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Development of the Two Heterogeneous Photosystem II Units in Etiolated Bean Leaves

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ABSTRACT

The development of the photosystem II units in relation to the heterogeneity of their photochemical centers was studied in etiolated bean leaves (*Phaseolus vulgaris* var. red kidney) greened under continuous or intermittent light. The study was done in order to see whether grana are the loci of the units with the efficient photosystem II activity (α units), while the stroma thylakoids are the loci of the units with the less efficient photosystem II activity (β units), as it has been proposed. In addition, the interrelations between α and β centers have been investigated. It was found that the α and the β centers of photosystem II were present in the first photosynthetic membranes irrespective of the mode of greening of the leaves. The magnitude of their respective photochemical rate constants, K'_α and K_β, increased with time in continuous light and it reached the steady-state values of the mature chloroplasts within 16 hours, while in intermittent light it remained smaller. The differentiation of the system II units in α and β centers containing units is more evident under conditions of intermittent illumination, *i.e.* when the rate of chlorophyll biosynthesis is the limiting step for chloroplast development.

It is concluded that the heterogeneity of the photochemical centers in system II is an endogenous property of the chloroplast lamellae. The α centers and the β centers develop independently of each other from the beginning of the light-induced greening. They do not share the same pigment beds. The presence of grana, chlorophyll *b*, and the chlorophyll-protein complex II is not a prerequisite for the formation or development of the α centers. The formation of these centers precedes grana formation in greening plastids.

Chloroplasts from higher plants are known to possess a unique organization consisting of areas of packed thylakoid membranes (grana) and areas of thylakoid membranes of lesser density (stroma thylakoids). Such a differentiation should reflect some functional and content differences. From past reports (2, 4, 20, 23), predomimant differences are the higher Chl *b* and complex II content and PSII activity of the grana in comparison to the stroma thylakoids.

The fluorescence induction curve of isolated chloroplasts reflects the light-dependent transition from a weekly fluorescent condition when all photochemical centers are open, to a more strongly fluorescent state as those centers become closed. According to Duysens and Sweers hypothesis (11), the fluorescence rise is due to the photoreduction of the primary electron acceptor Q, a fluorescence quencher, to the nonquenching form Q^-. For mature chloroplasts and in cation-containing media, the fluorescence induction is characterized by a sigmoidal type rise of fluorescence. The sigmoidal shape of the fluorescence induction has been attributed to the intersystem II unit-unit interaction when the PSII units are considered as statistical entities (statistical or lake model) (12, 15). The area over the fluorescence induction curve has been shown to be proportional to the number of quanta utilized by the reaction center of system II for photochemical work (7, 18, 21). For DCMU-poisoned chloroplasts and green algae, the growth of this area was found to be biphasic. A first order kinetic analysis of the area growth has revealed that it occurred in two distinct linear phases, which suggested a functional differentiation in PSII (18, 19). Of two kinetically distinguished types of PSII reactions, one was considerably less efficient in performing photochemistry than the other. It was concluded that there are two types of system II photochemical centers: the efficient centers, $(ZP_{680}\ Q)_{\alpha'}$ and the relatively less efficient centers, $(ZP_{680}\ Q)_\beta$. The differential effect of Mg^{2+} ions on the function of the two types of system II photochemical centers led to the hypothesis that the chloroplast grana are the loci of the efficient α centers, while the stroma thylakoids are the loci of the less efficient β centers (17).

The formation of the photosynthetic apparatus can be observed after exposure of etiolated leaves to continuous or intermittent illumination. Under conditions of continuous illumination the etioplasts quickly show the characteristics of developed chloroplasts (1, 13, 22). Intermittent illumination (2 min light and 98 min dark), however, transforms the etioplasts into "protochloroplasts" which show higher photochemical activity per Chl than chloroplasts (1), synthesize selectively Chl *a*, and are devoid of Chl *b* and grana (3, 6), contain "primary" thylakoids and are deficient in the Chl-protein complex II which is considered to be an essential component of grana stacks (4, 6).

In the present study we used developing chloroplasts, under conditions of continuous or intermittent illumination, in order to follow the formation of the photosynthetic units associated with the α and the β centers. We also examined the interrelations between the α and the β centers in view of the hypothesis that the α centers are located in the grana of the chloroplast. It was found that the α and the β centers were present in the first photosynthetic membranes, irrespective of the mode of greening. Their respective units developed independently of each other during greening. The absence of grana, Chl *b* and the Chl-protein complex II in protochloroplasts did not inhibit the development of the units associated with the α centers, indicating that the formation of grana is not a prerequisite for their formation.

MATERIALS AND METHODS

The experimental portion of this work included the growth of bean plants (*Phaseolus vulgaris* var. red kidney) in the dark. Five-day-old etiolated bean seedlings with one cotyledon removed were placed on wet filter paper in covered Petri dishes. The leaves were illuminated by either bright continuous light, or by intermittent light (2-min light and 98-min dark cycles). Other conditions for illumination were described by Argyroudi-Akoyunoglou and Akoyunoglou (3).

Active plastids were isolated in dim light and at 0 C from the developing bean leaves by a method described by Akoyunoglou and Michelinaki-Maneta (1). The isolation medium consisted of a 10 mM Tricine buffer (pH 7.8) containing 0.4 M sucrose, 5 mM

$MgCl_2$, 10 mM NaCl, 1 mM $MnCl_2$, and 1 mg/ml BSA. The isolated plastids were resuspended in the same buffer but containing 10 mg/ml BSA, and stored in complete darkness at 0 C. The Chl concentration was determined in 80% acetone solution according to Mackinney (16).

Fluorescence measurements were done with a set-up described by Argyroudi-Akoyunoglou and Akoyunoglou (5). Broad band blue actinic light, filtered from the light of a projection lamp, was admitted to the sample chamber through a photographic shutter (opening time about 1 msec).

The kinetics of the rise of the fluorescence at 685 nm was recorded on a storage oscilloscope (Tektronix 549) equipped with a Polaroid camera. The kinetic analysis of the chloroplast fluorescence has been done as described in detail by Melis and Homann (18, 19).

RESULTS

It has been shown (7, 21) that the area $\int_0^\infty (F_{max} - F[t])dt$ over the fluorescence induction curve of isolated chloroplasts is a measure of the total number of charge separations that occur at PSII during the fluorescence induction period. For DCMU-poisoned chloroplasts and green algae, the growth of this area was found to be biphasic (18), representing the progress of photochemistry in a heterogeneous pool of system II reaction centers. Kinetically distinguished were two types of system II photochemical centers, the efficient α centers and the relatively inefficient β centers (19). Figure 1 shows the kinetics of area accumulation over the fluorescence induction curve of DCMU-poisoned bean chloroplasts. The logarithmic plot shown in the same figure represents a first order analysis of the area accumulation. As observed, this accumulation is biphasic. The slope of the line corresponding to the first phase of the area growth gives the value of K_α which is the rate constant of a combination of events, namely the photochemistry of the α and the β centers (see ref. 18). The slope of the line corresponding to the second phase of the area growth gives the value of K_β which is the rate constant of the slow component corresponding to the photochemistry of the β centers. The intercept of this line with the logarithmic ordinate at zero time gives the log of β_{max}, the maximum value of the area accumulated due to the slow process. The value of β_{max} permits the calculation of the relative concentration of the α and β centers in the chloroplast. The kinetics of the area growth due to the photochemistry of the β centers is given by the equation:

$$\beta(t) = \beta_{max}(1 - e^{-K\beta t}) \quad (1)$$

A subtraction of the values of $\beta(t)$ from the corresponding values of the over-all area gives the kinetics of the area growth due to the photochemistry of the α centers. The kinetics of the two components, the slower β component ($\beta[t]$) and the faster α-component ($\alpha[t]$), is shown in Figure 2.

Since the term K_α is already used to describe the rate constant of the initial photochemical activity due to the function of both types of reaction centers, we have designated K'_α as the rate constant of the fast component of the area growth corresponding to the photochemistry of the α centers. In mature chloroplasts, the corresponding log plots reveal that the photochemistry of the α component is not an exponential process (Fig. 2). Inasmuch as a rate constant cannot be calculated, a comparable value for K'_α was taken from the half-time $t_{1/2}$ (α) of the growth of the α component. In the case of the developing chloroplasts and protochloroplasts it was found that the α component accumulated exponentially with time (first order reaction, see Fig. 6), and so the value of the rate constant K'_α was taken from the slope of the log plot of the α component of the area.

The rate of the photochemical reaction, be that of the α centers or of the β centers, depends on a lot of parameters.

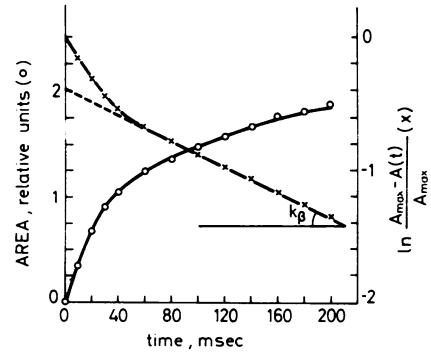

FIG. 1. Kinetics of area accumulation over the fluorescence induction curve of mature chloroplasts (O), and a first order analysis of the area as a function of time (×); 12 μM DCMU, exciting light intensity I_0 = 296 μw/cm^2, 4 μg Chl/ml. The total area A_{max} was equal to 2.5 relative units (r.u.); K_β = 5.0 sec^{-1}.

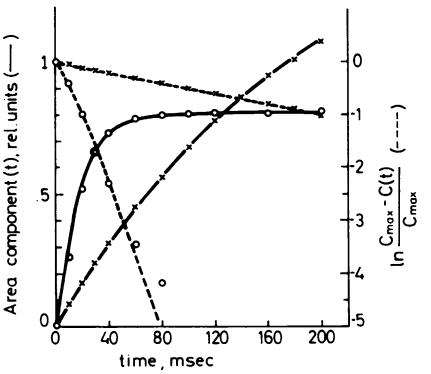

FIG. 2. Kinetics of the fast (O) and the slow (×) component of the area growth of isolated chloroplasts and a first order analysis of their time course. The area covered by the α component (O) was 0.8 r.u. while the area covered by the β component (×) was 1.7 r.u.; $K'_\alpha = 1/t_{1/2}$ (α) = 71.4 sec^{-1}. For other experimental conditions see Figure 1.

Under our experimental conditions, the rate-limiting step is the rate of Chl excitation in the photosynthetic units, and therefore, K'_α and K_β should be limited by the fraction of the light intensity absorbed by the pigments of the units. The fraction depends, among other things, on the size of the photosynthetic units. Therefore, the change of the values of the rate constants K'_α and K_β during greening should give an idea of the size change of the photosynthetic units.

Figures 3 and 4 show the values of the rate constants K'_α and K_β and the relative concentration of the α and β centers during greening in continuous light. The young chloroplasts (1-hr illumination) show a monophasic area growth indicating that at this stage of development there is a homogeneous pool of functioning system II centers (Fig. 3). This is expressed by the equality: $K'_\alpha = K_\beta$ at 1 hr of continuous illumination. Later on the initially small values of K'_α and K_β are increased to a maximum steady-state value which is different for both types of centers. Most of the increase is accomplished within 4 hr of continuous illumination and a plateau is reached after 16 hr. The time elapsed until the maximum value of the rate constants is reached is the time needed for the biosynthesis of an adequately large number of antenna Chl molecules (see Table I), and the organization of this Chl into the developing units. This is also supported from the results of our recent studies on the development of the PSII unit. We found that after 1 hr of continuous illumination the size of the system II unit in the developing chloroplasts is 4.5 times smaller than that of mature chloroplasts. In 4 hr of continuous illumination the size of the unit increased almost three times, and it becomes only 1.5 times smaller than that of mature

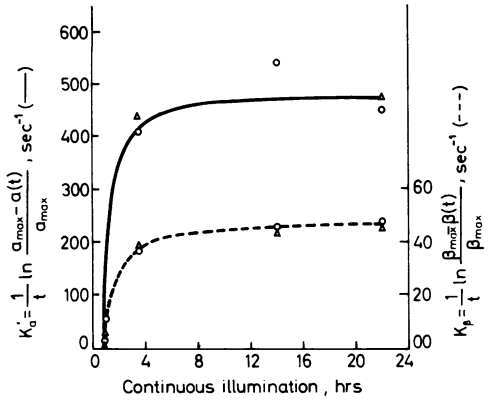

FIG. 3. Continuous illumination-induced change of the photochemical rate constants K'_α and K_β in developing chloroplasts; 12 μM DCMU (O), 12 μM DCMU and 1 mM NH_2OH(△), 4 μg Chl/ml, exciting light intensity I_0 = 2610 μw/cm^2.

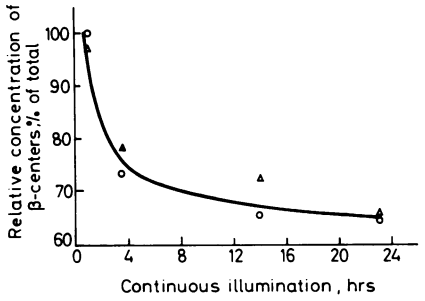

FIG. 4. Continuous illumination-induced change of the relative concentration of β centers in developing chloroplasts. The respective value of the green control was 65%. For other conditions see Figure 3.

Table I. Rate of chlorophyll a (Chl a) and chlorophyll b (Chl b) accumulation in 5-days old etiolated bean leaves greened in continuous light

Time in continuous light hr	Chl a	Chl b	Chl a/Chl b
	μg/gr fresh wt		
1	26	3.0	8.6
2	37	6.0	6.0
4	72	13.6	5.2
6	136	36.6	3.7
10	335	106.0	3.15
16	501	159.0	3.15
24	764	241.5	3.16

chloroplasts (G. Akoyunoglou, submitted for publication). Obviously, under conditions of continuous illumination there is a fast growth of the units around the α and the β-centers.

The change of the relative concentration of the α and the β centers during greening with continuous illumination is shown in Figure 4. During the first hours of greening we observe a higher relative concentration of the β centers. This concentration gradually decreases to the steady-state value of approximately 65%. One explanation is that the first photosynthetic membranes of the developing chloroplast contain a relatively higher amount of functioning β centers, and that the steady-state is established after the formation of additional membranes (possible grana) which contain functioning α centers as well. An alternative explanation is that in the developing photosynthetic membranes the α and the β centers are surrounded by a limited number of organized Chl molecules. The small size of the young photosynthetic units makes them kinetically similar and relatively inefficient. In this case, the intrinsic heterogeneity of photochemical centers cannot be detected in the very early stages of greening due to the small size of the photosynthetic units. This hypothesis is supported by the results of Figure 3 where the changes of K'_α and K_β appear to occur simultaneously. However, the kinetic differentiation of the system II photosynthetic units becomes evident during the early stages of the chloroplast development. The developing units reach their maximum size in a few hours after exposure to continuous illumination (Fig. 3).

Intermittent illumination of etiolated bean leaves causes the transformation of etioplasts to protochloroplasts (4). Since protochloroplasts do not contain any grana structures they can be used to ascertain whether the formation of the α centers is a phenomenon related to grana formation. Figure 5 shows the change of the relative concentration of the α and β centers during greening under intermittent illumination. It is seen that the originally high relative concentration of the β centers decreases, and finally reaches the green control steady-state value of 65%. The steady-state value is virtually reached after the administration of approximately 60 to 70 cycles of intermittent illumination. The results indicate that the kinetic expression of the α centers does exist in protochloroplasts and, therefore, that the formation of the α centers is a phenomenon that precedes the biosynthesis of Chl b, the Chl-protein complex II, and the formation of grana (3, 4, 6).

Figure 6 gives a detailed presentation of the kinetic analysis of the sample which received 23 light and dark cycles. As we see, the α and the β component of the area are exponential functions of time.

Figure 7 shows the change of the values of K'_α and K_β as a

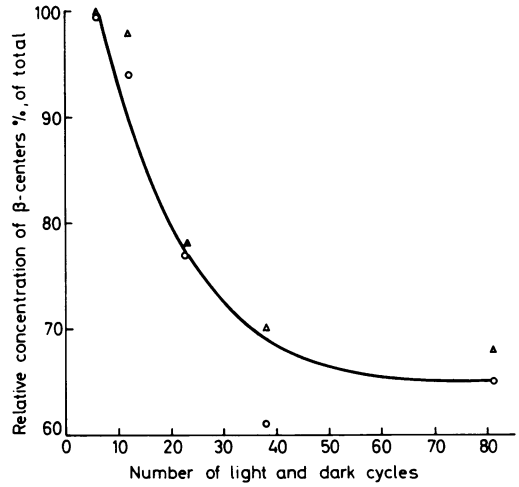

FIG. 5. Intermittent illumination-induced change of the relative concentration of β centers in developing protochloroplasts; 12 μM DCMU (O), 12 μM DCMU and 1 mM NH_2OH(△), 4 μg Chl/ml, exciting light intensity I_0 = 2610 μw/cm^2.

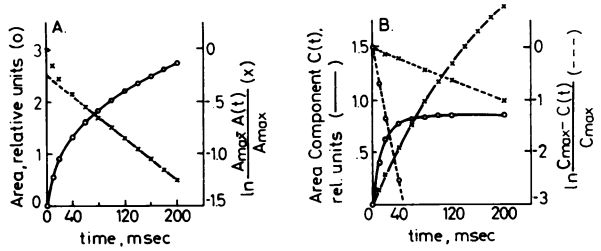

FIG. 6. A: kinetics of area accumulation over the fluorescence induction curve of young (23 light-dark cycles) protochloroplasts (O), and a first order analysis of the area as a function of time (×). The total area (A_{max}) was equal to 3.8 r.u. B: kinetics of the fast (O) and the slow (×) component of the area growth of isolated protochloroplasts (23 light-dark cycles) and a first order analysis of their time course. The area covered by the α component (O) was 0.8 r.u. while the area covered by the β component (×) was 3.0 r.u.; K'_α = 62 sec^{-1}, K_β = 5 sec^{-1}. For other experimental conditions see Figure 5.

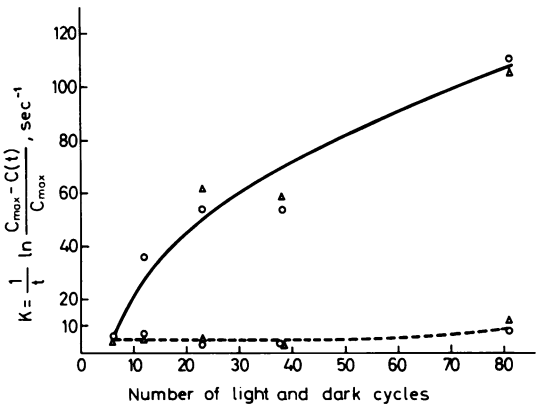

FIG. 7. Intermittent illumination-induced change of the photochemical rate constants K'_α (——) and K_β (- - -) in developing protochloroplasts. For other experimental conditions see Figure 5.

function of the number of light-dark cycles administered to the etiolated leaves. In the beginning of greening, a monophasic kinetics of the area growth over the fluorescence induction curve is observed. Subsequently, there is a dissimilar dependence of K'_α and K_β on the number of light-dark cycles, since their values do not increase in a parallel way. The value of K'_α increases steadily as a function of the number of light-dark cycles while the value of K_β remains almost constant.

It is known that under conditions of intermittent illumination the Chl biosynthesis is limited (3). Our results indicate that under this type of illumination the distribution of the antenna Chl molecules among photosynthetic units affects the photochemistry of the α and the β centers differently. The photochemistry of the α centers is favored in comparison to the corresponding photochemistry of the β centers (for more details see under "Discussion").

After 60 light-dark cycles, there is a steady-state in the relative concentration of the α and the β-centers (Fig. 5). After 60 light-dark cycles the values of K'_α and K_β are still much smaller than the corresponding ones of the green control (Fig. 7). The attainment of a steady-state between the relative concentration of the α and the β centers indicates that at that stage the differentiation between the photochemical centers of the photosynthetic membranes is already in an advanced state. However, the growth of the α and β units does not follow the same course. Judging from the values of K'_α and K_β at 60 light-dark cycles, we see that the α and β units have not reached their maximum size. From that we may conclude that the differentiation of the α from the β centers does not depend only on the amount of Chl accumulated. Other parameters that might be relevant include the intrinsic organization of the centers themselves.

In protochloroplasts, the values of K'_α and K_β never reach those of the green control, indicating that the existing units are never completely filled with Chl molecules. However, we can induce the transformation of protochloroplasts into mature chloroplasts by continuous illumination. Under these conditions, a fast and parallel increase of the values of K'_α and K_β up to the green control values is observed. The change is completed in less than 6 hr of continuous illumination.

The results of a representative experiment, where etiolated leaves were exposed first to 14 light-dark cycles and then to continuous illumination, showed that in 1 hr the K'_α and K_β increase almost five times, and in 3 hr they both reach the values of the mature chloroplast.

Kitajima and Butler (14) have reported that the nonvariable fluorescence emission F_0 and the variable fluorescence emission F_v of isolated chloroplasts are of the same type and that both are emitted from the bulk Chl of PSII. A simple theory of the mechanism of PSII events related to photochemistry (8, 15, 17) predicts that the ratio F_v/F_{max} of the total variable fluorescence to the maximum fluorescence yield is equal to the yield of primary photochemistry. Under our experimental conditions, the yield of primary photochemistry should depend on the degree to which the Chl molecules are organized into functionally effective units. Figures 8 and 9 show the F_v/F_{max} ratio of etiolated leaves greened under continuous and intermittent illumination, respectively. Figure 8 shows that the ratio F_v/F_{max} increases during greening with continuous light from the low value of approximately 0.29 at 1 hr of illumination to a steady-state value of approximately 0.72. Figure 9 shows the change of the system II primary photochemistry as a function of the number of light-dark cycles. We observed that the initially low value of the ratio, approximately 0.44 after 10 light-dark cycles, is increased to a value higher than that of the green control. The increase in the F_v/F_{max} ratio during greening in continuous or intermittent illumination reflects the change in the ratio between organized and unorganized Chl in the developing chloroplasts or protochloroplasts (G. Akoyunoglou, submitted for publication). However, the fact that in protochloroplasts the F_v/F_{max} ratio reaches values much higher than those of mature chloroplasts suggests that the protochloroplasts are more efficient in utilizing the absorbed light energy.

DISCUSSION

It was reported (1) that the PSII reaction centers appear very early during greening of young etiolated leaves. The present study shows that the α and the β centers of PSII are formed early during greening in continuous or intermittent light. Moreover,

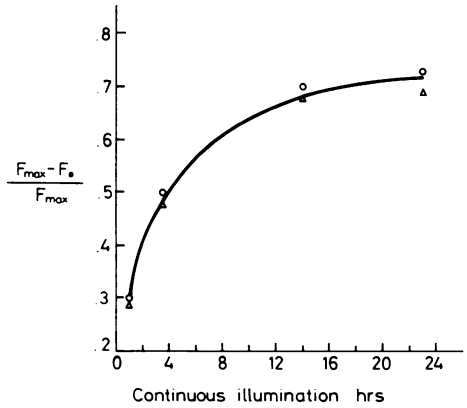

FIG. 8. Continuous illumination-induced change of the maximal yield of primary photochemistry, $(F_{max}-F_0)/F_{max}$ in developing chloroplasts. The respective value of the green control was approximately 0.72. For other conditions see Figure 3.

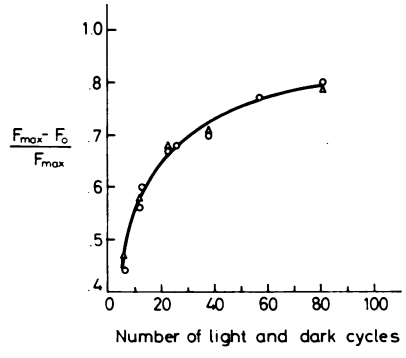

FIG. 9. Intermittent illumination-induced change of the maximal yield of primary photochemistry, $(F_{max}-F_0)/F_{max}$, in developing protochloroplasts. For other conditions see Figure 5.

the appearance and development of the α and β centers are independent of grana formation.

In a recent publication (19), the hypothesis was presented that the two types of system II reaction centers functioned independently in different pigment beds. The possibility existed, however, that the α and the β centers compete for the same exciton in one pigment bed. In the latter case, it is expected that the developmental changes of the photosynthetic units would affect the kinetic characteristics of the α centers and the β centers in the same way.

The differential effect of the process of greening on the rate constants K'_α and K_β under conditions of intermittent illumination (see Fig. 7) during which the slow rate of Chl biosynthesis limits the development of the photosynthetic units, supports the hypothesis that the α centers do not share the same pigment bed with the β centers. Otherwise, there should have been a parallel change of the values of K'_α and K_β.

In the light of our observations we propose the following changes in the development of the photosynthetic units from the etioplast to the chloroplast in beans. In the early stages of development, the α centers and the β centers are surrounded by a limited number of Chl molecules, thus the respective α and β units are of a small relative size. Development in continuous illumination induces a rapid biosynthesis of the Chl and all of the other components of the developing thylakoid. The Chl formed fill the open spaces of the appearing and growing α units and β units, hence the fast change of the value of K'_α and K_β (see Fig. 3). Under conditions of intermittent illumination, however, the rate of Chl biosynthesis is not fast enough to provide the adequate amount of Chl molecules needed for each one of the newly formed photochemical centers. In this case, each unit contains a limited number of antenna Chl molecules and this is reflected by the low relative values of K'_α and K_β. This suggests that either the developing chloroplast, when at limiting Chl concentration, use its Chl preferentially for the formation of small units, or that light is necessary for the formation of components necessary for the growth of the PSII units. From the dependence of K'_α and K_β on the number of light-dark cycles (see Fig. 7), it appears that the size of the β units does not change appreciably between 10 and 60 light-dark cycles. On the contrary, the size of the α units appears to be increasing. This means that either increasing amounts of Chl become available to the α centers, or that the α units are organized with time (Melis and Akoyunoglou, unpublished) so as to insure a more efficient utilization of the absorbed light energy. In the latter case, one could suppose that the α units tend to form larger aggregates of photosynthetic units (statistical or lake model) while the β units remain isolated from each other with no excitation energy transfer occurring between them.

That connected system II units are developed in protochloroplasts was also evident from the results of our recent studies on the development of the PSII unit (G. Akoyunoglou, submitted for publication). It was found that under intermittent illumination, the system II units formed are seven times smaller than those of mature chloroplasts. Moreover, the shape of the fluorescence rise is exponential early during exposure to light-dark cycles, but later it becomes sigmoidal, indicating the development of energy transfer and connection between units. This further showed that the connection of the system II units is not controlled by the concentration and type of Chl, or the presence of complex II and grana, but by the structural development and organization of the membrane.

Dubertret and Joliot (10) reached a similar conclusion studying the greening process of a dark-grown *Chlorella* mutant. They have shown that the induction kinetics of the fluorescence was exponential-like during the early stages of greening, but they later become sigmoidal. They suggested that the early PSII units cannot interact with each other in terms of excitation energy transfer. The change in the shape took place when the size of the system II units increased 2-fold by the addition of more Chl *b*, and the mutant became fully greened. Since the PSII unit in its two forms, *i.e.* isolated or connected with other units, was found to have almost the same size, they concluded that this is due to changes in the structure of the developing membrane, and not to the addition of the Chl *b* molecules.

We conclude that the α and the β centers are both present in the photosynthetic membranes from the beginning of the light-induced greening of bean etioplasts. The α centers and the photosynthetic units associated with them develop independently of the β centers. The presence of grana, Chl *b*, and the Chl-protein complex II is not a prerequisite for the formation of the α centers. Moreover, the heterogeneity of the photochemical centers in system II is an endogenous property of the chloroplast lamellae. The question that arises now is, "What are the structural differences between the α and β centers of the PSII units?"

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