harvesting pigments and the photochemical reaction centers.

This approach to investigating the greening processes was primarily introduced by Butler [18] and Diner and Mauzerall [16] by the use of fluorescence-induction measurements. We previously analyzed the evolution of light-harvesting, energy transfer and trapping properties of System II during greening of a dark-grown *Chlorella* mutant [19]. We interpreted transients of fluorescence inductions in the presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and of oxygen evolution activation in terms of size and general organization of System II units. More recently, Cahen et al. [20], Melis and Akoyunoglou [21] and Akoyunoglou [22] reported on System II development as analyzed by fluorescence-induction measurements. However, although they appear to be consistent with results obtained with conventional methods, the reported conclusions may be considered somewhat speculative inasmuch as interpretations of such transitory phenomena are difficult and controversial.

The validity of interpretations of fluorescence-induction parameters is analyzed in the present report by comparing them with results obtained with other methods on the same organism. Comparative oxygen and fluorescence measurements were performed during the transitory phases of chloroplast development throughout greening of dark-grown *Euglena gracilis*, whose functional and structural aspects were already described by Stern et al. [23,24], and Ben-Shaul et al. [25]; The pattern described for the organization of chlorophyll and photochemical center leading to functional System II units during greening of these cells will complete the results obtained by these authors and will serve as reference for the results in papers to follow.

Materials and Methods

Cell culture and preparation. Euglena gracilis Z cells, maintained in darkness for several years were grown in the dark at 26° C in a pH 3.5 medium containing 33 mM DL-lactate as sole utilizable carbon source [26]. After 3 days of exponential growth, 2 l of culture were aseptically mixed with 5 l of pH 6.8 resting medium [23], then stirred in the dark for 3 days in order to allow the cells to undergo residual divisions. At that time, cultures were exposed to 1200 lux of white light provided by banks of fluorescent tubes and samples were taken at different times of greening. After determination of cell numbers and chlorophyll concentrations the cultures were gently centrifuged at 1000 × *g* and the pellets were resuspended in appropriate volumes of supernatant in order to obtain algal suspensions equivalent to 5 μg chlorophyll/ml. These conditions led to minimal and standardized screening effects during oxygen or fluorescence measurements.

Oxygen measurements. Oxygen measurements were performed at 25° C with a YSI Clark-type electrode in a 1 cm diameter sample chamber containing 2 ml of suspension.Light intensity was selected by screening the actinic beam with neutral density filters.

For flashing light measurements, a xenon stroboscope (General Radio, type 1538 A, Strobotac) was used, equipped with an extension lamp allowing the flashing light source to be placed 2 cm from the sample chamber. Flash intensity and homogeneity were increased by coating the lamp and the sample chamber with a sheet of polished stainless steel. Two regimes of flashes were

used, from110 to 690 and from 670 to 4170 flashes/min, in each of which duration and intensity were constant, with values of 3 μs (at $\frac{1}{3}$ peak intensity) and $15 \cdot 10^6$ lux (measured at 1 m) in the first, and 1.2 μs and $1.5 \cdot 10^6$ lux in the second (manufacturer's specifications). Tests with neutral filters placed between the lamp and the sample chamber showed that the intensity of the flashes of the first range, but not those of the second, was saturating for cells throughout greening. Addition of low intensity background light of any wavelength had no effect on flash yield.

Fluorescence-induction measurements. Fluorescence-induction measurements were performed at room temperature on the same algal suspension as above in the presence of $2 \cdot 10^{-5}$ M DCMU. The apparatus consisted of a stabilized 250 W quartz-halogen lamp which provided a white light beam focused on a magnetic shutter (0.6 ms opening time) and which was filtered through a 20 mm broad band BG 38 blue Schott filter. The blue actinic beam was focused again on a 3 mm diameter, 1 mm deep sample cuvette specially built to minimize inhomogeneities of the light and screening effects by the cells [27]. Incident intensity was $1.8 \cdot 10^4$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. Fluorescence emission was filtered through two red cut-off filters with low intrinsic fluorescence (Rubilith Amberlith Ulano + Kodak wratten No. 70) before reaching an RTC XP 1002 photomultiplier. Variations of the signal as a function of time were recorded on the memory screen of a Tektronix D II 5103 N oscilloscope.

Samples taken at the early stages of greening required high cell concentrations in order to obtain the desired chlorophyll concentration. This could have been responsible for important perturbations of fluorescence parameters caused by light scattering by the cells. This was tested by comparing the fluorescence properties of whole green cells or ultrasonicated chloroplast fragments which were diluted either with buffer or with highly concentrated suspensions of etiolated cells or fragments. Variations induced by light scattering did not exceed 10% and were considered negligible.

Spectra of greening cells in vivo. Low temperature absorption spectra were recorded with a Cary 14 spectrophotometer equipped with an accessory for measuring light-scattering samples. Cells were deposited as thin layers on Millipore filters. For the early stages of greening, cells were replaced by concentrated suspensions of differentiating proplastids obtained by breaking greening cells with a French pressure cell (700 lb/inch^2) and then partially purifying the proplastids on a sucrose gradient. Low temperature was obtained by cold conduction through a metal strip from liquid nitrogen to the reference and sample Millipore filters which were held on windows in the upper portion of the metal strip and placed in the path of the analytical and reference beams.

Results

Chlorophyll synthesis and oxygen evolution

The time courses of oxygen evolution and chlorophyll accumulation during greening of etiolated, non-dividing *Euglena gracilis bacillaris* were previously described by Stern et al. [23]. Our results are very similar and are given in Fig. 1 as a basis for the understanding of subsequent measurements.

After exposure of etiolated cells to light, chlorophyll synthesis begins

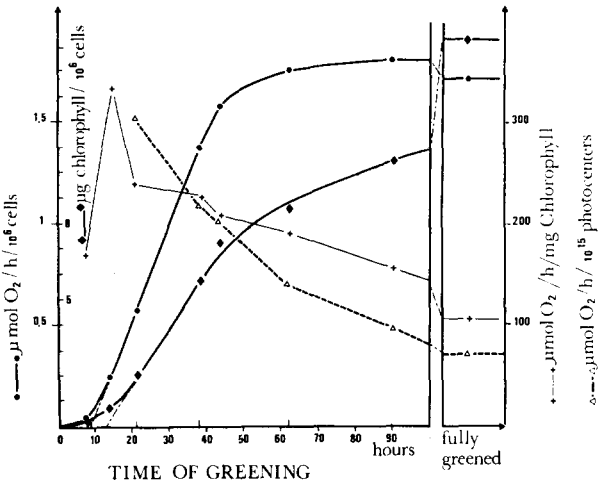

Fig. 1. Development of photosynthetic oxygen evolution during greening. Oxygen evolution is quantitatively measured under saturating continuous light ($8 \cdot 10^5$ ergs/s per cm^2) and expressed on a per cell, chlorophyll or reaction center basis. Reaction center concentrations were calculated from measurements of oxygen evolution under repetitive short flashes.

immediately at a low rate and then increases to a maximal linear rate. This lag of chlorophyll synthesis can be measured by extrapolating the linear phase to the time scale. In most experiments, this lag lasted for about 14 h, although a great variability can be observed, probably depending on the paramylum content of the cells at the onset of greening [28]. Such a variability might also be correlated with the cathepsine activity (peptide hydrolase) level obtained in the starved cells [29] which have to derive the amino acids required for chloroplast development from internal pools or from the degradation of preexisting proteins [30]. After this linear phase of maximal synthesis the rate of chlorophyll accumulation progressively decreases and maximal levels, ranging between 8 and 15 µg chlorophyll/10^6 cells, are obtained after 80—120 h. The chlorophyll *a* : chlorophyll *b* ratio decreases from 8 : 9, to 5 : 6 during the first 10—15 h following exposure to light, then stabilizes as greening proceeds.

The development of oxygen evolution, expressed on a per cell basis follows approximately the same pattern as chlorophyll accumulation, but with a significantly shorter lag and a much faster increase. Due to difficulties in measuring very weak photosynthetic oxygen evolution superimposed on high respiratory rates, we were not able to determine if this lag corresponded to an accelerating process from the onset of illumination or to a time delay in the acquisition of oxygen evolution ability by the cells. At any rate, after this lag and the linear increase, photosynthetic ability stabilizes after about 70—80 h of greening, independently of chlorophyll synthesis.

When expressed on a chlorophyll basis, oxygen evolution increases sharply during a phase whose duration seems to correspond to the lag phase of chlorophyll synthesis, and then decreases slowly from approx. 350 to 100 µmol $O_2 \cdot h^{-1} \cdot mg^{-1}$ chlorophyll, as greening proceeds. This evolution results from

the shorter lag and greater rate in the appearance of oxygen evolution ability, compared to chlorophyll accumulation in greening cells.

Light saturation points

The light intensities needed for saturation of oxygen evolution were measured at different stages of greening and were found to correspond to about $8 \cdot 10^5$, $4.3 \cdot 10^5$, $2.9 \cdot 10^5$ and $1.9 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} for cells greened for 7, 13, 37 and 63 h, respectively. Values of light saturation points thus markedly decreases during the early phases of greening, then progressively tend toward steady-state values after 63 h of illumination in this experiment.

Measurements of reaction center concentrations and of apparent size of photosynthetic units

The concentrations of reaction centers connected to the electron transport chains were calculated from measurements of oxygen evolution under a continuous regime of repetitive saturating short flashes, according to the method of Emerson and Arnold [4]. The total concentrations of active System II photochemical centers, including chlorophyll *P*-680, primary electron donor Z and acceptor Q, connected or unconnected to the plastoquinone pool, were estimated from areas defined by the fluorescence induction curves in the presence of DCMU and their asymptotes [31,32], normalized by using the F_{max} level, on the assumption that most of fluorescence emission originates from chlorophylls belonging to System II [33]. Results expressed on a per cell basis are compared with chlorophyll accumulation in Fig. 2.

The time-courses of accumulation of System II photocenters in greening cells, as measured by oxygen and fluorescence methods, appear to be similar in spite of slight differences in the length of the lags, which may not be significant, and show the same general pattern as that for chlorophyll accumulation. After a lag phase 7—10 h longer, thus lasting about 30 h, System II reaction centers undergo a linear increase in concentration in parallel with chlorophyll. The time shift of the curves remains constant until the end of greening, and is then eliminated by an earlier decay in chlorophyll synthesis. As a consequence of this difference in the duration of the lag phase of the two curves, important amounts of chlorophyll are already accumulated in the greening cells when the linear phase of maximal rate of System II reaction center formation begins. The reaction center : chlorophyll ratios thus are low within the first 30 h of greening, as shown in Fig. 3. During the subsequent phase of linear accumulation, chlorophylls and reaction centers are synthesized at the same relative rate. However, they are added to components already accumulated during the lag phase for reaction center formation, particularly to the large amounts of early synthesized chlorophylls almost devoid of reaction centers. These are responsible for the reaction center : chlorophyll ratio increasing instead of remaining constant as greening proceeds (Fig. 3). Maximal and steady-state values of this ratio are obtained when both accumulation curves stabilize at the end of greening.

Simultaneously, the size of the Emerson-type photosynthetic unit (expressed by the reciprocal of this ratio) dramatically decreases during the greening process, as shown in Fig. 3, and stabilizes at a value of approx. 1500 chlorophylls per reaction center.

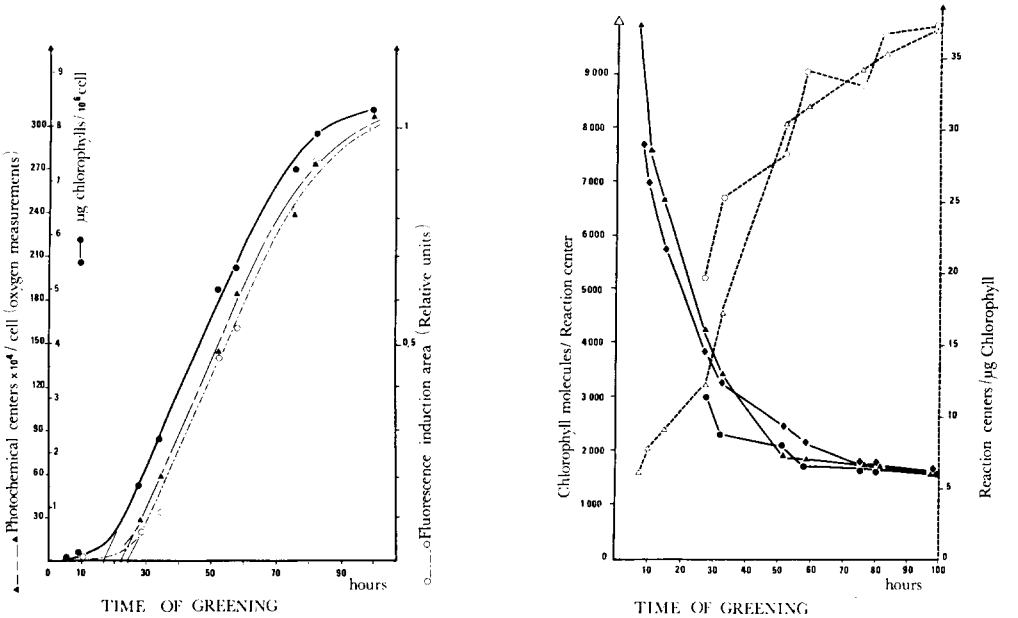

Fig. 2. Time course of chlorophyll synthesis and photocenter formation in greening cells. The extrapolations of the linear phases on the time scale measure lag phases of 16 h for chlorophyll synthesis, 24 and 26 h for photocenter formation when calculated from oxygen measurements (Emerson's method) or from normalized fluorescence inductions.

Fig. 3. Changes in photosynthetic unit size and photocenter concentration on a chlorophyll basis. Total chlorophyll per reaction center is calculated from flashing light experiments (●———●, quantitative values of chlorophyll molecules/reaction center), or from the reciprocal of the normalized induction areas (▲———▲, relative values); efficient chlorophylls per System II photocenter are estimated from the reciprocal of the half-rise time of fluorescence (◆———◆, relative values). These curves are normalized to a common final value. Reaction center concentrations are expressed as the reciprocal of the unit size determined by flashing light experiments (○- - - - -○, quantitative values in 10^{10} reaction centers/µg chlorophyll or by the normalized fluorescence induction area (△- - - - -△, relative values).

Estimation of the turnover rate of the overall photosynthetic reaction

It is surprising that the maximal rate of oxygen evolution per chlorophyll is obtained at about 16 h (Fig. 1) when the reaction center : chlorophyll ratio is the smallest (Fig 3). This lack of direct relationship between these two parameters is well expressed by the dashed curve in Fig. 1 which represents a progressive decrease of the maximal rate of oxygen evolution per reaction center as greening proceeds. Such evolution could originate from changes of the turnover rate of the overall photosynthetic reaction during greening.

Fig. 4 shows the oxygen yield per flash as a function of the length of the dark interval between flashes. The lack of detectable response did not allow significant measurements before 21 h of greening. Since the flash yield depends on the number of active centers, the minimal dark interval allowing maximal yield will be a measure of the time required for the regeneration of reaction centers after light excitation, or of the turnover rate of the overall photosynthetic reaction. For technical reasons, flashes in the 14—85 ms range were not saturating and, consequently, excited a fraction of the reaction centers, depending on the size of their efficient light-harvesting antenna. However, these

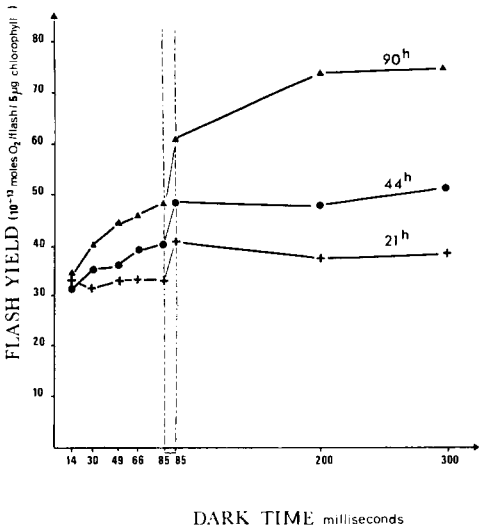

Fig. 4. Relationships between flash yield and dark interval on cells greened for 21, 44 and 90 h. The gap at 85 ms is due to flashes saturating in the 85—300 ms dark interval range but not in the 14—85 ms range.

results are qualitatively if not quantitatively significant. At 21 h of greening the flash yield appears to be saturated for dark intervals of approx. 30—50 ms; as greening proceeds, maximal flash yield is obtained for increasing dark times of about 85 ms at 44 h, and finally 200 ms for fully green cells. A marked decrease of the turnover rate of the overall photosynthetic reaction thus occurs during greening and accounts for the apparent contradiction between maximal rate of oxygen evolution and low concentration of reaction center on a per chlorophyll basis. On the other hand, it is to be noted that the jump of flash yield observed for the same 85 ms dark interval in the two flash intensity ranges increases significantly during greening.

Fluorescence induction properties

Fluorescence inductions in the presence of DCMU offer several data concerning light-harvesting, excitation transfer and trapping properties of the Photosystem II photochemical apparatus. We already mentioned the relationships between the fluorescence induction areas and the System II photochemical center concentrations. In a previous paper, we discussed time-courses and shapes of fluorescence induction curves [19]; half-rise times were taken as measurements of the sizes of the light-harvesting antennae of System II units [16], since they are inversily proportional to the number of excitations reaching System II photocenters per unit time, i.e., under a constant light intensity, they are inversily proportional to the number of light-harvesting chlorophylls actively associated with these centers. The shapes of the induction curves were interpreted as resulting from the probability P of excitation transfer from one unit to another; as P increases from 0 to the normal value of 0.6, the shape of the induction curves evolves progressively, from exponential to sigmoidal [34]. Variable fluorescence (F_v) depends on the redox state of the primary electron acceptor Q of System II photocenters [35]; when measured

in the presence of DCMU, F_v thus will be proportional to the amount of chlorophylls active in transferring absorbed energy to System II photochemical centers. Constant fluorescence (F_c) corresponds to non-trapped energy even if all System II photocenters are in an active state including an oxidized primary electron acceptor Q; constant fluorescence thus will measure the amount of chlorophyll inactive for the transfer of excitations to reaction centers, because they are either not connected to or are too for from these centers. As a consequence, the $F_v : F_{max}$ and $F_c : F_{max}$ $(1 - F_v : F_{max})$ ratios will reflect the proportion of chlorophylls active and inactive in transferring energy to System II reaction centers.

Fig. 5A shows the evolution of F_{max} levels as a function of the time of greening, measured in three different experiments. After a very fast decrease occurring during the first 15—30 h, depending on the experiments, which could be correlated to the lag phases in chlorophyll or System II photocenter accumulation, F_{max} progressively reaches a steady-state level representing 10—20% of the initial level measured at 6 h of greening. This evolution seems to be not directly time dependent since these curves are spread over a rather large time range; however, when expressed as a function of the normalized induction areas (Fig. 5A′), i.e., as a function of the System II photocenter

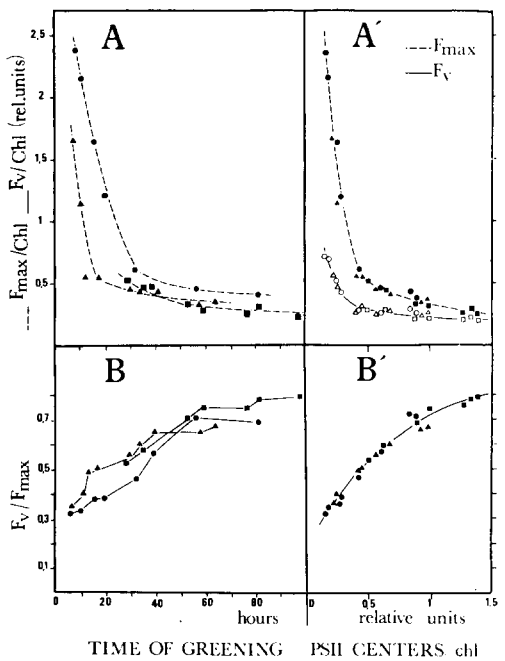

Fig. 5. Changes in fluorescence parameters observed in three different experiments (●, ■, ▲). The chlorophyll content of the samples measured was adjusted to the same final 5 μg chlorophyll/ml concentration. (A) Changes in F_{max} level as a function of time of greening: similar spreads of F_v on the time scale are not shown. (A′) Same changes of F_{max} and F_v expressed as a function of System II photocenter concentration per chlorophyll (normalized fluorescence induction). Variations of F_c correspond to the difference between F_{max} and F_v. (B) Increase of the $F_v : F_{max}$ ratio as a function of time of greening. (B′) Increase of the $F_v : F_{max}$ ratio as a function of the concentration of System II photocenters per chlorophyll (normalized fluorescence induction).

concentration per amount of chlorophyll, a single curve is obtained from the three experiments.

A similar change of F_{max}, originating in a decrease of F_c without significant change of F_v, was already observed during greening of a mutant strain of *Chlamydomonas* [20]. Surprisingly, the drop of F_{max} observed in the present case, occurs at the expense not only of F_c but also of F_v as the concentration of Photosystem II reaction centers on a chlorophyll basis increases during greening (Fig. 5A'). In other words, instead of increasing the amplitude of F_v per System II decreases during greening. This leads to the assumptions (1) that there must be a decrease of fluorescence quantum yield of both active and inactive chlorophylls responsible for F_v and F_c emission, respectively, during greening, and (2) that such a decrease is related to increasing concentrations of System II reaction center per chlorophyll. Anyhow, F_c decreases much faster than F_v, as is well expressed by the increase of the $F_v : F_{max}$ ratios as greening proceeds (Fig. 5B). $F_v : F_{max}$ evolution appears to be more dependent on the Photosystem II reaction center concentration than on time (Fig. 5B'). In other words, the proportion of chlorophylls which are inactive in the transfer of excitation to reaction centers decreases as the concentration of System II photochemical centers per amount of chlorophyll increases.

Simultaneously, the reciprocal of half-rise times of fluorescence inductions (which express the number of active light-harvesting chlorophylls associated with each System II photochemical center) increases about 4-fold and stabilizes at the end of greening. Finally, simultaneously with the increase of $F_v : F_{max}$ ratios, System II photocenter concentrations and half-rise times of fluorescence, the shape of the induction curves evolves progressively from exponential at the early stages of greening to sigmoidal, as shown in Fig. 6.

Reaction center concentration thus seems to control the evolution of the F_{max}, F_c and F_v levels, of the $F_v : F_{max}$ ratios, of the half-rise times of fluorescence and of the shape of the induction curves which all move together. Except for F_{max}, the evolution of these fluorescence-induction parameters during greening is the same as that observed with fully greened cells in which

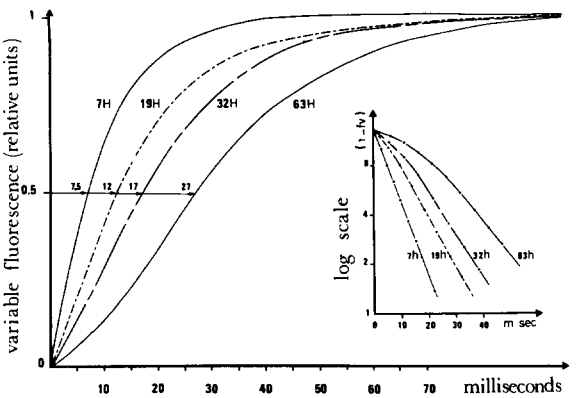

Fig. 6. Changes in the shape of fluorescence inductions in cells greened for 7, 14, 32 and 63 h. Variable fluorescence is normalized to the maximal level. Half-rise times increase from 7.5 to 12, 17, 27 ms, respectively. Insert: log plot of $(1 - F_v)$.

increasing proportions of reaction centers are allowed to regenerate during increasing dark periods, after having been closed by pre-exposure to light.

Low temperature absorption spectra

Changes in absorption spectra and difference spectra (Fig. 7) show that usual chlorophyll forms already described in *Euglena* [36] are visible throughout greening although their relative proportions vary. Most of the early synthesized chlorophylls are combined with their lipoproteic environment as the 673 nm chlorophyll *a* form, which seems to be the first formed in greening organisms [37]. As greening proceeds, one can observe a relative increase of the 680 nm absorbing chlorophyll *a* and of the 650 nm absorbing chloro-

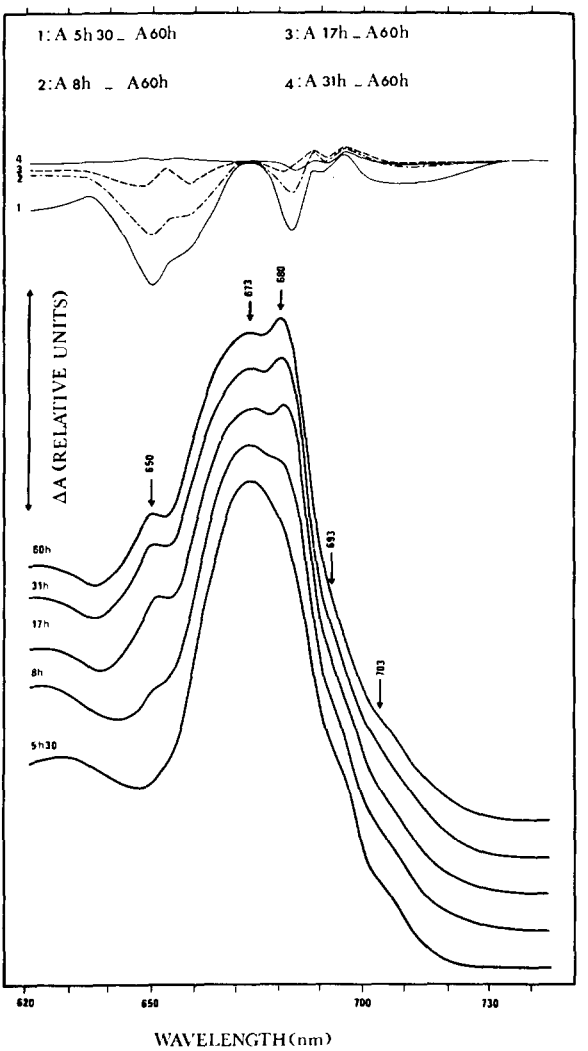

Fig. 7. Low temperature absorption spectra and difference absorption spectra of cells greened for 5.5, 8, 17, 31 and 60 h. Absorption spectra were recorded continuously and normalized to the 673 nm peak. Difference spectra were plotted from calculations.

phyll *b*, without important changes in the relative amounts of the chlorophyll forms which absorb around 693 and 703 nm. These changes are principally visible during the early phases of chloroplast development corresponding to the lag phase in reaction center formation. After 31 h of light exposure, the greening continues by a quantitative increase of chlorophyll per cell without significant qualitative changes in the proportion of their different forms. In other words, the different chlorophyll forms which constitute the light-harvesting systems are already present in nearly steady-state relative amounts when the phase of maximal rate of reaction center formation begins.

It is especially difficult during the transitory phases of chlorophyll organization in the developing thylakoids to assign these different chlorophyll forms to System I or System II. However one can retain the possibility that chlorophyll *a*-673 is associated with System II and chlorophylls *a*-680, *a*-693 and *a*-703 with System I [36]. On the other hand, it seems well established that the 685 nm fluorescence emission originates in the 673 nm-absorbing chlorophyll [36]. The initial high level of fluorescence emission and its subsequent decrease thus could be correlated to the decrease in the relative amounts of this chlorophyll form as greening proceeds.

Discussion

Formation of active System II reaction centers

System II reaction centers leading to variable fluorescence are functionally connected with the water-splitting systems and with primary electron acceptors Q, but not necessarily with the plastoquinone pools. On the contrary, reaction centers which keep the ability to evolve oxygen under a continuous regime of repetitive flashes are connected to complete electron transport chains. Fig. 2 does not show significant differences in reaction center concentrations when measured by fluorescence or oxygen methods at any stage of greening. Active System II reaction centers, including the primary electron donors and acceptors, thus appear to be connected to complete electron transport chains immediately or soon after formation. This can be related to their long lag phase which could be responsible for their being the last components of complete electron transport chains to be inserted.

This very long lag phase introduces a 7—10 h delay in active reaction center formation compared to chlorophyll synthesis. This delay is maintained constant throughout greening and leads the two curves to rise in parallel with a 7—10 h shift in time. Such a parallelism suggests a control of formation of active reaction center by chlorophyll even if System II can develop whithout concomitant chlorophyll synthesis, as shown by our unpublished results and those found by Gurevitz et al. [38] on the same organism. The nature of such control appears to be indirect and will be analyzed in subsequent reports.

Evolution of size of photosynthetic units

Whatever its origin, this shift in time of active reaction center formation compared to chlorophyll synthesis leads to a progressive decrease of the overall chlorophyll : reaction center ratio, i.e., of the Emerson-type photosynthetic unit, until a steady-state value is reached at the end of greening (Fig. 3).

Such an overall ratio does not give any indication of the size of the functional light-harvesting systems of Photosystems I or II. We do not have any data concerning Photosystem I but the decrease of the reciprocal of the half-rise times of fluorescence induction (Fig. 3) expresses a very marked decrease of the functional light-harvesting antennae of System II units. In addition, we observed that the difference in flash yield observed for the same 85 ms dark interval in the two flash intensity ranges increases significantly as greening proceeds (Fig. 4). In other words, even for the same intensity, the non-saturating flashes used excite a smaller fraction of reaction centers at the end than at the beginning of greening. This confirms the decrease of the size of the functional light-harvesting antennae of active reaction center during greening.

However, these results seem to contradict measurements of light-saturating points whose decrease suggests that the size of the functional light-harvesting antennae of active reaction centers increases during greening. On the other hand, we pointed out another contradiction between the high maximal rates of oxygen evolution per chlorophyll and the low reaction center : chlorophyll ratio observed at about 16 h of greening. In fact, such contradictions are apparent only since saturation curves for flash yield as a function of increasing dark interval between flashes (Fig. 4) show that the turnover rate of the overall photosynthetic reaction markedly decreases during greening from approx. 30 to 50 ms at 21 h of illumination to about 200 ms in fully greened cells. This could originate from high regeneration rates of NADP and ADP due to a relatively high CO_2 fixing ability of the developing chloroplasts as compared with their ability to produce NADPH and ATP. Anyhow, the measured high regeneration rate of the initially formed reaction centers largely balances their low concentration and accounts for the two contradictory points. The results obtained with the several methods used thus are concordant in demonstrating a progressive decrease of the size of the Emerson-type photosynthetic units as well as of the functional light-harvesting antennae of active System II units.

Organization of chlorophylls in the developing thylakoids

One of the most prominent characteristics of fluorescence inductions is the high F_c level as compared to F_v, and thus the very low value of $F_v : F_{max}$ ratio observed during the early stages of greening (Fig. 5B'). A large proportion of the early synthesized chlorophylls constituting the large initially formed Emerson-type photosynthetic units thus are unable to channel the absorbed energy to active reaction centers of Photosystem I or II.

As greening proceeds, F_c decreases; the simultaneous increase of $F_v : F_{max}$ expresses a decrease in the relative amount of these inefficient chlorophylls which then become associated to System I or II as an active part of light-harvesting antennae. A similar evolution was already observed by Cahen et al. [20] during greening of a mutant strain of *Chlamydomonas*; they interpreted a marked decrease of F_c, leading to a decrease of F_{max} without significant changes of F_v as resulting from a progressive reorganization and association of unorganized chlorophylls with light-harvesting systems of Photosystem I, accompanied by a decrease of their fluorescence yield. However, such an interpretation does not account in our case for the participation of F_v to the decrease of F_{max} as the concentration of reaction center per chlorophyll

increases (Fig. 5A′). It seems likely that this decrease of F_{max} originates in a decrease of fluorescence quantum yield of non-functional chlorophylls (responsible for F_c emission) as well as of functional chlorophylls intimately associated with System II light-harvesting antennae (responsible for F_v emission). Such a probable decrease of fluorescence quantum yield during greening could likely originate in the observed parallel relative decrease of the 673 nm absorbing chlorophyll (Fig. 7), which is already recognized to be responsible for the 685 nm fluorescence emission [36]. If variations of fluorescence intensity are omitted by normalizing the fluorescence induction at the same F_{max} level, the increase of $F_v : F_{max}$ ratio results from a decrease of F_c at the benefit of F_v. Such evolution thus would express a progressive conversion of inactive chlorophyll responsible for F_c emission into functional light-harvesting chlorophylls responsible for F_v emission, thus connected with active System II reaction centers.

These chlorophylls are not added to functional light-harvesting antennae of already present and active System II units since their size decreases during greening (Fig. 3). The dependence of this process on reaction center concentrations on a per chlorophyll basis (Fig. 5B′) rather suggests that these inactive chlorophylls are converted into functional light-harvesting antennae of newly formed System II reaction centers.

The question now arises as to why most of the early synthesized chlorophylls are inactive in transferring the absorbed energy to reaction centers. A first simple hypothesis is that early synthesized chlorophylls are not organized in the developing photosynthetic membrane in a way that allows energy migration and, consequently, energy transfer to reaction centers. Subsequent reorganization then would lead to their conversion into efficient light-harvesting chlorophylls functionally connected with newly formed active System II reaction centers. However, such reorganization should lead to an increase in the size of these developing System II units, which was not observed. On the other hand, it is difficult to imagine a simple explanation for such reorganization to be under the dependence of System II reaction center concentration. It seems likely that early synthesized chlorophylls, responsible for F_c emission, become rapidly organized in a way that allows energy migration, but remain inactive until functional connections are established with subsequently formed active System II reaction centers. Such an hypothesis is consistent with the 7—10 h delay in reaction center formation to chlorophyll synthesis and accounts for the dependence of $F_v : F_{max}$ on reaction center concentrations.

However, the question remains as to why these early synthesized chlorophylls, which seem to be organized in a way that allows energy migration, are not able to channel the absorbed energy to already present reaction centers. Two possibilities could a priori account for this property, depending on the structure and spatial organization of System II units in the developing photosynthetic membrane. If System II units are organized as structural entities according to the puddle model, but spatially isolated from each other, their physical separation will constitute a physical barrier to energy transfers from light-harvesting chlorophylls of inactive developing units to reaction centers of neighbouring, already active ones. In this case, however, completion of these

inactive developing System II units by addition of active reaction centers, would only increase $F_v : F_{max}$ ratios without affecting half-rise times or the exponential shape of fluorescence induction curves. Developing System II units are not organized as isolated structural entities.

On the contrary, System II units could be organized according to the same "puddle" model but as individual entities connected to each other, or according to the "lake" model, as entities of statistical meaning defined by an average ratio of functional light-harvesting chlorophylls to System II reaction centers. Anyhow, the greening process should be equivalent in this case to an increase of density of System II reaction center on a chlorophyll continuum allowing free migration of energy among System II units. However, the extent of this migration is limited by the increasing probability of the absorbed energy to be dissipated as heat or fluorescence (F_c) as the distance to travel before being trapped by reaction center increase. Within the framework of the "lake" model these distances should be very large during the first stages of greening when the reaction centers to chlorophyll ratios are very low; consequently F_c emission should be high and the few already present reaction centers should be functionally isolated. In addition, the chlorophylls of this continuum, shared among few active System II reaction centers, should lead to a very large optical cross sections of resulting System II units. As greening proceeds, the density of System II reaction centers on this continuum increases; their average distances decrease and should lead to a progressive decrease of F_c emission, and to a progression of energy transfer between units, and thus to a change of fluorescence induction curves from exponential to sigmoidal. Simultaneously, the average number of light-harvesting chlorophylls per System II reaction center should decrease.

We observed such concomitant changes in fluorescence parameters as greening proceeds. This dependency of harvesting, transfer and trapping properties on increasing System II reaction centers concentration appears to be similar to that found in green cells, in which increasing concentrations of active "open" System II reaction center are obtained by increasing the regeneration times in the dark after inactivation by preillumination. The general organization of fluorescent chlorophylls in the developing photosynthetic membranes seems not to change basically during greening. Thus, data are consistent with a chlorophyll organization according to a functional continuum allowing energy migration among several units. However, they do not allow determination of whether these chlorophylls are organized as structurally or as statistically defined light-harvesting antennae of System II units.

The development of functional System II during greening of *Euglena* is consistent with the generally accepted process of membrane growth by stepwise insertion and reorganization of newly synthesized components. However, in the present case, the sequence of events leading to the formation of active System II units appears to be different from that observed with similar fluorescence methods in other greening organisms, since it was shown on *Chlorella* [19], *Chlamydomonas* mutants [20] and on bean leaves greened under continuous or intermittent light [21,22], that light-harvesting antennae associated with active System II reaction centers reached mature size by progressive addition or reorganization of newly formed chlorophylls. On the other hand, a

stepwise process was also proposed for spatial organization of light-harvesting antennae in developing photosynthetic membranes of the same *Chlorella* mutant [19] and of bean leaves [21,22]. In both cases the progressive development of energy transfer between System II units was interpreted as resulting from late connection of light-harvesting antennae initially formed by a clustering of the first synthesized chlorophylls as a spatially isolated islet. The properties of energy migration among newly synthesized chlorophylls suggest that such transitory states do not occur during greening of *Euglena* and that thylakoids develop without basic changes in chlorophyll organization.

In conclusion, the complementary methods used provide results that confirm each others and support a model for chlorophyll organization in developing photosynthetic membranes of *E. gracilis* Z during greening under standard conditions. If, as previously proposed [19], System II units correspond to structural entities, individual and connected System II light-harvesting antennae would rapidly be achieved before being completed as active System II units by insertion of active reaction centers. The decrease in size of the Emerson-type photosynthetic unit would then correspond to a decrease in the proportion of not yet achieved System II units as greening proceeds. The decrease in size of functional System II units would only be apparent; it would result from a loss of energy transferred from the developing units when completed with active reaction center.

Acknowledgements

We want to thank Drs. P. Joliot and J.M. Briantais for helpful discussions and J. Diamond for reading the manuscript. This research was supported by a grant of D.G.R.S.T. (No. 74.7.0180).

References

1 Boardman, N.K., Anderson, J.M., Kahn, A., Thorne, S.W. and Teffry, E.T. (1971) in Autonomy and Biogenesis of mitochondria and chloroplasts, (Boardman, N.K., Linnane, W.A. and Smillie, R.M., eds), pp. 70—84, North Holland Publ. Co., Amsterdam
2 Ohad, I. (1975) in Membrane Biogenesis (Tzagoloff, A., ed.), pp. 279—350, Plenum Press, New York
3 Gaffron, A. and Wohl, K. (1936) Naturwissenschaften 24, 81—107
4 Emerson, R. and Arnold, A. (1932) J. Gen. Physiol. 16, 191—205
5 Schmid, G.H. and Gaffron, A. (1971) Photochem. Photobiol. 14, 451—464
6 Myers, J. and Graham, J.R. (1971) Plant Physiol. 48, 282—286
7 Schmid, G.H. and Gaffron, A. (1968) J. Gen. Phys. 52, 212—239
8 Wild, A. (1969) in Progress in Photosynthesis Research (Metzner, H., ed.), pp. 871—876, International Union of Biological Sciences, Tübingen
9 Herron, H.A. and Mauzerall, D. (1972) Plant Physiol. 51, 141—148
10 Egneus, H., Reftel, S. and Sellden, G. (1972) Physiol. Plant. 27, 48—55
11 Henningsen, K.W. and Boardman, N.K. (1973) Plant Physiol. 51, 1117—1126
12 Baker, N.R. and Hardwick, K. (1974) in Proceedings of the Third International Congress on Photosynthesis (Avron, M., ed.), pp. 1897—1906, Elsevier Scientific Publ. Co., Amsterdam
13 Baker, N.R. and Butler, W.L. (1976) Plant Physiol. 58, 526—529
14 Faludi-Daniel, A., Amesz, J. and Nagy, A.H. (1969) Biochim. Biophys. Acta 197, 60—68
15 Wieckowski, S. (1971) Photosynthetica 5, 44—49
16 Diner, B. and Mauzerall, D. (1973) Biochim. Biophys. Acta 292, 285—290
17 Pleniscar, M. and Bendall, D.S. (1973) Biochem. J. 136, 803—812
17 Butler, W.L. (1965) Biochim. Biophys. Acta 102, 1—8

19 Dubertret, G. and Joliot, P. (1974) Biochim. Biophys. Acta 357, 399—411
20 Cahen, D., Malkin, S., Shochat, S. and Ohad, I. (1976) Plant Physiol. 58, 257—267
21 Melis, A. and Akoyunoglou, G. (1977) Plant Physiol. 59, 1156—1160
22 Akoyunoglou, G. (1977) Arch. Biochem. Biophys. 183, 571—580
23 Stern, A.I., Schiff, J.A. and Epstein, H.T. (1964) Plant Physiol. 39, 220—226
24 Stern, A.I., Epstein, H.T. and Schiff, J.A. (1964) Plant Physiol. 39, 226—231
25 Ben-Shaul, Y., Schiff, J.A. and Epstein, H.T. (1964) Plant Physiol. 39, 231—240
26 Calvayrac, R. and Douce, R. (1970) FEBS Lett. 7, 259—262
27 Delosme, R. (1967) Biochim. Biophys. Acta 143, 108—128
28 Freyssinet, G. (1976) Plant Physiol. 57, 824—830
29 Bertini, F., Brandes, D. and Buetow, D.E. (1965) Biochim. Biophys. Acta 107, 171—186
30 Schantz, R., Schantz, M.L. and Duranton, H. (1965) Plant Sci. Lett. 5, 313—324
31 Murata, M., Nishimura, M. and Takamiya, A. (1966) Biochim. Biophys. Acta 120, 23—33
32 Malkin, S. and Kok, B. (1966) Biochim. Biophys. Acta 126, 413—432
33 Kitajima, M. and Butler, W.L. (1975) Biochim. Biophys. Acta 376, 105—115
34 Joliot, A. and Joliot, P. (1964) C.R. Acad. Sci. Paris 258, 4622—4625
35 Duyssens, L.M.N. and Sweers, H.E. (1963) in Studies in Microalgae and Photosynthetic Bacteria (Japan Society of Plant Physiology), pp. 353—372, University of Tokyo Press, Tokyo
36 Brody, M. (1968) in The Biology of Euglena (Buetow, D.E., ed.), Vol. II, pp. 215—283, Academic Press, New York
37 Butler, W.L. (1966) in The chlorophylls (Vernon, L.P. and Seely, G.R., eds.), pp. 343—398, Academic Press, New York
38 Gurevitz, M., Kratz, H. and Ohad, I. (1977) Biochim. Biophys. Acta 461, 475—488