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STRUCTURE AND ORGANIZATION OF SYSTEM II PHOTOSYNTHETIC UNITS DURING THE GREENING OF A DARK-GROWN *CHLORELLA* MUTANT

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SUMMARY

The formation and the organization of Photosystem II photosynthetic units during the greening of a dark-grown *Chlorella vulgaris*, mutant 5/520, have been investigated by analysing the kinetics of the "activation" of oxygen evolution and of the fluorescence induction.

1. The existence during the early stages of the greening of a stationary photosynthesis demonstrates the presence of active Photosystem II at these initial stages, which are integrated in a functional whole, leading to overall photosynthesis.

2. The rise-time of oxygen evolution has been measured using far-red and green light in order to estimate the relative number of chlorophylls per unit. The amount of chlorophyll *a* remains relatively constant during the greening, while the progressive addition of chlorophyll *b* causes the size of the units to increase approx. 2-fold.

3. The induction kinetics of the fluorescence are exponential during the early phases of greening and later become distinctly sigmoidal; this suggests that the first units synthesized on the surface of the membrane are isolated from each other by obstacles preventing electronic excitation transfers and that such obstacles which might correspond to some distance between such units, can disappear at later stages, allowing energy transfers to occur.

These observations suggest that the Photosystem II units represent organized functional entities. They apparently consist of a relatively constant number of chlorophyll *a* molecules, which during the greening is complemented progressively by the addition of chlorophyll *b*.

INTRODUCTION

Emerson and Arnold [1], measuring the photosynthetic yield, found that for a regime of repetitive saturating flashes, one oxygen was evolved for each 2500 chlorophylls. They concluded that the greater number of chlorophylls are not directly involved in the photochemical process.

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

This finding was elaborated by Gaffron and Wohl [2]. They proposed a model of a photosynthetic unit built by the close association of approx. 2500 chlorophylls cooperating in the absorption of a light quantum and in its transfer to the reactive center, or active photochemical center, where the photochemical process leading to the reduction of one CO_2 against the formation of one O_2 occurs. But the requirement of about eight quanta absorbed for the evolution of one O_2 implies that eight photoacts are necessary for one O_2 evolved. This suggests that each photochemical center is associated with about $2500 : 8 \cong 300$ chlorophyll molecules. On the other hand, the evidence for the existence and cooperation of two photosystems in photosynthesis leads one to suppose the existence of two different types of units (Photosystem I and II units), in which about 300 chlorophylls serve one photochemical center.

But the comparison of the total active photocenter concentration to the total chlorophyll concentration, even if very useful, does not allow one to determine if the chlorophylls are all effectively connected with active centers. As a matter of fact, such a determination of the number of chlorophylls per active unit ought to be associated with measures of the quantum requirement, which only with great difficulty approach precision. Further, such size determinations are possible only when the measured values of the quantum requirement approach the theoretical value of eight quanta absorbed for one O_2 evolved, in which case all chlorophylls are associated with photochemical centers with a configuration allowing a transfer efficiency of 100 %.

In the case of quantum requirements higher than eight, for instance in the special case of differentiating chloroplasts for which it is questionable whether all the synthesized chlorophylls are immediately connected with active centers, this method of quantitative determination of the number of chlorophylls per unit becomes hazardous.

On the other hand, there are at present no data allowing a definition of the structure of these units and their organization in the membrane. The notion of a quantasome [3], resulting from morphological observations by electronic microscopy, had to be abandoned. The excitation transfers between units, systematically observed with the functional methods, does not allow the authors the choice between a photosynthetic unit model in which the photochemical centers are dispersed on a chlorophyll continuum with a statitiscally determined ratio [5], and another model in which the units are organized in functionally interconnected entities, with the possibility of transfer of energy [4].

In this paper some properties of the photosynthetic units (Photosystem II) of a greening dark-grown *Chlorella* mutant (5/520) are analyzed. The methods used, including measures of the kinetics of fluorescence induction and of the "activation of oxygen evolution", allow the direct determination of the number of photons reaching the Photosystem II photoactive centers during a given time. The capture and transfer efficiencies are thus measured and since these efficiencies are determined by the pigment environment of the centers, certain properties of the unit are revealed. Moreover, these highly sensitive methods are especially suitable for relative measures of the number of chlorophyll molecules per unit of various materials already differentiated or in course of differentiation. They also can reveal possible interactions between these units.

The dark-grown *Chlorella vulgaris* mutant was chosen because it allows the analysis of different developmental stages of the chloroplasts during greening. This

chloroplast development might allow one to study the initial processes such as structural development and photosynthetic units organisation which eventually yield a fully functional chloroplast.

MATERIALS

Cell culture

The ultraviolet light induced mutant of *C. vulgaris*, 5/520, was isolated by Claes [6]; the cells were grown in 10-l flasks at room temperature in the dark on a glucose mineral medium containing 0.5 mM KNO_3, 0.1 mM $MgSO_4$, 0.1 mM KH_2PO_4, added with 22.3 mg ferric EDTA, oligo-elements (Arnon formula) and 2 g glucose per liter. The culture was agitated and aerated by bubbling air. The mutant cannot synthesize chlorophyll in a depleted or "resting medium". The greening of the dark-grown cells was thus obtained by exposing the cells in the late exponential stage of growth (10^6 cells/ml) to 500 lux light provided by a fluorescent tube (Mazda Fluor 20 W "Blanc Industrie"). Higher intensities cause photooxidation of chlorophylls in the early stages of greening. Wild-type *Chlorella pyrenoidosa*, Emerson strain, was cultivated at 25 °C in Roux flasks containing a Knop solution added with oligo-elements (Arnon Formula). The culture was exposed to continuous illumination at 5000 lux. Aeration and agitation of the culture was done by bubbling with air : CO_2 (95 : 5, v/v).

Cell preparation

The algae were centrifugated at $2000 \times g$ for 5 min and concentrated by resuspending in a Sörensen buffer (0.05 M $NaHPO_4$, 0.05 M KH_2PO_4, pH 6.8, with 0.05 M KCl). For oxygen measurements, the cells were concentrated to $7 \cdot 10^8$ cells/ml buffer and for fluorescence measurements they were concentrated to obtain a final concentration of 5–10 μg chlorophyll/ml suspension. For technical reasons, an unavoidable delay of at least 5 h elapsed between cell preparations and measurements.

Chlorophyll determinations

An aliquot of these preparations was extracted with 90 % acetone in water and the chlorophyll *a* and chlorophyll *b* content was determined by the kinetics of the acid-catalyzed pheophitinization [7].

METHODS

Fluorescence measurements

Principle of the method. The photochemical reaction occurring at the level of the active Photosystem II centers can be symbolized as,

$$Z\ \mathrm{Chl}\ Q \dashrightarrow Z^+\ \mathrm{Chl}\ Q^-,$$

where Z represents the primary electron donor and Q the primary acceptor (quencher). Normally, Q is reoxidized by the electron transfer chain.

Light excitation reaching a unit whose photochemical center includes a primary acceptor in the reduced, or non-quenching form, Q^-, is dissipated as fluorescence [8]. The redox state of the acceptor thus determines, as a first approximation, the yield of

chlorophyll fluorescence in vivo. If by any means (inhibitors, low temperatures, very high light intensity) the reoxidation rate of Q^- by the electron transfer chain is stopped or considerably reduced, the kinetics of fluorescence induction now reflect the photodestruction kinetics of the quenchers.

Considering a hypothetical model with units including only one photochemical center, these units being isolated from each other, a progressive reduction of the primary acceptor Q according to first-order kinetics can be predicted, resulting in a exponential rise of the fluorescence. Important perturbations have always been noted in the fluorescence induction kinetics suggesting that this model is insufficient: while typically photochemical they display a sigmoidal character. This has been explained as due to a cooperative activity of the units [4]. The light quanta collected by an already "closed" unit, including a primary acceptor in the Q^- reduced form, instead of being dissipated as fluorescence, can be transferred to a neighboring "open" unit and reduce its oxidized Q acceptor. This increased efficiency of the reduction of Q, as the number of active units diminishes, will be reflected in a lower transient fluorescence yield, with sigmoidal kinetics.

Moreover, since each quencher is irreversibly destroyed by one photochemical reaction, in the presence of inhibitors, the time necessary for the reduction of the whole of the photochemical complexes, i.e. the time course of the fluorescence rise, is proportional to the number of photons reaching the centers per unit time. For practical reasons a relative estimate is made of the rate of photon reception by the centers by measuring the half rise-time of the fluorescence.

Briefly, we consider that the half rise-time of fluorescence is inversely proportional to the number of photons reaching the photochemical centers per unit time; the more or less sigmoidal character of these kinetics reflects the efficiency of energy transfers between photosynthetic units.

Experimental conditions. The fluorescence measurements were performed at room temperature as previously described [9]. The primary acceptors were reduced by a low intensity, actinic and detector light beam, and their reoxidation by the electron transfer chains was blocked by the addition to the algal suspension of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to a final concentration of $5 \cdot 10^{-5}$ M. The screening effect of chlorophyll could entail heterogeneity of the exciting light at the level of the cells suspended in the cuvette and thus cause disturbances in the shape of the fluorescence induction kinetics. To overcome this problem, the cuvette was built in a glass tube (internal diameter 2 mm, external diameter 6 mm), whose external surfaces were metal coated in order to increase the reflectivity of light in the interior of the cuvette. This insured the best possible homogeneity of the actinic light, and a good collection of the fluorescent light. Furthermore the concentration of chlorophyll in each suspension of a given greening culture was held constant by appropriately diluting the cells with buffer. In no case was the chlorophyll concentration greater than 10 μg/ml of cell suspension. Under these conditions, the intensity of fluorescence is directly proportional to the concentration of chlorophyll, while the rise-time of fluorescence kinetics is independent of the chlorophyll concentration. Comparative measures on the various samples then become possible in spite of slight disturbance due to the unavoidable absorption of the actinic and fluorescent light within the cells.

Oxygen measurements

Principle of the method. When photosynthetic material is exposed to weak illumination after having been kept in the dark for several minutes, the rate of oxygen evolution increases progressively and reaches a stationary value after a certain interval called the "activation time", which is inversely proportional to the light intensity [10]. The oxygen yield per flash under a sequence of short saturating flashes, measured by different authors [11, 12], led Kok et al. [12] to propose a coherent interpretation of the "activation process". They suggested that the formation of one oxygen molecule involves four successive photoreactions occurring at the same center; these reactions would correspond to the accumulation of four charges, or oxidizing equivalents, by the electron donors:

$$ZQ \dashrightarrow Z^{+}Q \dashrightarrow Z^{2+}Q \dashrightarrow Z^{3+}Q \dashrightarrow Z^{4+}Q$$

$$Z^{4+} + 2\,H_2O \dashrightarrow O_2 + Z + 4\,H^{+}$$

where Z is the electron donor and Q the electron acceptor.

Between each photoact, a non-photochemical reaction occurs, corresponding to the withdrawal of one negative charge from the primary acceptor Q by the electron transfer chain (these dark steps which have not been represented in the scheme, are not limiting at low light intensities).

The Z^{2+} and Z^{3+} states are unstable and a material returned to darkness for several minutes has all its centers in states Z^{+} (80 %) and Z (20 %). The "activation period" reflects the time of transition from the initial state to the steady state in which the centers are distributed equally among the four photochemical states ($Z = Z^{+} = Z^{2+} = Z^{3+} = 25\,\%$). Mathematical analysis shows that the duration of this transition depends on the number of photons reaching the centers per unit time [12]. It then becomes possible to compare the measurements of various photosynthetic materials, provided that the distribution of centers between the states Z and Z^{+} is identical in the "deactivated" state preceding the measurements.

The half rise-time for oxygen evolution is, for the same material and the same actinic light intensity, of the same order of magnitude as the half rise-time of fluorescence in the presence of inhibitors. Moreover, during the "activation process", which is always measured under low light intensity, all of the centers remain in a photochemically active state. Under such conditions the energy transfers between photosynthetic units, as described above, are not preceptible and do not disturb the measures.

Experimental conditions. Oxygen measurements were performed with a modulated polarograph, described by Joliot and Joliot [13]. The algal suspensions were concentrated in the Sörensen buffer to a density of $7 \cdot 10^{8}$ cells/ml in order to obtain a saturated cell monolayer on the platinum electrode, and to avoid screening effects. It must be mentioned that the number of cells deposited on the platinum cannot be obtained with precision and only relative rates of oxygen evolution can thus be conveyed. On the other hand, for a given intensity of the actinic beam ,the intensity of the light reaching the photochemical sites of the samples to be compared can vary due to the intracellular absorption by chlorophylls, this mainly occurring when the samples are of very different pigmentation. To overcome this problem, the wavelengths were chosen in the region of relatively low absorption by chlorophylls (550 and 706 nm).

Under these conditions, the intracellular screening was minimized, and the photosynthetic units to be compared were exposed to relatively homogeneous illumination.

These kinetic considerations of fluorescence and oxygen evolution "activation" allow relative estimations of the number of photons reaching active photochemical centers per unit time. This number is proportional to the intensity of the incident light, to the average number of collecting chlorophylls which are associated with the photoactive centers, and to the efficiency of the energy transfers between these collecting chlorophylls and the centers. The very high quantum yield observed in normal photosynthetic organisms leads one to accept a transfer yield close to one, so that we will provisionally ignore this third parameter.

This being so, and the light intensity being determined, the measurement of the half rise-times of fluorescence and of oxygen "activation" will allow a relative estimation and comparison of the number of chlorophyll molecules per photosynthetic unit of various materials.

RESULTS

Chlorophyll synthesis during greening

Cells of the *Chlorella* mutant 5/520 in the exponential phase of growth in darkness contain about 5 % of the chlorophyll found in the fully green cells, with a chlorophyll *a*/chlorophyll *b* ratio of about 10. The synthesis of carotenoids is blocked in darkness at the level of precursors and only phytoene, phytofluene, ζ-carotene and prolycopene accumulate [14].

When cells are exposed to low light intensity, as the greening process starts the cell growth stops or is considerably reduced. Chlorophyll is synthesized after a lag phase whose duration could vary from 0 to 3 h (Fig. 1). 18 h later, the chlorophyll content per cell is 20–30 % that of the fully greened mutant. The rate of chlorophyll synthesis then diminishes considerably, and the complete greening of the algae

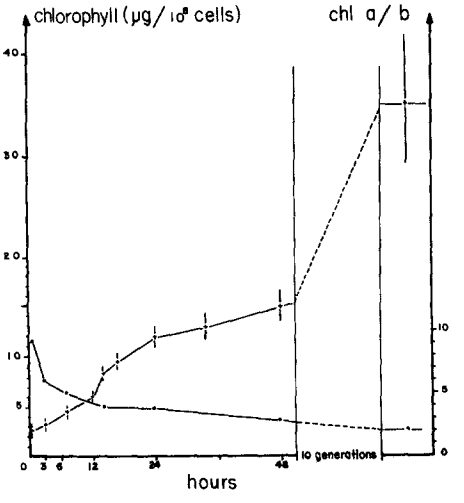

Fig. 1. Chlorophyll synthesis and evolution of the chlorophyll *a*/chlorophyll *b* ratio during greening of the *C. vulgaris* mutant 5/520. Dark-grown cells were exposed to 500 lux at time zero. chl, chlorophyll.

TABLE I

Chlorophyll content and fluorescence induction of wild-type *Chlorella pyrenoidosa* and of the mutant 5/520 during greening

	Mutant 5/520			Wild-type control
	3-h illuminated	24-h illuminated	fully greened	
Chlorophyll concentration				
μg/ml suspension	5.7	8.5	9.6	8.4
μg/10^8 cells	3.2	12	42	64
Chlorophyll *a*/chlorophyll *b*	5.8	4.8	2.3	4
Curve	exponential	exponential	sigmoidal	sigmoidal
Half rise-time of fluorescence (ms)	73	38	36	48

requires approx. 10 generations. Then the final concentration of chlorophyll per cell is of the same order as wild-type and normal level of carotenoids is attained. Simultaneously the chlorophyll *a*/chlorophyll *b* ratio falls from 10 to 4–5 within the seven first hours of illumination of the algae then decreases slowly and stabilizes at 1.5–2 after 48 h. Since the chlorophyll *a*/chlorophyll *b* ratio of the wild-type is about 3–4, the chlorophyll *b* accumulation is much higher in the green mutant.

When exposed to light intensities higher than 700 lux, the greening process is considerably reduced if not stopped. Preliminary experiments suggest that this can be the result of photodestruction of newly synthesized chlorophylls, since photoprotective carotenoids are not yet synthesized [15].

Fluorescence measurements

The Curves 1 and 4 (Fig. 2) represent the fluorescence rises of the mutant illuminated for 3 h and of the wild-type control, with linear and semi-logarithmic coordinates. The curves plotted with semi-logarithmic coordinates demonstrates that, while the kinetics for wild-type are sigmoidal, those for the mutant illuminated for 3 h are exponential. There are thus (see Methods) no energy transfers between the units developed in the early greening stages, while the photosynthetic units of the wild-type can exchange light excitation. In addition, a comparison of the half rise-times of the two kinetics leads one to conclude that the rate of photons reaching the photochemical centers is 1.7–2 times lower for the early greening stages of the mutant than for the wild-type.

As greening progresses, the fluorescence kinetics become sigmoidal, as can be seen with the fully greened mutant (Curve 3, Fig. 2), suggesting that just as for wild-type, energy transfers now occur. In the same period the fluorescence half rise-times decrease by about 2, to values approaching those of the wild-type control.

After 24 h of illumination, when the chlorophyll content is 30 % that of wild-type, the kinetics still show an exponential character (Curve 2, Fig. 2), even though the half rise-time is close to that observed for both the fully greened mutant and the control. These results show that the appearance of energy transfers between units is independent of the fluorescence rise velocity.

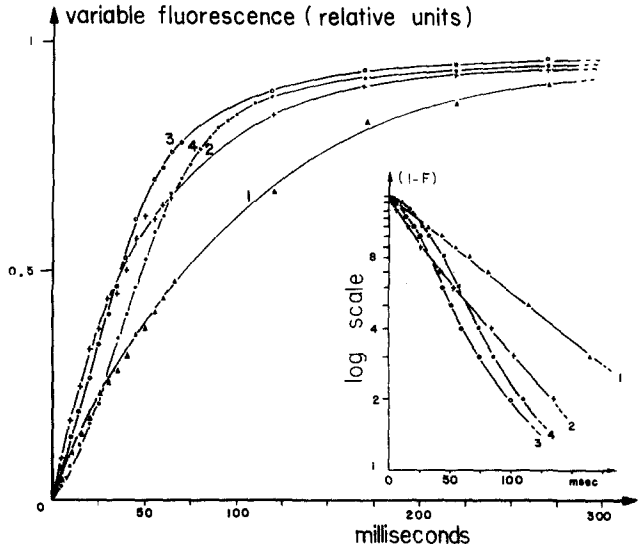

Fig. 2. Fluorescence induction in the presence of 50 μM DCMU with wild-type and greening mutant 5/520. Room temperature. Curve 1, 3-h illuminated mutant; Curve 2, 24-h illuminated mutant; Curve 3, fully greened mutant; Curve 4, wild-type control. Curves are normalized to the same maximum fluorescence (For experimental details see Table I). Insert: Log plot of (1-F) for the same curves.

Fluorescence induction times were measured in a population of cells and so the possibility of a size heterogeneity of photosynthetic units exists. Since bigger units would show slower response times, the curves resulting from such a mixed population would deviate markedly from the exponentials that we have observed. If such heterogeneity does exist in the cell populations studied, it is of minor importance.

Oxygen measurements

Fig. 3a represents the oxygen evolution at the onset of the illumination, i.e. the "activation phase", normalized for the sake of comparison of the mutant illuminated for 3 h and of the wild-type control.

With an actinic light of 506 nm, absorbed by both chlorophylls *a* and *b* the half rise-time of the mutant is 1.7–2.0 times greater than that of wild-type. Thus fewer excitations per unit time reach the active centers of the mutant than those of the control, confirming similar results obtained by fluorescence measurements. With, however, actinic light of 706 nm, exciting essentially only chlorophyll *a*, the approximate equality of the half rise-times (Fig. 3b) indicates that the rate of photons reaching the photoactive center for the two samples is approximately equal. Thus it appears that the quantum-collecting efficiency of chlorophyll *b* is lower in the mutant than in the control. This can be due either to a lower chlorophyll *b* content in the mutant or to a decreased functional role. The values of the chlorophyll *a*/chlorophyll *b* ratio during the greening confirm the hypothesis that newly formed Photosystem II units are deficient in chlorophyll *b*.

The stability of the rate of oxygen evolution after the "activation" period

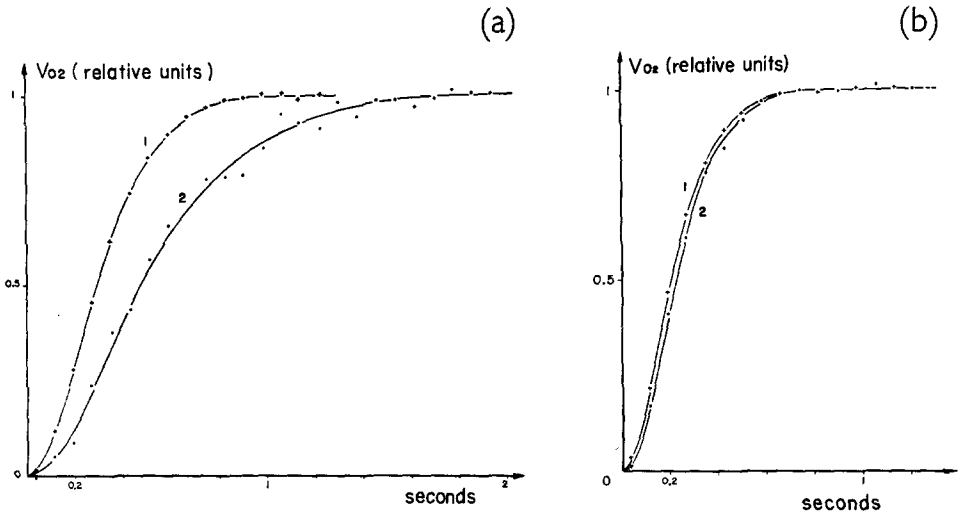

Fig. 3. Kinetics of oxygen evolution of wild-type control and of the mutant illuminated for 3 h with 500 lux white light. (a) The wavelength of the modulated actinic light is 550 nm, exciting both chlorophyll *a* and chlorophyll *b*. (b) The wavelength of the actinic light is 706 nm, exciting essentially only chlorophyll *a*. Curves 1, wild-type control; Curves 2, mutant illuminated for 3 h. Curves are normalized to the same stationary rate of oxygen evolution (For experimental details see Table II).

TABLE II

Chlorophyll content and activation of oxygen evolution of wild-type *Chlorella pyrenoidosa* and of the mutant 5/520 after 3 h greening

	Wild-type control	Mutant
Chlorophyll concentration ($\mu g/10^8$ cells)	62	2.1
Chlorophyll *a*/chlorophyll *b*	3.1	4.4
Half rise-time of oxygen evolution (ms)		
550 nm	290	574
706 nm	200	232

demonstrates the existence of a persistent photosynthesis from the onset of greening. Repeated experiments did not allow us to establish a relationship between the rate of constant photosynthesis and the chlorophyll concentration in the greening cells; variations were observed ranging from 20 to 100 % of the figure obtained for wild-type. Such variations which cannot be attributed to the method show the existence of variable amounts of chlorophylls inactive for oxygen evolution. Such inactive chlorophylls could be due to a proportion of developing units still inactive; a progressive 1.5–2.0-fold increase of the stationary oxygen evolution rate, observed during 15 min of illumination and due to the appearence of new active photochemical centers could correspond to photoactivation of previously inactive centers, for example by the incorporation of manganese [16]. In view of the magnitude of these variations in O_2 evolution, however, it is more probable that a significant destruction

of photochemical centers has occurred, perhaps during preparation of the cells, or due to the long time elapsed between cell preparation and measurements.

It is to be noted that whatever the causes of these variations of photosynthetic efficiency, the variable amounts of inactive chlorophyll do not disturb the half rise-times of oxygen evolution; these remain independent of the final oxygen evolution rates at all stages of greening.

We found, however, that half rise-time values could be altered by exposing the dark-grown mutant cells to 500 lux for 3 h followed by 10 000 lux for 1 h. Under these conditions the number of photons collected per unit time by the photosynthetic units was reduced by six. Considering the greatly reduced carotenoid content in the mutant at this early greening stage we have explained this phenomenon as probably resulting from the photooxidation of collecting chlorophylls [15]. One can also invoke the possibility of differences in photosynthetic membrane architecture between wild-type and mutant, resulting in a photolability of chlorophyll in the mutant.

DISCUSSION

As already emphasized (see principle of the method), the half rise-times of fluorescence induction and of "oxygen evolution activation" kinetics are inversely proportional to the number of light quanta reaching photoactive centers per unit time. If the light intensity of the actinic beam is kept constant, differences in half rise-time values of different samples can be interpreted as reflecting differences in the number of photon-collecting chlorophylls associated with the photoactive centers of these samples. Pursuing this hypothesis, the 2-fold decrease of the half rise-time of fluorescence and oxygen evolution kinetics observed with the green exciting light reflects a 2-fold increase of the total number of chlorophylls *a* and *b* in the Photosystem II units during greening; the chlorophyll content of the fully greened mutant is then close to that found for the wild-type. The nearly constant value of the half rise-time of oxygen evolution observed using far-red actinic light suggests that the chlorophyll *a* content of active Photosystem II units at the beginning of greening is the same as that in fully differentiated units present in the green mutant as well as in the wild type. The observed increase of the photon-collecting chlorophylls of the Photosystem II units during greening thus appears to result from chlorophyll *b* addition to an approximately constant chlorophyll *a* content of the units.

These changes in chlorophyll *b* content of the units undergoing formation have been confirmed by Photosystem II action spectra (Bennoun, P. and Dubertret, G., unpublished) which have shown an increasing contribution of chlorophyll *b* to photosynthesis as greening progresses, while simultaneously, the chlorophyll *a*/chlorophyll *b* ratio decreases. The evolution of the chlorophyll *a*/chlorophyll *b* ratios during greening can thus by no means reflect solely the evolution of the Photosystem I/Photosystem II ratio, but also the increase of chlorophyll *b* in the Photosystem II units during formation.

These interpretations of rise-times of fluorescence and oxygen evolution rest on the hypothesis that the yield for the transfer of light quanta collected by the chlorophylls of the units to the photocenters is constant and close to one; it can be imagined that the observed variations in the rate of arrival of photons at the reactive centers are not determined by variations in the number of collecting chlorophylls associated

with these centers, but by their organization, entailing a lower transfer efficiency due to the loss of a part of the captured photons. Quantum requirement with values higher than eight in the above case do not allow one to estimate these transfer efficiencies, since although they can determine the magnitude of the number of photons lost for photosynthesis, they do not reveal whether they have been lost by capture at the level of photosynthetically inactive chlorophylls, or due to the inefficiency of their transfer toward the reacting centers. Yet, the following indirect argument supports our interpretation of the observed data: we could establish that throughout the successive stages of the greening, the centers were reached at a constant rate by photons absorbed by the chlorophyll *a* of the units. With the hypothesis of a possible variation of the transfer efficiencies, such variation ought to be imagined to be strictly compensated by concomitant inverse variations of the quantities of chlorophyll *a* associated with the reactive centers. Such a process is hard to imagine, while, interpreted as we did, the observed size variations of the units by the enrichment of the antenna by the addition of chlorophyll *b* appears the simpliest.

The shape of the curves of fluorescence induction shows marked variations during greening. Of sigmoidal type for both the fully greened mutant and for the wild-type, they furnish evidence for connections and energy transfers between units (see Methods). Of exponential type for the greening mutant, and at least until 24 h of greening, they suggest that the organization of the Photosystem II units in the newly formed photosynthetic membranes is different from that of fully green cells by virtue of a lack of connections, resulting in the absence of energy transfer.

As already mentioned the mutant after 3 h of greening exhibits, from one experiment to another a 5-fold variability in oxygen-evolving ability on a chlorophyll basis, probably due to the destruction of a variable proportion of photochemical centers. If units were connected, we would expect the half rise-time of both the oxygen evolution and the fluorescence induction to diminish due to transfers of energy collected by adjacent inactive units to the remaining photoactive centers [17]. The observed constancy of these half rise-times, from one experiment to another argues against connections and energy transfers between newly formed units and confirms our interpretation based on an analysis of the shape of the curves.

These observations suggest the existence of barriers between newly formed units which oppose energy transfer and which originate in the architecture of the photosynthetic membrane. It is difficult to specify the nature of these barriers, but it is possible that they correspond to some distance between units, which is too great to permit energy transfers. As the greening progresses, reorganisation of the photosynthetic membranes might cause these barriers to disappear resulting in the connection of the photosynthetic units, manifest by the appearance of energy transfers.

This connection process between formerly isolated units can be explained by the increase of the overall dimensions of the units due to the addition of chlorophyll *b*; the exponential shape and the half rise-time of fluorescence (Curve 2, Fig. 2) demonstrate that the units present after 24 h of illumination are not connected in spite of dimensions approaching those of the units of the control. Thus chlorophyll *b* probably does not contribute to the connection process; this could, however, originate by the intercalation of newly formed units between the preexisting ones, or by another type of reorganization of the membrane structure during greening.

Whatever their nature, the existence of barriers to energy transfers at the early

stages of greening of the 5/520 mutant contradicts the model invoking the photosynthetic units as having only a statistical meaning, corresponding to a distribution of photocenters on an uniform layer of chlorophyll. Our results, however, are consistent with the model supposing the units to correspond to organized functional entities: a photochemical center then would be associated with an antenna composed of a given number of chlorophylls. In the case of the 5/520 mutant, the Photosystem II units appear to reach "adult" size by 24 h of greening, the maturation process involving mainly the addition of chlorophyll *b* to an approximately constant amount of chlorophyll *a*. A further process, that of the connection of these mature units occurs by other means which somehow reduce the distance (either physical or functional) between units.

CONCLUSION

By applying kinetic methods for the analysis of fluorescence induction and "oxygen evolution activation" to a particular biological material, the greening 5/520 mutant of *Chlorella vulgaris*, we were able to elaborate on the notion of photosynthetic units (System II). They seem to consist of organized functional entities composed of a photochemical center associated with photon-gathering antenna, the latter formed by a determined quantity of chlorophyll *a* and *b*. In the case of the 5/520 mutant these quantities of antenna chlorophyll are relatively stable, increasing only 2-fold during greening, largely due to the almost exclusive addition of chlorophyll *b*.

These kinetic analyses have indicated that the process of connection of photosynthetic units occurs by means other than enlargement of antenna.

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