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Cyclic Changes in Thylakoid Membranes of Synchronized *Chlamydomonas reinhardi**

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Abstract. Cyclic variations in the activity of photosystems I and II occur during the cell cycle of wild-type *Chlamydomonas reinhardi*. These variations have been localized to chloroplast thylakoid membranes and correlated with corresponding changes in the ratio of chloroplast cytochromes to chlorophyll.

Introduction. Although much is known about the biochemical composition,[1] structural organization,[2] and functional activity[3] of chloroplast thylakoid membranes, it is not known whether these parameters remain stable or undergo changes during rapid membrane biogenesis.

Previous studies dealing with this problem have focused attention primarily on the structural aspects of thylakoid membrane formation in higher plants.[4, 5] A more detailed morphological and biochemical analysis of membrane biogenesis has been performed in the y-1 mutant of the green alga *Chlamydomonas reinhardi*.[6–8] Dark-grown y-1 cells retain a chloroplast which contains almost no thylakoid membranes but relatively normal levels of many important photosynthetic enzymes.[6] Thylakoid membranes are rapidly produced upon exposure to light. Previous studies[6] with the y-1 mutant revealed that the Hill activity of these membranes remains relatively constant during this period of rapid biogenesis. It was therefore suggested that thylakoid membranes are produced by a single-step assembly process (i.e., by a process in which all components forming a functional membrane unit are fitted into the membrane at the same time). Subsequent work indicated that there are definite changes in both the biochemical composition[8] and the functional activity[7, 9] of thylakoid membranes during the regreening process. These recent results have been considered more compatible with a multi-step assembly hypothesis.[7, 8] However, since membrane formation in the y-1 mutant may represent a rather special case, generalization of these findings to wild-type cells remained open to question.

To explore thylakoid membrane biogenesis in further detail and under more physiological conditions, we have extended these studies to synchronous cultures of wild-type *Chlamydomonas reinhardi*. The data presented in this paper indicate that thylakoid membranes undergo cyclic variations in both photoreductive capacity and biochemical composition during the normal cell cycle.

Materials and Methods. Wild-type *Chlamydomonas reinhardi* (#90, mt-) obtained from the Indiana University collection were maintained on Sager and Granick medium I essentially in the manner described by Ohad *et al.*[6] Synchronous cultures were

produced by subjecting the cells to a programmed cycle of 12 hr light and 12 hr dark at 25°.[10]

The specific activities of photosystems I and II (PS I and PS II) were determined in disrupted cell suspensions. Cells were harvested from synchronous culture at various times, washed once in 0.01 *M* Tris-HCl buffer, pH 7.2, and resuspended in about 5 ml of the same buffer to give a cell suspension having a final chlorophyll concentration of approximately 10 μg/ml. The washed cells were then disrupted by one passage through a French pressure cell at 6000 psi. All washing and breakage procedures were conducted at 4°C.

PS I specific activity was determined by a procedure slightly modified from Hoober *et al.*[7] Each 3.0-ml reaction mixture contained Tris-HCl buffer, pH 7.2 (150 μmoles); dichloroindophenol (DC1) (0.6 μmoles); ascorbic acid (3.5 μmoles); 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) (0.06 μmoles); methyl red (0.16 μmoles); and 0.5 ml of pressurate. The ascorbic acid and pressurate were added just prior to illumination. Cuvettes containing 1.0 ml of reaction mixture were illuminated with a 500 watt incandescent bulb fitted with a red filter transmitting light with wavelength longer than 600 mμ. The reduction of methyl red was determined by following the change in optical density at 430 mμ after 30-sec illumination periods using a Zeiss model M4 QIII spectrophotometer. Optical density changes in dark controls were subtracted from each measurement.

PS II specific activity was measured by the reduction of dichloroindophenol dye.[7] Each 3.0 ml reaction mixture contained Tris-HCl buffer, pH 6.6 (30 μmoles); KCl (15 μmoles); dichloroindophenol (0.1 μmoles), and 0.5 ml of pressurate. Reduction of dichloroindophenol was measured by the change in optical density at 600 mμ after 30-sec illuminations. This reaction is completely inhibited by DCMU.

All photoreductive activity measurements were conducted at saturating light intensities to insure direct proportionality to chlorophyll content.

Chlorophyll concentration was measured in 80% acetone extracts[11] and DNA as described by Chiang and Sueoka.[14]

To determine the concentration of chloroplast cytochromes, synchronized cells were harvested, washed once in 0.1 *M* Tris-HCl buffer, pH 7.2, and frozen. When a sufficient number of samples were collected, they were thawed and resuspended in the same buffer. Cell solubilization was obtained by adding Triton X-100 (1.0% final) to the buffer followed by a 10-min incubation at room temperature and one passage through the French pressure cell at 6000 psi. Cytochrome content was determined on aliquots of this preparation using the Cary model 15 recording spectrophotometer.[12]

Cytochrome 563 was measured by the dithionite-reduced minus ascorbate-oxidized (D-A) difference spectrum, while both cytochromes 559 and 553 were measured by the ascorbate-reduced minus ferricyanide-oxidized (A-F) difference spectrum. The cytochromes measured in the D-A and A-F spectra were unambiguously identified by spectra taken at liquid nitrogen temperature (ref. 13; see also insert in Fig. 4). However, for quantitative determinations it was necessary to record difference spectra at room temperature. Under these conditions, cytochrome 559 and cytochrome 553 are not resolved, a single peak at 556 mμ being observed. In each case, 50 μl of either 0.1 *M* ferricyanide or 0.1 *M* ascorbic acid (pH 6.0) or H_2O plus solid dithionite were added to 1.0 ml of pressurate in 1.0 ml cuvettes. Molar concentrations were determined assuming a molar extinction coefficient of 2×10^4 for all cytochromes.

Results. Wild-type *Chlamydomonas reinhardi* undergoes synchronous growth when exposed to a repeating program of 12-hr light and 12-hr dark.[10] There is no increase in cell number during the entire light period. Division into either four or eight daughter cells takes place in the latter half of the dark period giving an approximate five- to sixfold increase in cell number at the end of each cycle (see Fig. 1).

There is a 3- to 4-hr lag at the beginning of each light period before the

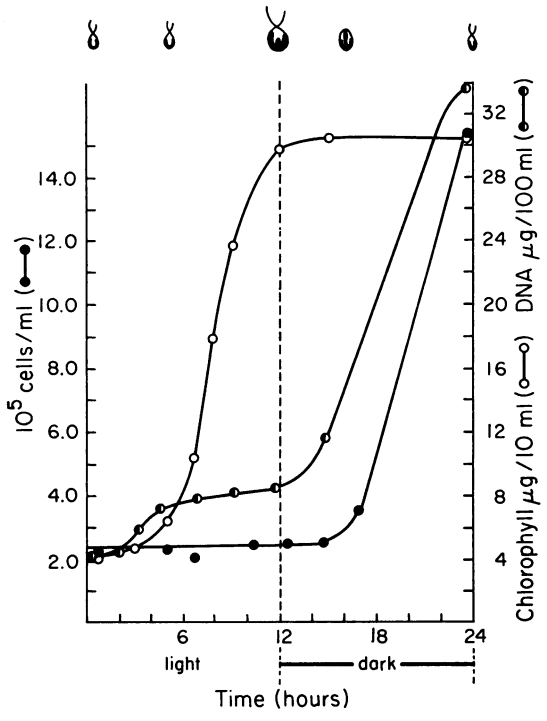

FIG. 1.—Various parameters in the synchronous culture of *Chlamydomonas reinhardi*.

Appearance of the cells at various times during the cycle is diagrammed on top of the figure.

chlorophyll content per milliliter (or per cell) begins to increase. The chlorophyll content then rises rapidly during the second half of the light period and remains stationary during the entire dark period. Since the cells undergo division in the dark, the chlorophyll content per cell is the same at the beginning and end of each 24-hr cycle.

The DNA content per ml (or per cell) increases in a two-step manner; the first occurring after 3 to 4 hr in the light and the second shortly prior to cell division in the dark. The DNA replicated in the light period accounts for about 14% of the total increase and has been identified as chloroplast DNA by Chiang and Sueoka.[14] Proportionate growth is again indicated by the fact that the DNA content per cell is the same at the beginning and end of each cycle.

PS I and PS II specific activities (expressed per mg chlorophyll) can be measured in broken cell suspensions during the course of synchronous growth. PS I specific activity is determined by the photoreduction of methyl red, and that of PS II is determined by the photoreduction of dichloroindophenol.[7] PS I specific activity undergoes an approximate 40 to 50% relative increase after about 3 hr in the light, then decreases during the remainder of the cycle and reaches a minimum value at about 4 hr in the dark. PS II specific activity undergoes a more pronounced cyclic variation; it increases by 100 to 125% during the first 3 hr of illumination and then decreases both during the remainder of the light period (when the chlorophyll content rapidly increases) and during the dark period (when the chlorophyll content is stationary). Figure 2 gives the results of a representative experiment in a series of ten.

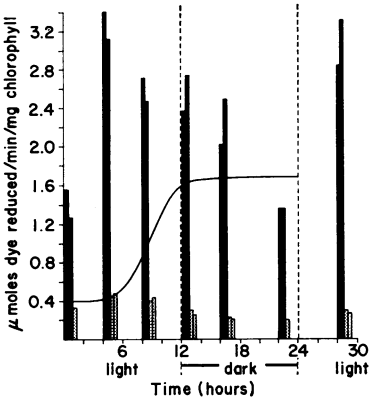

FIG. 2.—Changes in photoreductive activity during synchronous development. PS I specific activity is measured by the photoreduction of methyl red (*stipled bars*), and PS II specific activity by the photoreduction of dichloroindophenol (*black bars*). Each bar represents activity determinations made with a different disrupted cell suspension. chlorophyll (μg/ml);—.

Accordingly, there are two periods in the synchronous cycle in which PS II specific activity changes with no corresponding change in total chlorophyll content. The first period corresponds to the rapid increase in activity during the first 3 hr in the light and the second to the gradual decrease in activity in the dark. Since these determinations were made on broken cell suspensions, it can reasonably be assumed that they reflect functional-structural modulations of thylakoid membranes rather than regulatory processes at the whole cell or chloroplast level.

To check this assumption and to eliminate possible effects of soluble chloroplast or cytoplasmic components, PS II specific activity was measured in thylakoid membranes isolated from cells at various times during synchronous growth. Such a thylakoid membrane fraction was prepared by a series of centrifugations in discontinuous sucrose gradients as described by De Petrocellis *et al.*[8] It corresponds to the thylakoid membrane fraction II of their procedure and consists mainly of intact thylakoid membranes still partially organized in grana. In addition, it contains small amounts of vesicles derived from the chloroplast envelope and the pyrenoid tubules. The chloroplast matrix and ribosomes are lost during the purification procedures. The fraction is contaminated by a few disrupted mitochondria. When PS II specific activity is measured on such a purified preparation, there is an unavoidable 50–70% loss in activity compared to control measurements in whole cell pressurates. However, as can be seen in Figure 3, isolated thylakoid membranes

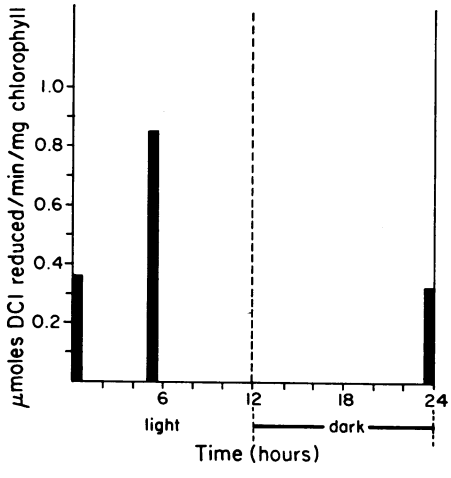

FIG. 3.—Specific activity of PS II of thylakoid membranes isolated during synchronous development.

still exhibit the same relative change in PS II specific activity during the cell cycle.

On the basis of these results it can be concluded that the photoreductive activity of the thylakoid membranes changes during the cell growth cycle. This change could result from either a chemical alteration of the membrane (insertion or deletion of specific molecules) or form a reorganization of existing components into different functional arrays. As a first step in testing these alternatives, we have measured the ratios of the various cytochromes to chlorophyll during synchronous growth. The path of electron flow from water to NADP in *Chlamydomonas reinhardi* has been well documented.[15] Cytochrome 559 is located near the reducing side of PS II and cytochrome 553 is located near the oxidizing side of PS I. There is an additional cytochrome of the *b* series, cytochrome 563, which is believed to function in cyclic electron flow around PS I.

Figure 4 shows a typical experiment in which PS II specific activity and the ratios of the various cytochromes to chlorophyll have been measured throughout

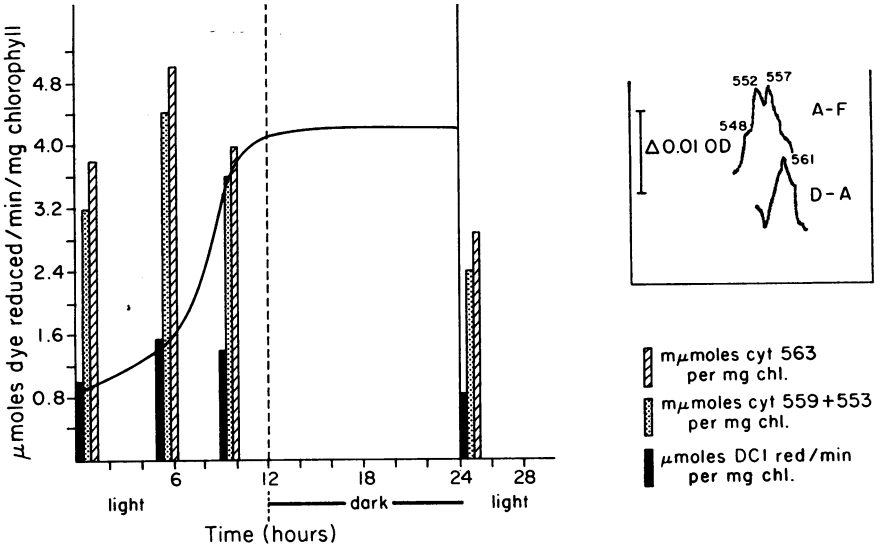

FIG. 4.—Changes in PS II specific activity (*black bars*) and cytochrome to chlorophyll ratios during synchronous growth. The chlorophyll content (μg/ml culture) is indicated by the solid line. The insert shows liquid nitrogen spectra used to identify cytochromes. The small relative increase in PS II specific activity (compared to the data in Fig. 2) is caused by the high initial cell density used to insure adequate samples for the cytochrome assays.

synchronous growth. The ratio of cytochromes 559 + 553 to chlorophyll changes in a manner exactly parallel to PS II specific activity. In this particular experiment, the ratio of cytochrome 563 to chlorophyll follows a similar course.

Data from five such experiments are summarized in Figure 5. The ratio of cytochromes 559 + 553 to chlorophyll is well correlated with PS II specific activity. There is a comparable general trend in the ratio of cytochrome 563 to chlorophyll, but the correlation with PS II specific activity appears to be less rigorous.

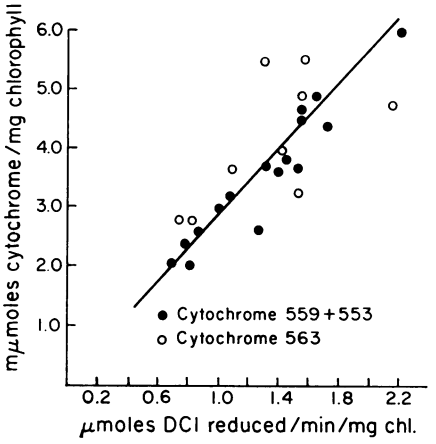

FIG. 5.—Correlation of PS II specific activity to cytochrome/chlorophyll ratios. Each point represents an individual determination performed at various times during synchronous growth. The line gives least mean square determination for cytochrome (559 + 553)/chlorophyll.

Discussion. The results presented here indicate that PS I and PS II specific activities undergo cyclic changes during the synchronous development of wild-type *Chlamydomonas reinhardi*. It is of interest to note that Senger and Bishop[16] have observed cyclic variation in the percentage of Emerson enhancement during synchronous growth of *Scenedesmus*. From quantum yield determinations, they have inferred that this variation results from a change in PS II and not PS I activity. Our direct determinations show that in *Chlamydomonas* the specific activities of both photosystems change, with that of PS II certainly being more pronounced.

Our results further indicate that the changes in PS II specific activity (as determined in broken cell preparations) closely parallel changes in the ratio of cytochromes 559 + 553 to chlorophyll. Since we have localized the changes in PS II specific activity to thylakoid membranes, we assume that they reflect parallel changes in the cytochrome content of the same membranes. This assumption is supported by published evidence which indicates that cytochrome 559 is tightly bound to chloroplast membranes[17] and that the same applies to cytochrome 553 over a wide range of concentrations.[8]

On the basis of these considerations, we have interpreted our data in the following manner:

(*a*) There is an increase both in the ratio of cytochromes 559 + 553 to chlorophyll and in PS II specific activity during the first 3 hr of illumination. Since there is at this time no increase in the total chlorophyll per cell, the change in the cytochrome to chlorophyll ratio most probably reflects an insertion of additional cytochrome molecules into pre-existing thylakoid membranes.

(*b*) Conversely, there is a decrease both in the ratio of cytochromes 559 + 553 to chlorophyll and in PS II specific activity during the dark period when there is again no increase in total chlorophyll. This decrease in the cytochrome to chlorophyll ratio most probably reflects a loss of cytochrome molecules from thylakoid membranes.

(*c*) The chlorophyll content increases rapidly during the second half of the light period. The decrease both in the ratio of cytochromes 559 + 553 to chloro-

phyll and in PS II specific activity seen at this time could also result from a rapid insertion of chlorophyll molecules into pre-existing membranes. These molecules may represent antenna chlorophyll associated with PS II reaction centers. One would therefore predict that the average size of a PS II reaction unit would increase during this time.

The data presented in this communication therefore indicate that chloroplast thylakoid membranes undergo a definite series of functional and biochemical alterations during the growth-division cycle of *Chlamydomonas reinhardi*. Initial electron microscopic observations of cells in thin section have not revealed any correlated changes in these membranes with respect to either general morphology or degree of stacking in grana.

Since there are modulations in both membrane functional activity and biochemical composition, it would be of great interest to determine by what means these modulations are controlled. This problem can be investigated by the use of chloroplast-specific inhibitors of RNA and protein synthesis.[7, 18, 19] Although many thylakoid membrane proteins appear to be produced in the cytoplasm, several, including cytochrome 559, are most probably synthesized within the chloroplast.[18] The changes in the ratio of cytochromes 559 + 553 to chlorophyll observed during synchronous growth could be explained by the arrest in the dark of protein synthesis within the chloroplast. If cytochrome 559 is made in the chloroplast, and has a high turnover rate, one could explain why the ratio of cytochrome to chlorophyll decreases in the dark and increases again upon exposure to light. Preliminary data do indicate that protein synthesis in chloroplasts indeed occurs only when the cells are exposed to light.

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