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Appearance of Membrane-bound Iron-Sulfur Centers and the Photosystem I Reaction Center during Greening of Barley Leaves[1]

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ABSTRACT

Dark-grown barley (*Hordeum vulgare*) etioplasts were examined for their content of membrane-bound iron-sulfur centers by electron paramagnetic resonance spectroscopy at 15K. They were found to contain the high potential iron-sulfur center characterized (in the reduced state) by an electron paramagnetic resonance *g* value of 1.89 (the "Rieske" center) but did not contain any low potential iron-sulfur centers. Per mole of cytochrome *f*, dark-grown etioplasts and fully developed chloroplasts had the same content of the Rieske center. During greening of etioplasts under continuous light, low potential bound iron-sulfur centers appear. In addition, the photosystem I reaction center, as measured by the photooxidation of P700 at 15K, also became functional; during greening the appearance of a photoreducible low potential iron-sulfur center paralleled the appearance of P700 photoactivity.

These findings indicate the close association of the low potential iron-sulfur centers with the photosystem I reaction center; they also support the concept that the development of stable charge separation in the photosystem I reaction center requires, in addition to P700, a low potential iron-sulfur center.

In recent years there have been numerous reports of the characterization during the greening process of electron carriers in higher plants and algae. In chloroplasts, Cyt *f*, b_6, and a low potential form of Cyt b_{559} are found in dark-grown etioplasts (8, 13, 23, 25). Other electron carriers of fully developed chloroplasts, such as plastocyanin (14), chloroplast ferredoxin (15, 26), and ferredoxin-NADP reductase (26) have also been found in etioplasts before greening. The high potential form of Cyt b_{559}, not found in etioplasts, appears during greening (16).

In addition to these electron carriers, which were known to be present in fully developed chloroplasts, a new group of electron carriers, the membrane-bound iron-sulfur centers, has recently been found in chloroplasts (19); these electron carriers have been characterized on the basis of their EPR[2] properties. Two of these centers have extremely low oxidation-reduction potentials and are associated with the reaction center of chloroplast PSI: one center, center A (EPR *g* values of 2.05, 1.94, and 1.86; E_m about −530 mv [10, 17]) is photoreducible at cryogenic temperatures (5, 6, 19) and has been proposed as the PSI primary electron acceptor (5, 6); there is a second center (center B, EPR *g* values of 2.05, 1.92, and 1.89; E_m about −600 mv [10, 17]) whose function is not known at this time. The latter is known to be closely associated with the photoreducible center (9, 10) and it has been proposed that both centers may be in one protein, as in the case of the bacterial ferredoxins which contain two iron-sulfur clusters/molecule (22).

A third iron-sulfur center, with different properties, recently was identified in chloroplasts (18). The oxidation-reduction potential (E_m = +290 mv) of this center, known as the "Rieske-type" center, is more positive than the potentials of the other centers. This center also has different EPR properties (EPR *g* values of 2.02, 1.89, and 1.78 [18]). The function of this third center is not known, but an analogous iron-sulfur protein in the mitochondrion may be involved in electron transfer in the Cyt *b*-*c* region of the respiratory chain (24).

The purpose of the present work was to investigate changes in these membrane-bound iron-sulfur centers during the greening process. EPR measurements at cryogenic temperature have been used to follow these three centers during the formation of chloroplasts. Our results indicate that the etioplasts contain only the "Reiske" center and that the remaining low potential iron-sulfur centers appear during greening; the appearance of an iron-sulfur center that is photoreducible at 15K correlates with the appearance of the PSI reaction center during greening.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare*) seeds were scattered thickly over sterilized vermiculite (about 2 cm deep) and soaked with nonsterile distilled H_2O. Subsequent watering was done with distilled H_2O. Plants for greening experiments were grown undisturbed for 6 to 8 days in a dark, sealed, paper chromatography drying chamber. The temperature was 25 ± 2 C. For greening, the chambers were opened and placed under a bank of seven 40 w cool-white fluorescent lamps about 70 cm above the level of the leaves. The light intensity at plant level was 1×10^4 ergs·cm^{-2}·sec^{-1}. Wet filter paper lined opposite sides of the chambers to insure high humidity. Other plants were grown for 6 to 8 days on a laboratory bench under room fluorescent lights and also received a small amount of direct sunlight.

Plastid Isolation. Plastids were isolated as described below in dim light. Leaves were harvested, cut into 1- to 2-cm pieces, and blended in a Waring Blendor with as small a volume as possible of the following blending solution: 0.3 M sucrose, 0.05 M tris-HCl buffer (pH 7.8), and 0.01 M NaCl. Leaves from partially greened or dark-grown plants were blended for 10 to 20 sec and leaves from light-grown plants required 30 to 60 sec for blending. The slurry was filtered through two layers of filtering silk and the filtrate was centrifuged at 3,000*g* for 2 min. The pellet was resuspended in a minimum volume of blending solution and stored in the dark. EDTA to a final concentration of 2 mM and sodium ascorbate to a final concentration of 10 mM were added

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[2] Abbreviation: EPR: electron paramagnetic resonance.

to the suspension before it was frozen in liquid N_2 for subsequent EPR studies.

Determination of Chlorophyll. Chlorophyll was determined after 80% acetone extraction. The concentrations of Chl *a* and *b* in the acetone extracts were calculated using the formulas of Anderson and Boardman which take into account absorbance due to Pchl (3).

Determination of Cytochromes. Reduced minus oxidized difference spectra were recorded from 530 nm to 580 nm in the split beam mode of an Aminco DW-2 spectrophotometer. Plastids were diluted in 0.05 M K-phosphate buffer (pH 7.2) for assay. Hydroquinone-reduced minus ferricyanide-oxidized difference spectra were used to estimate the concentration of Cyt *f*. An extinction coefficient of 20.6 $mM^{-1}cm^{-1}$ (554 nm minus 538 nm) was used in the calculation of the concentration of Cyt *f* (21).

Chemical Determination of Membrane-bound Iron-Sulfur Centers. For the determination of the Rieske iron-sulfur center, samples containing ascorbate were used directly with no other additions. From the preparation, 0.3 ml was removed and frozen to 77K in a 3-mm i.d. quartz EPR tube.

For estimation of low potential iron-sulfur centers, samples were mixed with glycine buffer (pH 10) (final concentration, 0.1 M) and methyl viologen (final concentration, 0.02 mM) at 4 C in quartz EPR tubes. A small amount of solid sodium dithionite was added and the suspension was incubated for 1 min in the dark prior to freezing to 77K.

EPR spectra were recorded at 15K on a modified JEOL X-band spectrometer operated at a microwave frequency of 9.21 GHz, as previously described (5, 6).

Determination of Light-induced P700 Photooxidation and Bound Iron-Sulfur Center Photoreduction at 15K. Samples reduced with ascorbate were incubated in the dark at 4 C for 10 min prior to freezing to 77K in matched, calibrated quartz EPR tubes. Spectra in the P700 $g = 2.00$ region (3,285 ± 25 gauss) and in the region of the $g = 1.94$ bound iron-sulfur centers (3,400 ± 250 gauss) were recorded in the dark. Samples were then illuminated with red light (Corning 2-64 filter; intensity, 1×10^6 ergs·cm^{-2}·sec^{-1}) in the EPR cavity at 15K for 30 sec and spectra were recorded after the cessation of illumination. Light minus dark difference spectra were obtained from data stored in an on-line PDP-8 digital computer. These data could be scaled to correct for different amplifier gains with different samples and it therefore was possible to correct for any differences in EPR conditions.

The extent of the light-induced P700 change was estimated from the amplitude (peak-to-trough) of the light minus dark $g = 2.002$ free radical signal; the extent of the light-induced iron-sulfur center change was calculated from the amplitude (peak-to-trough) of the $g = 1.94$ resonance line of the light minus dark signal from the reduced iron-sulfur center. These amplitudes are expressed in arbitrary units. Because of concentration differences between the samples, the magnitude of the light-induced changes was normalized on the basis of content of Cyt *f* in the respective samples (see below).

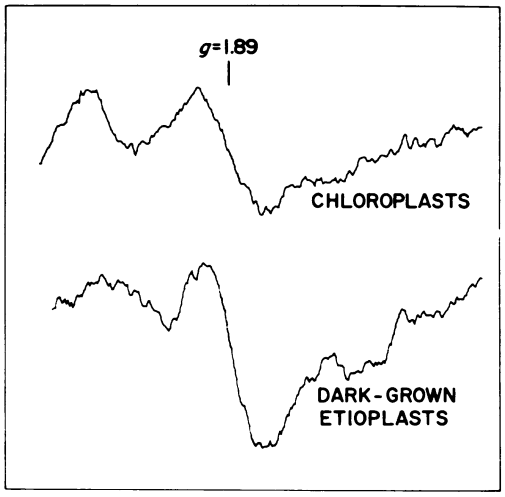

FIG. 1. Rieske iron-sulfur center in chloroplasts and dark-grown barley etioplasts. Chloroplasts from fully developed barley and etioplasts from 7-day-old dark-grown barley were isolated as described under "Materials and Methods." The samples were diluted with blending solution to give a Cyt *f* concentration of 1.5 μM. Samples were reduced with ascorbate prior to freezing to 77K for subsequent EPR analysis. EPR conditions: frequency, 9.21 GHz; modulation amplitude, 10 G; microwave power, 10 mw; temperature, 15K; amplifier gain, 360.

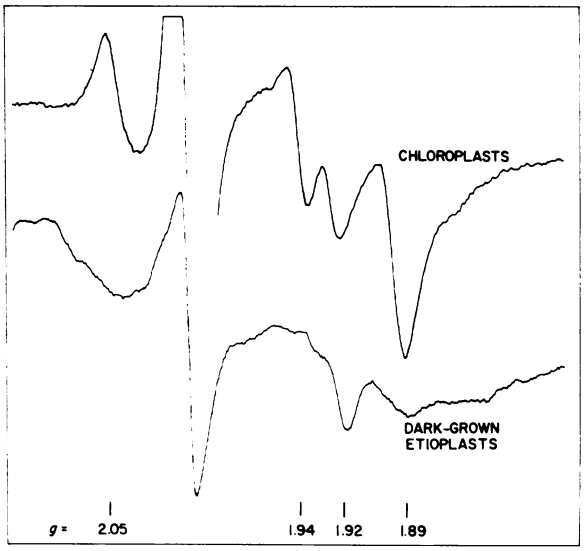

FIG. 2. Low potential iron-sulfur centers in chloroplasts and dark-grown barley etioplasts. Chloroplasts and etioplasts from dark-grown plants were prepared as described under "Materials and Methods." Samples were diluted to give a final Cyt *f* concentration of 1.5 μM and reduced at pH 10 with sodium dithionite in the presence of methyl viologen prior to freezing to 77K. EPR conditions as in Figure 1 except that an amplifier gain of 120 was used.

RESULTS

Iron-Sulfur Centers in Dark-grown Etioplasts. Ascorbate reduction of chloroplasts from fully developed plants and from dark-grown etioplasts results in the reduction of the Rieske iron-sulfur center (Fig. 1), and in both preparations EPR analysis shows the $g = 1.89$ EPR signal of the reduced form of this protein. For a direct quantitative comparison, the two samples contained the same concentration of Cyt *f* (1.5 nmol Cyt *f*/ml) and on this basis it appears that there is slightly more of the Rieske center in the dark-grown etioplasts than in chloroplasts.

Dithionite reduction (in the presence of methyl viologen) of chloroplasts results in the appearance of EPR signals from membrane-bound, low potential iron-sulfur centers. As shown in Figure 2, in chloroplasts these centers are characterized by *g* values of 2.05, 1.94, 1.92, 1.89, and 1.86; these *g* values have been interpreted as arising from two different centers (see above). When dark-grown etioplasts are reduced with dithionite in a similar manner, a resonance line appears at an apparent *g* value of 1.91 but no other resonance absorption lines are apparent in this spectral region. In addition, in chloroplasts the temperature dependence of the signals from the reduced iron-sulfur centers shows marked broadening at about 30K and the iron-sulfur center signals disappear above this temperature; the signal in etioplasts shows no such temperature dependence and persists at temperatures above 40K. On the basis of this analysis, it does

not appear that the signal in etioplasts originates from a low potential iron-sulfur center or that dark-grown etioplasts contain such centers. The origin of the signal at $g = 1.91$ in etioplasts is not yet known.

Development of Iron-Sulfur Centers during Plastid Greening. Because etioplasts did not contain any low potential iron-sulfur centers and because these centers were found in fully developed chloroplasts, the development of the centers was examined during the greening process. The time course for the appearance of Chl under our experimental conditions is shown in Figure 3. Chlorophyll appearance showed a lag of about 2 hr and then proceeded at a rapid rate, finally leveling off after 20 hr. The amount of Chl is expressed on the basis of the Cyt *f* content of the plastids (see under "Discussion") in this figure, and after about 24 hr, a Cyt *f* to Chl ratio of about 1:350 was attained; this value is similar to that observed in light-grown plants and to that reported for other photosynthetic systems from higher plants (7). The Chl *a* to Chl *b* ratio is extremely high during the first 4 hr of greening but then remains constant for most of the remaining greening period. The time courses we have observed for the appearance of Chl are similar to those reported by other workers (1, 2, 4) and give some definition of the greening process under the specific experimental conditions of this work.

Figure 2 indicates the low potential iron-sulfur centers are absent from dark-grown etioplasts, although they are found in fully developed chloroplasts. These centers therefore must appear during the process of greening. As shown in Figure 4, chemical reduction of plastid samples after different periods of greening indicates the development of the dithionite-reducible iron-sulfur centers (compare also with Fig. 2). It was not possible to detect these centers in samples taken at times shorter than 4 hr of greening because of low signal intensity. After approximately 4 hr, the centers are visible by our method and progressively increase in amount. The content of low potential centers in the 24-hr greened sample is identical to that in the fully developed chloroplast sample, when compared on the basis of Cyt *f*. The EPR signals of the low potential centers are somewhat broader at the early stages of greening and appear to become narrower in the latter samples; this change in linewidth may reflect alterations in the environment of the centers during the developmental process.

It was not possible to quantitate the two low potential iron-sulfur centers as a function of illumination time because of the

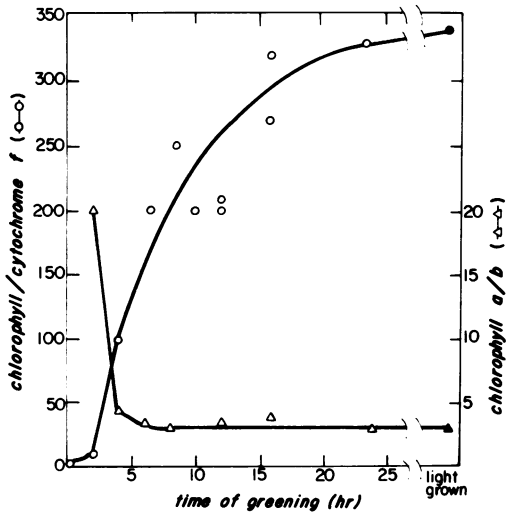

FIG. 3. Time course of the development of Chl during greening of barley. Plastid preparation was as described under "Materials and Methods" for plants greened for the indicated times. Analysis of Chl *a*, Chl *b*, and Cyt *f* was as described under "Materials and Methods."

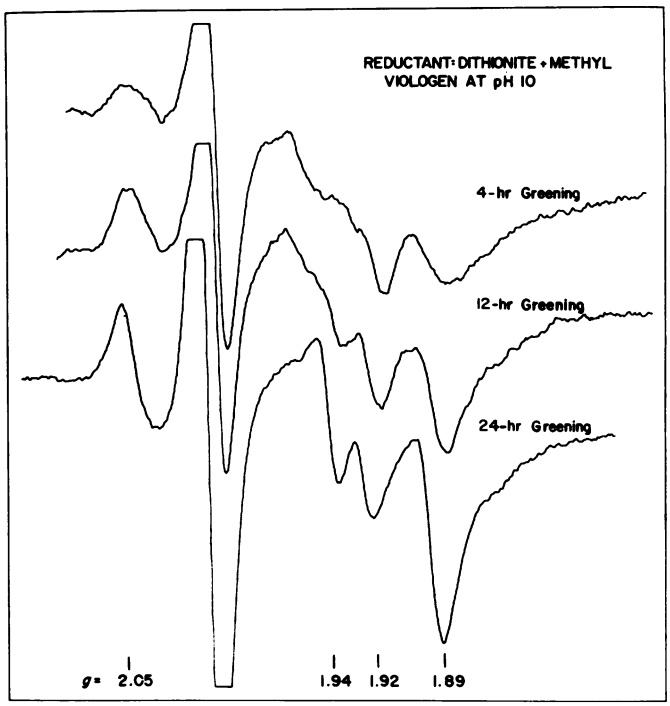

FIG. 4. Appearance of low potential iron-sulfur centers during greening of barley. Plastid preparation was as described under "Materials and Methods" for plants greened for the indicated times. Plastid preparations contained the following concentrations of Cyt *f*: 4-hr greened, 1.3 μM; 12-hr greened, 1.6 μM; 24-hr greened, 1.5 μM. Reduction at pH 10 with sodium dithionite and methyl viologen was as described under "Materials and Methods." EPR conditions as in Figure 2.

overlapping resonance lines. This type of data could be obtained only by complete chemical titration of the two centers at each greening point, and sufficient material for this type of measurement was not available. The center characterized by EPR *g* values of 2.05, 1.92, and 1.89 is clearly discernible and the resonance line at $g = 1.94$ indicates that the second center is also present. The ratio of these two centers appears to be relatively constant over the course of greening.

The appearance of one of the two low potential iron-sulfur centers could be followed by analysis of light-induced EPR changes of the center which can be photoreduced at cryogenic temperatures. By this technique it was possible to monitor also the appearance of the PSI reaction center, as measured by the $g = 2.002$ EPR free radical signal of P700, the PSI reaction center Chl, and to compare the appearance of these two PSI components on a more quantitative basis.

The light minus dark difference spectra for P700 and for the bound iron-sulfur center at different stages of greening are shown in Figure 5. P700 could be detected as early as 2 hr of greening, but the sensitivity of our technique was not sufficient for the detection of any reduced iron-sulfur center at that time. The extent of the P700 change and bound iron-sulfur center change in this figure is presented at different concentrations of Cyt *f* in each sample; for a direct comparison of samples at different times, the data were normalized to the same concentration of Cyt *f* in the respective samples. These data were used to plot time course curves for the appearance of P700 and the bound iron-sulfur center during greening (Fig. 6). A small lag (about 1-2 hr) is apparent, and it is followed by a rapid increase in the development of the PSI reaction center. As measured by the magnitude of the P700 signal, the reaction center appears to be fully developed after about 10 to 12 hr of greening, even though there is an increased synthesis of Chl beyond that time

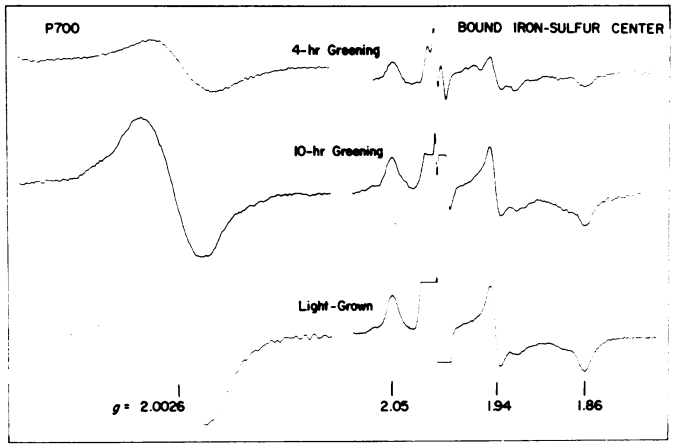

FIG. 5. Light minus dark difference spectra of P700 and bound iron-sulfur center during greening of barley. Plastid preparation from plants greened for the indicated times was as described under "Materials and Methods." The preparations had the following Cyt *f* concentrations: 4-hr greened, 2.3 μM; 10-hr greened, 3.9 μM; fully developed, 3.6 μM. Light-induced changes of P700 and the bound iron-sulfur center were measured at 15K by the EPR method. Spectra shown are light minus dark difference spectra obtained by computer subtraction.

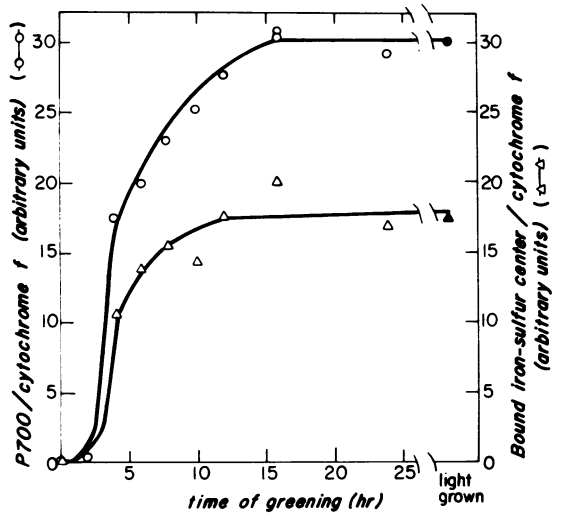

FIG. 6. Time course of the appearance of P700 photooxidation and bound iron-sulfur center photoreduction at 15K during greening of barley. Light minus dark difference spectra of photooxidized P700 and photoreduced bound iron-sulfur center were obtained from plastids isolated at different times of greening, as described under "Materials and Methods." Cyt *f* content of each sample was obtained from chemical difference spectra. Ratios of P700 to Cyt *f* and bound iron-sulfur center to cytochrome *f* were normalized to a concentration of 1 μM Cyt *f*.

(see Fig. 3). The appearance of the reduced iron-sulfur center closely parallels that of light-induced P700 (Fig. 6). Over the entire time course of the greening process (from 4 hr until 24 hr), the ratio of the light-induced P700 signal to that of the reduced iron-sulfur center signal is constant (Fig. 7). The data obtained from a sample greened for 24 hr were identical to those obtained from chloroplasts of light-grown plants and indicated the complete development of the PSI reaction center under our greening conditions.

DISCUSSION

Our results indicate that the multiple bound iron-sulfur centers in chloroplasts develop at different times during the greening process. One iron-sulfur center, the Rieske center, is found in dark-grown etioplasts and the amount of this component shows little change during greening. In this study, we have used as an internal reference the Cyt *f* content of the plastids in the samples. The use of Cyt *f* as a standard is based on previous findings (16, 23) that during greening there is little change in the Cyt *f* content on a fresh weight basis. Based on an equimolar concentration of Cyt *f*, etioplasts contain about 20% more of the Rieske center than do fully developed chloroplasts, but this difference is not considered significant.

The remaining bound iron-sulfur centers, the low potential centers, were not found in etioplasts but these centers developed during greening. It was not possible to follow these centers in a quantitative manner, but our analysis gives some indication that these two centers appear at approximately the same time and that the ratio of the two is relatively constant. Our studies of light-induced changes of the PSI reaction center indicate that P700 and the photoreducible iron-sulfur center parallel each other in the time course of appearance. The PSI reaction center can be detected after 2 hr of illumination and the reaction center increases thereafter. A recent report by Baker and Butler (4) has shown that P700 can be detected as early as 1 hr after greening, but our detection methods are not as sensitive as those used by Baker and Butler and hence we do not believe that our results are at variance with that report.

The parallel appearance of P700 and the photoreducible iron-sulfur center during greening strengthens the close association of these two electron transfer components. Although the proposed role of this bound iron-sulfur center as the PSI primary electron acceptor has been questioned recently by two groups (11, 12, 20), our present results indicate that the development of a functional PSI reaction center, as evidenced by P700 photooxidation, includes the participation of a bound iron-sulfur center. These data do not, however, preclude the involvement of a functional transient intermediate before the iron-sulfur center, but they do indicate the key role of the iron-sulfur center in a stabilized charge separation in PSI.

Our results also indicate that during greening, in addition to the development of Chl, functional reaction centers are synthesized and assembled. The absence of low potential iron-sulfur centers in etioplasts implies either that these centers are synthesized *de novo* in the light or that their active centers, the iron-sulfur core, are assembled in a light-dependent process. Further studies are anticipated in which we hope to characterize the formation of these functional iron-sulfur centers during the development of the chloroplasts.

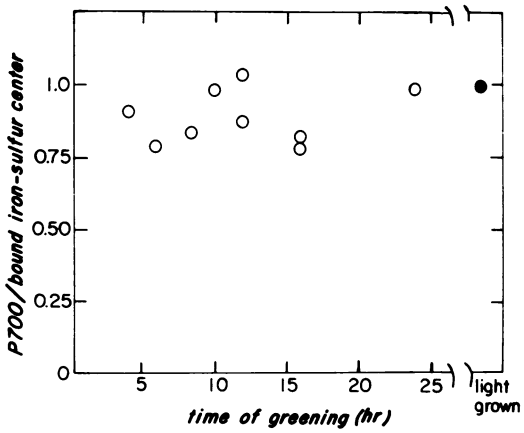

FIG. 7. Ratio of photooxidized P700 to photoreduced bound iron-sulfur center during greening. Data taken from Figure 6 with the ratio in the light-grown sample set equal to unity.

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