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THE INFLUENCE OF CYTOCHROME b_{559} ON THE FLUORESCENCE YIELD OF CHLOROPLASTS AT LOW TEMPERATURE

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SUMMARY

The maximum light-induced fluorescence yield, F_M, of spinach chloroplasts at −196 °C was less when the chloroplasts were oxidized with ferricyanide prior to freezing; the minimum fluorescence yield, F_0, of the dark-adapted chloroplasts at −196 °C was unaffected. The ratio of the fluorescence yields, F_M/F_0, measured at 695 nm at low temperature was 4.5–5.0 for normal chloroplasts and 2.0–2.5 in the presence of ferricyanide. The oxidative titration curve of F_M followed a 1 electron Nernst equation with a midpoint potential of 365 mV and followed closely to the oxidation of cytochrome b_{559}. The photoreduction of C-550 at low temperature was the same at all redox potentials over the range of 200–500 mV. It is suggested that a relatively strong oxidant associated with the water-splitting side of Photosystem II, possibly the primary electron donor, can quench chlorophyll fluorescence of Photosystem II as well as the primary electron acceptor.

INTRODUCTION

Knaff and Arnon[1,2] showed that irradiation of chloroplasts at liquid nitrogen temperature causes the photooxidation of cytochrome b_{559} and the photoreduction of a new component C-550. Erixon and Butler[3] demonstrated that C-550 was the same as, or was closely related to, the primary electron acceptor of Photosystem II by showing a one-to-one correspondence between absorbance changes of C-550 and fluorescence yield changes at low temperature. They also demonstrated that photooxidation of cytochrome b_{559} at low temperature requires the concomitant photoreduction of C-550 (chemical reduction of C-550 prior to freezing blocks the photooxidation of cytochrome b_{559} because of the absence of the electron acceptor[3]) which confirms the primary role of C-550 as the electron acceptor and further establishes that cytochrome b_{559} is oxidized by the same photoreaction that reduces C-550. Cytochrome b_{559}, however, does not appear to be the primary electron donor, even though the reaction occurs at low temperature, because chemical oxidation of cytochrome b_{559} prior to freezing does not limit the photoreduction of C-550 (refs 2, 4). The primary

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photoact reduces C-550 and oxidizes the primary electron donor (assumed to be a reaction center chlorophyll, P_{680}) and the oxidized primary donor subsequently oxidizes cytochrome b_{559} in a dark reaction at low temperature.

The correspondence between the redox state of C-550 and the fluorescence yield of the chloroplasts was established in low temperature measurements under a variety of experimental conditions[3] (at room temperature influences other than the primary photochemistry of Photosystem II, *e.g.* membrane potentials or ion gradients, may affect C-550 and fluorescence yield and obviate the meaningful correlations which result from the primary photochemical events[5]). Redox titration curves for the light-induced fluorescence yield changes and for the light-induced absorbance changes of C-550 measured in chloroplasts at −196 °C were identical over the redox potential region from +100 to −150 mV where C-550 was chemically reduced[3]. However, preliminary observations made during experiments on the redox properties of cytochrome b_{559} (ref. 6) suggested that the light-induced fluorescence yield changes were less at higher potentials where cytochrome b_{559} was oxidized even though the photoreduction of C-550 was unimpaired. Those observations are confirmed and extended in the present paper. At redox potentials where cytochrome b_{559} is reduced the fluorescence yield at low temperature may increase 5-fold during irradiation whereas at higher potentials where cytochrome b_{559} is oxidized the fluorescence yield may increase only by a factor of 2. These results reveal that the maximum fluorescence yield obtainable at −196 °C by reducing C-550 is determined by the redox state of a component, possibly the primary electron donor, on the oxidizing side of Photosystem II.

METHODS

Spinach chloroplasts[7] were suspended at 10 μg chlorophyll/ml in 40 ml of buffer medium (0.05 M Tris–HCl, 0.01 M NaCl, 0.4 M sucrose, pH 7.8) and potassium ferrocyanide was added to 0.1 mM. The addition of the ferrocyanide lowered the redox potential of the medium somewhat and stabilized the potential readings. The suspension was stirred gently in an open beaker near 0 °C in the dark. Aliquots (1–10 μl) of oxidant (0.5 M potassium ferricyanide) or reductant (0.5 M potassium ferrocyanide or 1 M neutralized sodium ascorbate) were added in the oxidative and reductive titrations. The redox potential of the medium was measured with a combination Pt, Ag–AgCl electrode (Instrumentation Lab 15010). Aliquots of the chloroplast suspension were taken at known redox potentials and frozen in a 35 mm × 15 mm × 3 mm cuvette to −196 °C in darkness.

Fluorescence excited with a steady weak (20 μW/cm²) monochromatic (630 nm) beam was measured with a 500-mm Bausch and Lomb monochromator (grating of 1200 lines/mm blazed at 500 nm) and a 9558C EMI phototube. A Corning 2-64 (2030) cut off filter was placed in front of the phototube. For measurements of the time course of fluorescence changes induced by the measuring beam, the monochromator was set at 695 nm with an 8.3-nm pass band and the output of the amplifier was taken directly to a recorder. For measurements of emission spectra, the monochromator with a 5.0-nm pass band scanned the spectrum and the signal from the amplifier was taken to a Fabritek 1072 computer which was indexed to the wavelength drive of the monochromator to accept readings every 0.5 nm. Up to four emission

spectra could be stored in the computer and these spectra or difference spectra between a given pair could be played out on an X–Y recorder. The fluorescence spectra reported here were measured four times and averaged to improve the signal-to-noise ratio. Absorption difference spectra were measured by methods described previously[3].

RESULTS

The time course of fluorescence measured at 695 nm during irradiation at −196 °C with 630-nm light is shown in Fig. 1 for chloroplasts frozen at different redox potentials (247, 369 and 439 mV). The intensity of fluorescence of the dark adapted chloroplasts, F_0, is independent of the redox potential but the maximum level of fluorescence, F_M, reached during irradiation at low temperature is markedly influenced; the fluorescence yield increased about 4.5-fold during irradiation of the

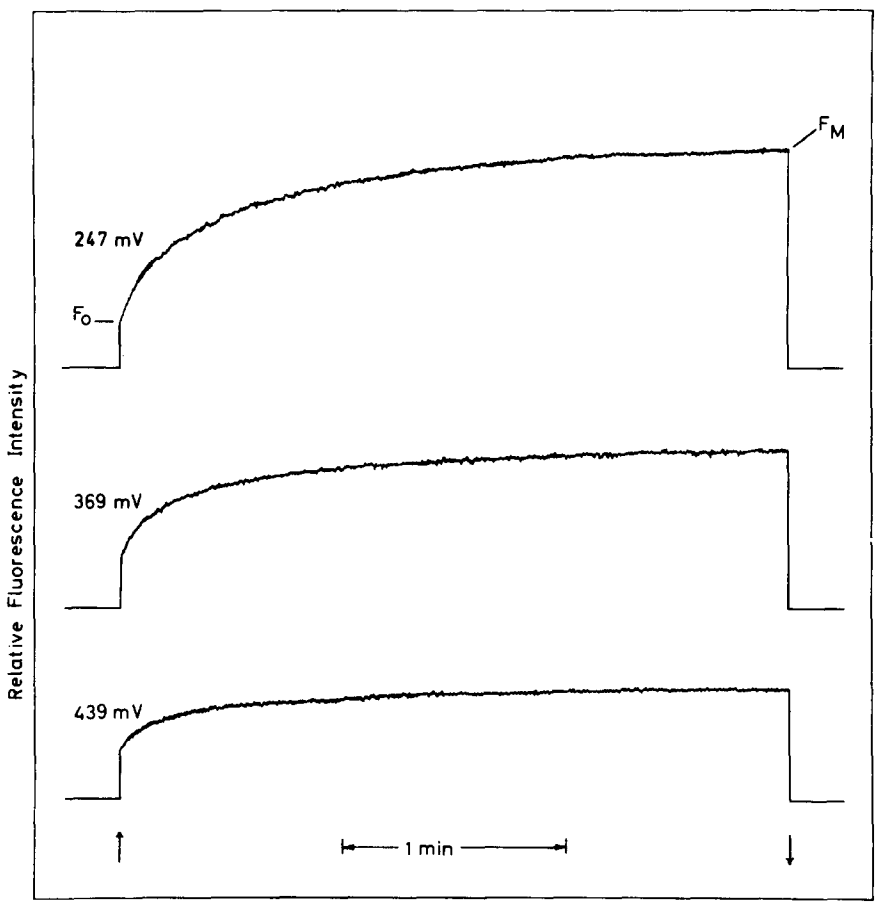


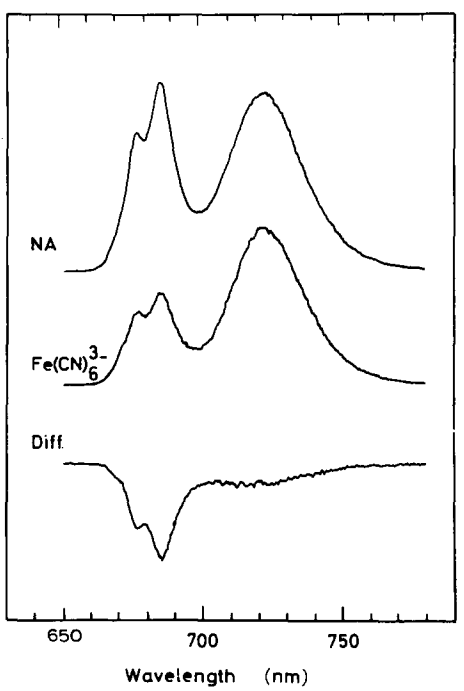
Fig. 1. Time course of fluorescence excited at 630 nm (20 μW/cm^2) and measured at 695 nm from dark adapted spinach chloroplasts at −196 °C. Excitation light on at upward arrow, off at downward arrow. Prior to freezing the redox potential of the chloroplast medium was set to the indicated values with mixtures of ferricyanide and ferrocyanide.

sample frozen at 247 mV, about 3-fold with the 369-mV sample and 2.5-fold with the 439-mV sample. Even with samples oxidized at 500 mV and frozen in darkness the fluorescence yield always increased at least 2-fold during irradiation.

The fluorescence emission spectra at −196 °C of a sample of chloroplasts frozen with no additions and of a sample frozen after the addition of 1 mM ferricyanide are shown in Fig. 2. Both of these spectra represent the emission from the high fluorescence yield state, F_M. The difference between the two emission spectra shows that the quenching imposed by oxidizing conditions is limited to the 686- and 695-nm emission bands associated with Photosystem II. The light-induced fluorescence yield changes are also limited to the 686- and 695-nm bands; the large 720-nm emission band associated with Photosystem I remaining constant[8,9].

Titration curves for the fluorescence of variable yield at −196 °C are shown in Fig. 3. The redox potential of the chloroplasts was established and measured at 0 °C prior to freezing. The circles represent the experimental values of F_0 and F_M at various redox potentials; the dashed curves are theoretical plots of the Nernst equation for one electron transitions with midpoint potentials of 365 mV for the oxidative titration (open circles) and 345 mV for the reductive titration (closed circles).

The titration curves for the light-induced fluorescence yield changes follows the oxidation and reduction of cytochrome b_{559}. The light-induced absorbance changes



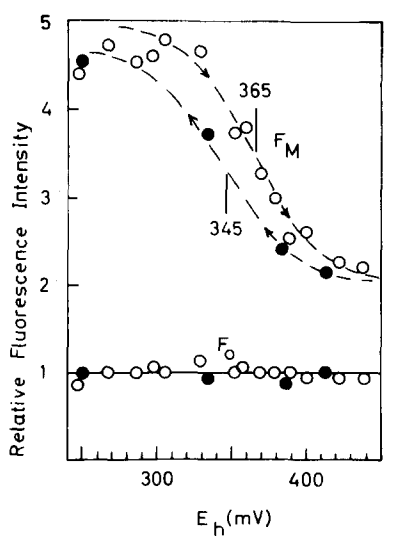


Fig. 2. Emission spectra of spinach chloroplasts at −196 °C frozen with no addition (NA) and with 1 mM ferricyanide and the difference spectrum between the two emission spectra.

Fig. 3. Redox titration curves of F_0 and F_M measured at −196 °C (see Fig. 1) as a function of the redox potential of the chloroplast medium before freezing. ○, values obtained in an oxidative titration; ●, values obtained in the reverse reductive titration. – – – –, plots of 1 electron Nernst equations with midpoint potentials of 365 and 345 mV.

of chloroplasts frozen to −196 °C at various redox potentials are shown in Fig. 4A. A normal photooxidation of cytochrome b_{559} and photoreduction of C-550 are demonstrated by the sample frozen at 227 mV. As the redox potential of the chloroplast medium becomes more positive cytochrome b_{559} is chemically oxidized so that less can be photooxidized. Difference spectra between samples frozen at different redox potentials in Fig. 4B show that cytochrome *f* (split α band with maxima at 548 and 552 nm) and a part of the cytochrome b_{559} (maximum at 556 nm) are oxidized on going from 227 to 313 mV. The rest of the cytochrome b_{559} is oxidized on going more positive from 313 to 485 mV (most of the oxidation was complete at 381 mV). The difference spectrum between the two light-induced difference spectra at 485 and 227 mV of Fig. 4A shows that only the photooxidation of cytochrome b_{559} is affected in this redox potential region. In confirmation with earlier measurements[4,6] the photoreduction of C-550 is independent of the oxidation state of cytochrome b_{559}.

It was noted that the light-induced fluorescence yield increase of Tris-washed chloroplasts at −196 °C was about half of that of normal chloroplasts. Since the Tris

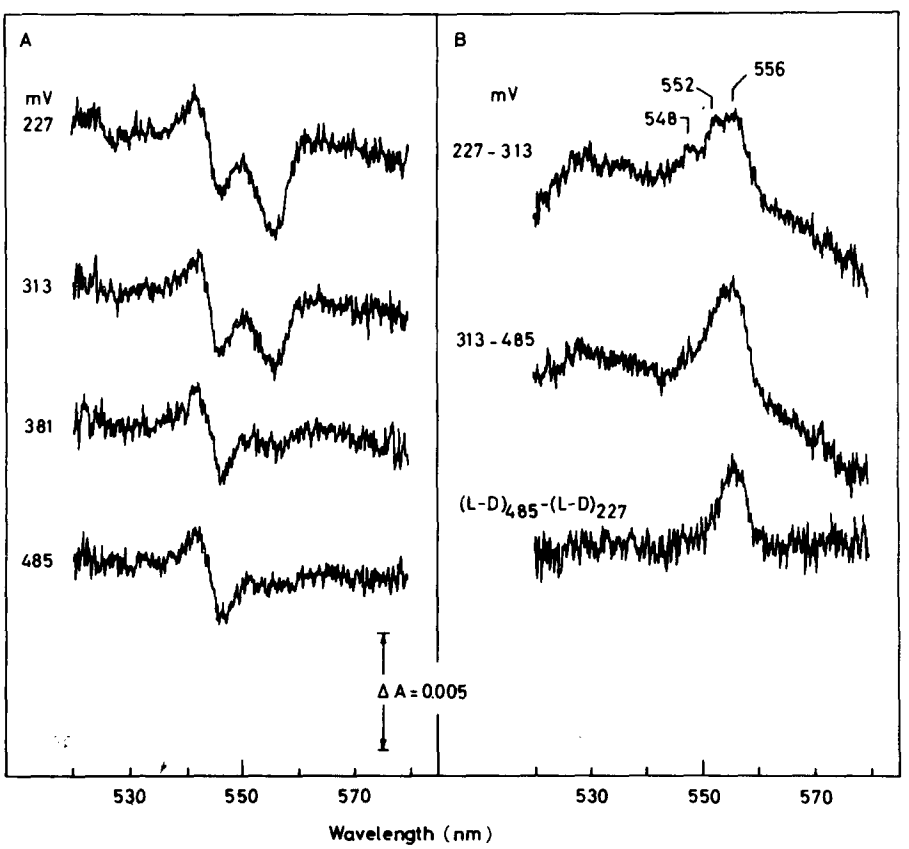


Fig. 4. A. Light-*minus*-dark difference spectra due to irradiation of the chloroplasts at −196 °C with actinic red light for 30 s. The redox potentials indicated were established in the chloroplast medium prior to freezing. B. Difference spectra between unirradiated samples frozen at the redox potentials indicated and the difference spectrum between the two light-*minus*-dark difference spectra taken at 227 and 485 mV shown in Part A.

washing treatment was shown to modify the normal high potential cytochrome b_{559} to a lower potential, autooxidizable form[6] we suspected that the low fluorescence of variable yield of Tris-washed chloroplasts at −196 °C was due to the cytochrome b_{559} being oxidized. This was confirmed by showing that the fluorescence of variable yield of Tris-washed chloroplasts at low temperature could be restored to the normal level if the chloroplasts were suspended with 1 mM ascorbate prior to freezing. Redox titration curves of F_M measured with Tris-washed chloroplasts were shifted markedly to lower potentials and spread out over a larger potential range in accordance with the redox titration curves of cytochrome b_{559} in Tris-washed chloroplasts[6].

The marked influence of oxidizing conditions on the F_M level was not found in measurements at room temperature. Addition of 1 mM ferricyanide to chloroplasts at room temperature in high light intensities and in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to prevent electron transport generally caused the F_M level to decrease slightly but by never more than 10%.

DISCUSSION

We assume that the primary electron donor in Photosystem II is the reaction center chlorophyll, P_{680}, analogous to the P_{700} of Photosystem I, in accordance with the proposals of Floyd *et al.*[10]. This is the same chlorophyll denoted chlorophyll a_{II} and measured near 690 nm in flash-induced bleaching experiments by Döring *et al.*[11,12]. (The possible relationships of chlorophyll a_{II} as measured by Döring *et al.* and of P_{680} as measured by Floyd *et al.*[10] to the primary photochemistry of Photosystem II have been discussed in detail elsewhere[13].) Cytochrome b_{559} appears to be a part of the Photosystem II reaction center[14] and we assume it can be oxidized at low temperature by the oxidized P_{680}. Under normal conditions where cytochrome b_{559} is reduced we would represent the low-temperature light-induced reactions as:

$$\text{cytochrome } b_{559}\cdot P_{680}\cdot C\text{-}550 \xrightarrow{h\nu} \text{cytochrome } b_{559}\cdot P^{+}{}_{680}\cdot C^{-}\text{-}550$$

with an immediate subsequent dark reaction:

$$\text{cytochrome } b_{559}\cdot P^{+}{}_{680}\cdot C^{-}\text{-}550 \longrightarrow \text{cytochrome}^{+}\ b_{559}\cdot P_{680}\cdot C^{-}\text{-}550$$

the final product resulting in a high fluorescence yield from Photosystem II. However, when cytochrome b_{559} is oxidized prior to freezing the photoreaction is:

$$\text{cytochrome}^{+}\ b_{559}\cdot P_{680}\cdot C\text{-}550 \xrightarrow{h\nu} \text{cytochrome}^{+}\ b_{559}\cdot P^{+}{}_{680}\cdot C^{-}\text{-}550$$

In this case $P_{680}{}^{+}$ may be stabilized at low temperature and result in a lower yield of Photosystem II fluorescence. On the other hand $P_{680}{}^{+}$ might oxidize another component when cytochrome b_{559} is already oxidized and this alternative secondary donor might quench fluorescence. The lower value of F_M under oxidizing conditions at −196 °C does not appear to be due to increased energy transfer from Photosystem II to Photosystem I since the 720-nm emission band does not show a concomitant increase.

The results demonstrate that the yield of chlorophyll fluorescence from Photosystem II is affected by the oxidation state of a component associated with the

oxidizing side of Photosystem II. The primary electron acceptor of Photosystem II (C-550 or Q) also acts as a quencher of fluorescence and that quenching is relieved when the acceptor is reduced. The component on the oxidizing side, however, appears to exert an independent quenching which limits the fluorescence yield obtainable when the acceptor is reduced. It is questionable whether the strong oxidant assumed to be responsible for this quenching attains sufficient concentration to exert appreciable quenching under normal conditions at room temperature.

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REFERENCES

1 D. B. Knaff and D. I. Arnon, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 956.
2 D. B. Knaff and D. I. Arnon, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 963.
3 K. Erixon and W. L. Butler, *Biochim. Biophys. Acta*, 234 (1971) 381.
4 K. Erixon and W. L. Butler, *Photochem. Photobiol.*, 14 (1971) 427.
5 W. L. Butler, *FEBS Lett.*, 20 (1972) 333.
6 K. Erixon, R. Lozier and W. L. Butler, *Biochim. Biophys. Acta*, 267 (1972) 375.
7 T. Yamashita and W. L. Butler, *Plant Physiol.*, 43 (1968) 2037.
8 N. Murata, *Biochim. Biophys. Acta*, 162 (1968) 106.
9 S. Okayama and W. L. Butler, *Plant Physiol.*, 49 (1972) 769.
10 R. A. Floyd, B. Chance and D. DeVault, *Biochim. Biophys. Acta*, 226 (1971) 103.
11 G. Döring, H. H. Stiehl and H. T. Witt, *Z. Naturforsch.*, 22b (1967) 639.
12 G. Döring, G. Renger, S. Vater and H. T. Witt, *Z. Naturforsch.*, 24b (1969) 1139.
13 W. L. Butler, *Biophys. J.*, 12 (1972) in the press.
14 L. P. Vernon, E. R. Shaw, T. Ogawa and D. Raveed, *Photochem. Photobiol.*, 14 (1971) 343.