Energy and Electron Transfer Systems of
Chlamydomonas reinhardi

II. TWO CYCLIC PATHWAYS OF PHOTOSYNTHETIC ELECTRON TRANSFER IN THE PALE GREEN
MUTANT

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ABSTRACT

Light- and oxygen-induced changes of cytochromes f, b_{563}, and b_{559} and ferredoxin-flavoprotein were studied by a
double beam spectrophotometer with combinations of inhibitors and lowered temperatures in the whole cells of the
pale green mutant of Chlamydomonas reinhardi (ATCC 18302). At room temperature, the steady state changes of
cytochrome f and ferredoxin-flavoprotein are small, but at low temperature slightly above 0°C, they are clearly defined.
Phenylmercuric acetate inhibits photoreduction of ferredoxin-flavoprotein and cytochrome f simultaneously but not
that of cytochrome b_{563}. 2-Heptyl-1-hydroxyquinoline-
N-oxide shows a crossover point between cytochromes f and
b_{559} and partially inhibits photoreduction of cytochrome f.
Two cyclic pathways operating in C. reinhardi are postu-
lated: (a) photosystem I → x → b_{563} → f → photosystem I;
and (b) photosystem I → x → ferredoxin-flavoprotein → f →
photosystem I.

On the other hand, it has also been suggested that cytochrome
f is directly reduced by flavoprotein (ferredoxin-NADP reductase)
instead of cytochrome b_{563} (13, 20, 24).

In this report, using the pale green mutant of Chlamydomonas
reinhardi, utilizing two types of inhibitors (HOQNO4 and
PMA), and lowering the temperature to slightly above 0°C,
we obtained results which suggest that two types of different
cyclic pathways are in operation in this organism.

A preliminary report of the present paper has been presented
elsewhere (12).

MATERIALS AND METHODS

Cultivation of Organisms. The pale green mutant of C. rein-
hardi (ATCC 18302) was cultivated in acetate medium of the
following composition: 7.4 mm sodium acetate; 1.7 mm sodium
citrate; 13 mm sodium potassium phosphate buffer (pH 7.0);
3.8 mm ammonium nitrate; 1.2 mm magnesium chloride; 0.36
mm calcium chloride; 37 mm ferric chloride; and trace amounts
of BO_{3}^{4-}, Li^{+}, Mn^{2+}, Co^{2+}, Cu^{2+}, MoO_{4}^{2-}, final pH 6.8. Usually
cultivation was done in a 6-liter round bottom flask containing
4 liters of medium with gentle stirring by a magnetic stirrer for
3 to 4 days in the dark at room temperature (approximately
25°C). No further aeration was necessary. Harvested cells were
washed once by growth medium and suspended in the same
medium supplemented with 0.25 mm sucrose to prevent sedimenta-
tion of the cells during spectrophotometric measurement.

Cultivation of wild type C. reinhardi (ATCC 18798) and ac-
206 mutant (kindly supplied by Dr. R. P. Levine) was done in
the same system with the pale green mutant, except that it was
illuminated with incandescent lamps.

Absorbance change of the cell suspension was monitored by a
double beam spectrophotometer (2). Most measurements of
cytochrome change were done in Soret band with 445 nm as
the reference wave length. The absorbance change in the aerobic
state was measured shortly after aeration of the cell suspension
in a 1-cm cuvette. The aerobic state referred to in this series of
reports is defined as the plateau region of absorbance change
between aeration and aerobic-anoxic transition, which is
usually observed 30 to 80 sec after aeration. Though the oxygen
concentration of the cell suspension decreases linearly to ana-
obiosis because of respiration, the redox levels of cytochromes
stay in a steady state during this aerobic state until the transition

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Abbreviations: HOQNO: 2-heptyl-1-hydroxyquinoline-N-oxide;
PMA: phenylmercuric acetate; CMU: 3-(4'-chlorophenyl)-1,1-di-
methylurea; BIMU: 5-bromo-3-isopropyl-6-methyluracil; CCP: car-
bonylcyanide m-chlorophenylhydrazone.
tration. The anaerobic state is defined as the steady state achieved after the transition from aerobic state. The spectrum of absorbance change in aerobic-anaerobic transition has been reported in the previous paper (13). The time required for achieving anaerobiosis from aerobic state varied from culture to culture and was probably due to the age and to the amount of endogenous substrates in the cells.

The measuring cuvette was cross-illuminated on the front surface at the incident angle of approximately 60° by an incandescent illuminator with a far red cut-off filter (>670 nm, VR-69, Toshiba, Tokyo). Measurement at low temperatures slightly above 0°C was achieved by circulating ice-cold water through an aluminum jacket holding the cuvette. The temperature of the cell suspension was measured directly by inserting a thin thermistor thermometer.

Other Methods. Delayed light emission from chlorophyll was measured with a multipurpose phosphoroscope (22). Chlorophyll was excited by blue light with the maximum at 460 nm (obtained by the combination of Toshiba VB-46 and Corning 9782 filters). The duration of flash was 104 msec, and the total light-dark cycle was 417 msec. The intensity of the flash was 4.01 × 10^14 photons cm^–2 sec^–1. Delayed light emission was monitored by a photomultiplier (EMI 9558), the output of which was displayed on an oscilloscope (Tektronix 564). Other details are described in Reference 22.

Chlorophyll concentration was determined by the method of Mackinney (16).

Chemicals. HOQNO, CMU, BIMU, and CCP were dissolved in ethanol. PMA (practical grade, Eastman Kodak) was also dissolved in ethanol and used after filtration of undissolved materials. Final ethanol concentration in the cell suspension was less than 0.4%.

RESULTS AND DISCUSSION

Effect of Low Temperatures. In order to shift the steady state of redox level of electron transfer components, light-induced absorbance changes were measured at low temperature. Typical kinetics at 2.5°C are shown in Figure 1. The most remarkable is a large light-induced deflection seen in the Soret band of cytochrome f. This situation is clearly seen in a spectrum of total absorbance change in the aerobic state (Fig. 2), which indicates that by illumination cytochrome f reaches a more oxidized level than that at room temperature (Fig. 3A).

It should be noted that there is a broad peak around 470 nm.

Judging from its maximum, it might be attributed to the reduction of flavoprotein (ferredoxin-NADP reductase) or ferrredoxin or, more likely, their complex as reported in vitro (5, 6, 17–19, 23). Therefore, it may be concluded that at lowered temperatures the light-induced level of cytochrome f shifts to a more oxidized level. This may be due to the deceleration of the rate of dark oxidation of ferredoxin-flavoprotein and of the dark reduction of cytochrome f. A similar conclusion was obtained from the analysis of rates and steady state level of light-induced cytochrome f/oxidation in the red alga Porphyra in the temperature range of 1 to 34°C (21). A further attempt to confirm this flavoprotein change by fluorometry has been unsuccessful so far; no light-induced change of fluorescence intensity has been observed. One possible explanation for this is that the fluorescence excitation light used (380 or 460 nm) also activates photosystem I and induces electron flow, so that further excitation by red light gives no additional change of fluorescence from the flavoprotein (or its ferredoxin complex). Indeed, fluorescence of chlorophyll (720 nm) was detected to the same extent upon excitation by 380 nm as by 440 nm (unpublished data).

Another point to be noticed here is that a small but significant shoulder is seen around 430 nm in the spectrum of Figure 2. Aerobic light-off decay has a biphasic nature, as can be seen at
432 nm (Fig. 1). By separately plotting the amplitudes of rapid (half-decay = 1 sec) and slow (half-decay = 10 sec) phases of light-off recovery against wave length, we obtained two spectra which are attributable to cytochromes f and b, respectively (Fig. 4). The light-on kinetics in aerobic state also has two phases. Since the rapid and slow phases of the light-on change have similar spectral shapes to those of the light-off change, it may be concluded that cytochrome f is rapidly oxidized and reduced upon the start and cessation of illumination, respectively, while the b-type cytochrome is slower in both reactions. The kinetics of the anaerobic light-on change also has a biphasic nature (Fig. 1), which can be resolved into two spectra (Fig. 5). In this case, rapid light-on change is attributable to cytochrome b_{553}.

The light-off reduction of cytochrome f under these conditions (aerobic low temperature) was quite rapid, whereas the apparent rate of the light-induced oxidation was slow, representing the balance of rates of oxidation and reduction. As for the small shoulder at 430 nm, it is tentatively attributed to a b-type cytochrome, which might not be cytochrome b_{553} but a different one, probably 359. This possibility will be discussed later.

**Effect of HOQNO.** The effect of HOQNO on light-induced absorbance change of cytochrome b has been reported (10, 11, 13). A more detailed study of the effect of HOQNO at different concentrations was carried out. At considerably higher concentrations, oxygen- and light-induced oxidations of cytochrome b_{553} diminished, and light-induced reduction of this cytochrome was observed (Fig. 6). The kinetics of aerobic-anaerobic transition was remarkably affected by HOQNO, though only 20% of total oxygen uptake by respiration was blocked by the inhibitor. The difference in redox levels between the aerobic and anaerobic states was quite small in the transitory cycle (Fig. 6, left bottom). The steady state oxidation level of cytochrome f under illumination increased in the presence of HOQNO. It should be noted, however, that despite its striking effect on cytochrome b, HOQNO does not affect the light-induced kinetics of cytochrome f so much as could be expected if cytochrome b were the only photoreducing agent of cytochrome f. The difference spectrum of light-induced change in aerobic state in the presence of HOQNO (Fig. 3B) clearly shows that the site of HOQNO inhibition is between cytochrome f and b_{553}. Total changes of absorbance at different wave lengths and under different conditions plotted against concentration of HOQNO (Fig. 7) indicate a close relationship between photoreduction and oxidation of cytochrome b and that of cytochrome f and confirm the HOQNO site mentioned above. It should be remarked that some other factors which photoreduce cytochrome f more rapidly than cytochrome b should exist in this system.

**Effect of PMA.** Phenylmercuric acetate induces a large shift of redox level of cytochrome f during illumination to a more oxi-
dized state (13). Further study of this effect revealed that the concentration of PMA to cause this effect is quite critical (Fig. 8); higher concentrations of PMA usually induce unspecific inhibition of any type of electron transfer. The most important point, however, is that at the particular concentration, at which absorbance change at 475 nm at low temperature occurred no longer, the change at 422 nm reached its maximum. The inhibition curve of the light-off reduction rate of cytochrome f by PMA was parallel to that of the absorbance change at 475 nm (Fig. 8). This result would indicate that PMA inactivates the ferredoxin, flavoprotein, or their complex, and consequently photoreduction of cytochrome f is blocked and its redox level goes up to a highly oxidized state. This explanation also seems likely in considering the chemical nature of PMA as a strong sulphydryl blocking agent (25).

On the other hand, at this particular concentration of PMA, further addition of HOQNO caused the same effect as has been seen in untreated cells (Fig. 6, third row left, and Fig. 9C), i.e., a rapid photoreduction of cytochrome b$_{563}$ (Fig. 9D). PMA alone did not cause such an effect (Fig. 9B). This result indicates that PMA does not affect the photoreduction of cytochrome b but blocks that of cytochrome f. In other words, cytochrome b is photoreduced by a different pathway from that of cytochrome f; despite using the electrons derived from the same origin of photosystem I. In the pale green mutant, contribution of photosystem II can be ruled out.

Photosystem II of the Pale Green Mutant. Contribution of photosystem II to the light-induced change cited in the preceding sections of the present paper could be ruled out for the following reasons. (a) None of the photosystem II inhibitors, such as CMU, DCMU, or BIMU, has affected any light-induced changes of cytochromes. (b) No appreciable oxygen evolution was observed under red light (broad band) illumination. (c) No enhanced delayed light emission of chlorophyll, which is considered to be derived from the pigment of photosystem II (21), was observed upon addition of specific inhibitors (Fig. 10, right), in contrast with the much increased levels of delayed light emission by these inhibitors in wild type cells (Fig. 10, left). In another type of mutant strain (ac-206) lacking cytochrome f (9) but still maintaining oxygen evolution activity (1/3 of the wild type), the effect of these inhibitors was not observed. However, in this case, CCP blocked delayed light emission almost completely, as in the case of the wild type (Fig. 11). On the other hand, the effect of CCP in the case of the pale green mutant was much less in degree. Indeed, there was no significant level of delayed light emission from the uninhibited pale green mutant, whereas there was a low but significant level of delayed light emission from unpoisoned cells of ac-206 (Fig. 11).

Fluorescence emission spectra of the pale green mutant excited by 430 nm light have a small peak at 715 nm either in the presence or absence of CMU, and no difference in intensity. On the other hand, in the wild strain, CMU enhanced fluorescence intensity to twice the control level (unpublished observation).

Effect of HOQNO on Cytochrome b Kinetics. At low temperature in the aerobic state in the presence of HOQNO, the kinetics

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**Fig. 7.** Effect of HOQNO on the light-induced changes of cytochromes b and f, and aerobic-anaerobic transition of cytochrome b. Chlorophyll concentration, 8.5 µg/ml; optical path, 10 mm; temperature, 24°C. Cytochromes f and b were measured at 422 and 432 nm, respectively. Reference wave length was 445 nm. Open circles: Anaerobic light-induced oxidation of cytochrome b; triangles: aerobic-anaerobic transition (reduction) of cytochrome b; solid circles: light-induced reduction of cytochrome b in aerobic state; squares: aerobic light-induced oxidation of cytochrome f.

**Fig. 8.** Effect of PMA on cytochrome f and ferredoxin-flavoprotein. Chlorophyll concentration, 4.2 µg/ml; optical path, 10 mm; temperature, 2.5°C. Open circles: Light-induced steady state change of cytochrome f; closed circles: light-induced steady state change of ferredoxin-flavoprotein (measured at 475 nm with reference to 510 nm); triangles: rate of cytochrome f dark reduction. All the values shown here are relative to the value of the control experiment without inhibitor. All the measurements were done within 10 min after addition of the inhibitor.

**Fig. 9.** Effects of PMA and HOQNO on aerobic light-induced change of cytochrome b. Chlorophyll concentration, 5.7 µg/ml; optical path, 10 mm; temperature, 23°C. Measured at 432 nm with the reference of 445 nm. The shift of the redox level of cytochrome f by PMA during illumination was observed under the same conditions (see Fig. 8 and Ref. 13).
of the cytochrome $b$ change were different and complicated compared with those at room temperature: a rapid transient reduction followed by a slow oxidation and a small light-off change (Fig. 12). Although seemingly complicated, these kinetics can be resolved into two components. Subtracting the kinetics of the control (Fig. 12A) from that of the HOQNO experiment (Fig. 12B), kinetics with rapid light-on reduction and light-off oxidation similar to that of HOQNO-treated cells at room temperature can be obtained. That is to say, the kinetics are composed of two components; one is slowly oxidized and attributable to a slow factor of Figure 4 seen as a shoulder peak; the other is attributable to the same cytochrome rapidly photoreducible at room temperature (Fig. 9C), probably cytochrome $b_{553}$. Apparently, HOQNO does not affect the former, slow component; on the other hand, low temperature is also not very effective in slowing down the rapid component.

At room temperature in anaerobic state, HOQNO-treated cells gave another, different kinetics, as can be seen in Figure 6 (2nd from the left). These kinetics appear to have two different components: one is slowly oxidized upon illumination; the other, rapidly oxidized upon cessation of illumination. In explaining this, a combination of two types of kinetics could be postulated; one component is rapidly oxidized upon illumination and slowly reduced in the dark; and the other, rapidly reduced upon illumination and rapidly oxidized in the dark. The difference spectra thus resolved (Fig. 13) indicate a small but significant difference in absorbance maxima and shape (B and C, D and E). A tentative conclusion of this section is that in the pale green mutant, besides cytochrome $b_{553}$, there is another cytochrome $b$, presumably $b_{551}$, which is not susceptible to HOQNO. The actual content or activity of this cytochrome does not seem to be high in this particular mutant with deficient noncyclic electron transfer.

**Proposed Scheme of Electron Transfer.** Summarizing the results, we postulate a tentative scheme of photosynthetic electron transfer system in *C. reinhardi* as shown in Figure 14.

Essentially similar to our previous scheme (13), the present one has some new features. First is the postulation of two different
pathways of cyclic electron transfer operating in parallel. In this particular mutant, the “cytochrome b pathway” seems to be narrower than the “ferredoxin-flavoprotein pathway,” judging from the observation that HOQNO does not induce such large shift of photooxidized level of cytochrome f as observed in the presence of PMA or at low temperature. It is, however, to be investigated in the future whether it is also the case for normal photosynthetic algae and chloroplasts of higher plants. Secondly, the electron donor to cytochrome b430 is not ferredoxin-flavoprotein but something closer to photosystem I, for example, so-called “x”. Laser pulse excitation of the pale green mutant has shown that the half rise time of the cytochrome b563? photooxidation is less than 1 msec (10), indicating that the cytochrome might be located fairly close to a primary photoreductant of photosystem I. On the other hand, reduction of cytochrome b563 by NADPH2 was reported in spinach chloroplasts (4). However, its relative contribution to photoreduction of cytochrome b563 does not seem to be very significant in vivo in this particular mutant.

The present scheme is not inconsistent with that of Levine (15) and lends additional support for his scheme, which has resulted from a series of extensive studies with a number of mutant strains of C. reinhardtii. However, the possibility of another type of cyclic pathway mentioned here is also to be taken into consideration as a process in photosynthetic electron transfer in green plants and algae.

Photophosphorylation coupled to cyclic electron flow is commonly observed in isolated chloroplasts with redox dyes. Many observations have been accumulated in favor of the presence of cyclic photophosphorylation in vivo (see the review of Levine [15]). Though the present study does not give any idea on the sites of phosphorylation, it seems that at least one of the sites is located on the cyclic pathways, since it has been shown that the chloroplasts isolated from the pale green mutant have energy-coupling sites, which are not sensitive to CMU (3).

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LITERATURE CITED