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Fluorescence of the reaction centre of photosystem II in cells of green alga *Chlamydomonas reinhardtii*

N.N. Lebedev and Irina V. Barskaya

A.N.Bakh Institute of Biochemistry, Moscow 117071, USSR

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Cells of green alga *Chlamydomonas reinhardtii*, mutant ASS66, which only exhibit the spectra and polypeptide composition of photosystem II, possess a weak fluorescence at about 673 nm along with two main emission bands at 686 and 698 nm, after cultivation in the dark. The 673 nm fluorescence exhibits an excitation spectrum similar to the absorbance of the isolated reaction centre of photosystem II (D1/D2 protein complex). It contains chlorophyll *a*, pheophytin *a*, and carotenoid bands with the chlorophyll:pheophytin ratio of about 3. Under illumination, the intensity of the 673 nm emission band decreases with concomitant increase in the emission band at 698 nm, which is thought to belong to the core antenna of photosystem II. Under illumination, in the presence of dithionite (strong reducing conditions), the relative intensity of 673 to 686 nm emission does not change, but the intensities of the pheophytin bands in its excitation spectrum were reduced two-fold. That the relative intensity of the 673 nm band in the emission spectrum under illumination in the presence of dithionite does not change indicates that it originates from an emitter other than pheophytin (possibly reaction centre chlorophyll *a*).

Pheophytin; Reaction center 2; Fluorescence; Green alga

1. INTRODUCTION

Pheophytin participation in primary charge separation in the reaction centre of PS II was shown in 1977 [1] and then confirmed by others [2,3]. But up to now there are no data on the mechanism of Pheo formation or the insertion into the RC apoprotein in vivo. For some time RC2 was thought to be incorporated in CP, emitting at 698 nm [4,5]. Indeed, the isolated CP47 protein with emission at about 694 nm possesses RC2 activity, as was revealed by difference absorption spectra. But, two years ago a new preparation was obtained which was composed of D1, D2 and cytochrome *b*-559 proteins and possessed RC2 activity [6–8]. This preparation exhibited an emission maximum at 684 nm and a shoulder at ~675 nm [7,8]. But the same emission was not detected in living cells, as the 686 nm emission band of whole cells is thought to belong to CP43 [4,5,8].

In the low-temperature fluorescence spectrum of a cyanobacterium, *Gloeotrichia raciborski*, we detected an emission band at about 673 nm, which exhibits an excitation spectrum similar to the absorbance reported for the isolated RC of PS II [9]. The increase of this emission in the dark follows the kinetics of the formation of D1 protein. Under illumination this band quickly transforms into the 698 nm emission which is known to belong to the core antenna of PS II. Thus, the 673 nm emission has features of the reaction centre of PS II, detached from its internal antennae. In the present study we tried to detect the RC2 emission in cells

Correspondence address: N.N. Lebedev, A.N. Bakh Institute of Biochemistry of the Academy of Sciences of the USSR, Leninsky prospect 33, Moscow 117071, USSR

Abbreviations: Car, carotenoid(s); Chl, chlorophyll; CP, chlorophyll-protein complex; CPLH, light-harvesting chlorophyll-protein complex; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; Pheo, pheophytin; PS, photosystem; RC, reaction centre; SDS, sodium dodecyl sulfate

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of another photosynthetic organism, a green alga. We used the unicellular green alga *Chlamydomonas reinhardtii*, mutant ASS66, which is devoided of CPLH and PS I [10,11]. This allowed us to exclude a possible interference of the RC fluorescence with the emission of CPLH at about 681 nm and to carry out a study on the simplest system, without additional CPs, except that of PS II. We also checked with dithionite, the known reductant of Pheo in RC2 [1], the redox activity of Pheo, which was observed in the excitation spectrum of the 673 nm emission.

2. MATERIALS AND METHODS

The green alga *Chlamydomonas reinhardtii*, mutant ASS66, was cultivated on a solid agarose medium with Na acetate [10,11,13] under continuous illumination with 'white' luminescent lamps (500 lx at the surface of Petri dishes) and at a temperature of 22–24°C. After about 100 h of cultivation (at the phase of logarithmic growth), the alga was transferred into darkness for 20 h and then used for measurements. For sample preparations cells were gently harvested from the agar, suspended in a liquid medium with 0.2% Na acetate and 0.3 M phosphate buffer, pH 7.3, and spreaded on a Synpor-3 membrane filter with a pore diameter of 1.5 μm at a Chl concentration of about 1 $\mu g \cdot cm^{-2}$. All these procedures were undertaken in the dark. In order to check the RC redox activity, the dark adapted cells suspended in the same liquid medium but with about 1 mg/ml solid Na dithionite were illuminated with incondensed white light (100 $W \cdot m^{-2}$) [12].

The low-temperature (77 K) fluorescence emission and excitation spectra were recorded with home-made equipment, described elsewhere [9,13], except that a 100 W stabilized tungsten lamp was used as the light source.

3. RESULTS AND DISCUSSION

The low-temperature fluorescence spectrum of the ASS66 mutant of *Chlamydomonas reinhardtii* cultivated in the light consists of two main emission bands at 686 and 698 nm (fig.1). Both bands have excitation spectra without Chl *b* maxima at about 470 nm (fig.2) which belong to the pigment, incorporated in CPLH [13]. However, there are pronounced Car maxima at about 464 and 498 nm, which are specific for the pigment, incorporated in CPa [5]. The band at ~545 nm, which may belong to a Pheo, is also seen [1,8]. So, the spectra show that the mutant mainly incorporates internal antennae of PS II without any other CPs of the photosynthetic membrane. Indeed, nondenaturating SDS-PAGE shows that this mutant is devoid of

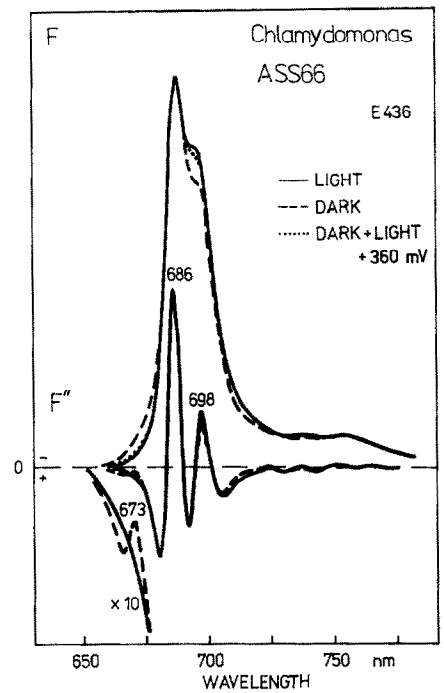

Fig.1. Low-temperature fluorescence emission spectra of *Chl. reinhardtii*, mutant ASS66, cells, before (——) and after (– – –) cultivation in the dark. Emission spectrum for the dark-adapted sample was measured before (– – –) and after (···) additional illumination for 20 min. Excitation at 436 nm.

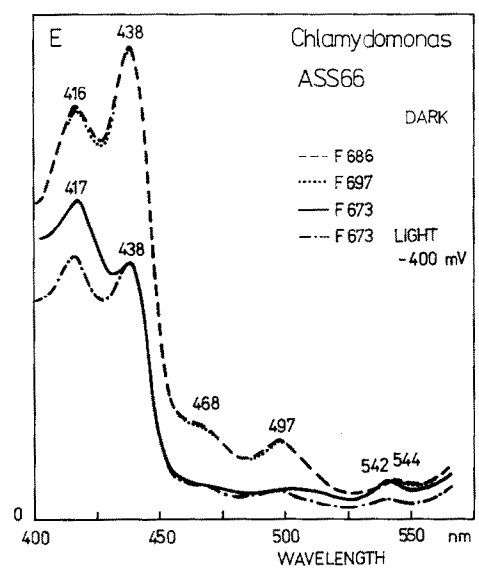

Fig.2. Excitation spectra for the low-temperature emission bands at 673 (—·—), 686 (– – –) and 698 nm (···) of dark-adapted *Chl. reinhardtii*, mutant ASS66, cells. Excitation spectrum for the emission at 673 nm was measured before (——) and after (-----) dithionite addition and illumination.

CPLH1–CPLH3, as well as CPLHO and CP1 [10,11]. Our study on its polypeptide composition also confirms this result (Lebedev et al., to be published). There are no pronounced differences in excitation spectra of the 686 and 697 nm bands (fig.2) indicating an efficient energy exchange between the CPs.

The dark cultivation of the mutant leads to the development of a new emission at about 673 nm, which is more pronounced in the second derivative of the spectrum (fig.1). At the same time the relative intensity of the 698 nm band decreases, similar to the changes observed for cyanobacterium cells cultivated in the dark [9]. The excitation spectrum of the 673 nm band shows an intensity increase of Pheo bands at 545 and 513 nm, as well as at 417 nm. The intensity of the Car bands in the excitation spectrum of the 673 nm fluorescence somehow diminished. The development of the new band in the dark is rather slow and completed in several hours. Under illumination it disappears with concomitant increase of the 698 nm emission (fig.1).

If one assumes, that the band at 438 nm mainly belongs to Chl and that the one at 417 nm belongs to both Chl and Pheo, and takes into account the molar extinction coefficients of the monomeric pigments [14,15], then the relative contribution of these pigments to the excitation spectrum of 673 nm emission can be calculated [9]. The results show the ratio to be about 3, which could be related to Chl dimer, Chl monomer and Pheo monomer in the photochemical active branch of D1/D2 protein [6,7] if there is a full energy exchange among the pigments. But it is also possible that there is an additional band (for example of a cytochrome) in this spectral region. Some features in the 500–600 nm region of the excitation spectrum prove this possibility (see below).

If Pheo maxima in the excitation spectrum of the 673 nm band belong to that of the RC2 pigment, they should be diminished under illumina-

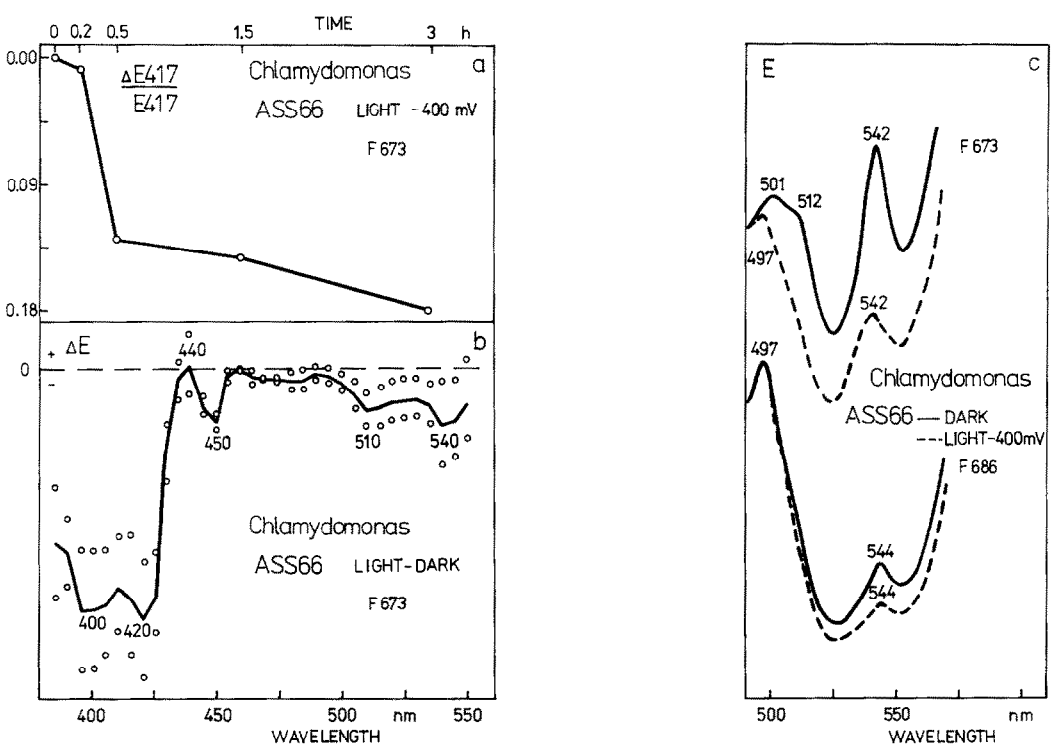

Fig.3. Time course for intensity changes at 417 nm in the excitation spectrum (a) and difference excitation spectrum (b) for the 673 nm emission band under illumination in the presence of dithionite of dark-adapted cells of *Chl. reinhardtii*, mutant ASS66. (c) A part of the excitation spectra for 673 nm and 686 nm emission before and after illumination with dithionite (expanded scale).

tion in the presence of dithionite [1]. Indeed, dithionite addition to the sample and illumination leads to a great decrease of the Pheo bands in the excitation spectrum (fig.2). But illumination does not considerably change the ratio of 673 to 686 nm emission band despite of the overall diminishing of the fluorescence.

As dithionite is a self-oxidizable compound, it is hard to determine directly its concentration in the cells. In order to be sure that the reaction was completed after its addition we measured the time course of the disappearance of the Pheo band after dithionite addition and illumination. There was a lag phase of about 10 min, possibly due to dithionite penetration into the cells, then the reaction was 75% complete in about 0.5 h and continued rather slowly during the subsequent 3 h (fig.3a). The difference excitation spectrum shows (fig.3b) that dithionite addition and illumination results in the disappearance of all three Pheo bands at ~420, 510 and 540 nm and the development of a new one at ~440, which could belong to a reduced pigment.

The maxima at 513 and 542 nm in the excitation spectrum of 673 nm fluorescence remaining after illumination in the presence of dithionite (fig.3c) indicate that despite saturation of the reaction, not all Pheo had disappeared. The amount of change at 417 nm (fig.3a) also indicates reduction of about 1/6 of all RC pigments. Using the same extinction coefficients as in previous calculations, the amount of disappearing Pheo was found to be about 1/2 of all Pheo. The same was true for excitation spectra of the other two emission bands (at 686 and 698 nm). Thus the results are in good agreement with the conclusions that (i) Pheo, which could be seen in the fluorescence excitation spectra of all three emission bands, is that from RC2, and (ii) the emission at 673 nm could belong to RC2 in vivo. As the relative intensity of the 673 nm band in the emission spectrum does not change after illumination under strong reducing conditions, the fluorescence at 673 nm probably originates from the RC2 pigment other than Pheo: possibly monomeric Chl *a*.

In order to determine the Pheo/Chl ratio in the excitation spectrum of the internal antennae of PS II (emission at 686 and 698 nm), we used the same extinction coefficients, as described above. The results show it to be about 1:30. This result is also in good agreement with the more precise determination of the Pheo/Chl ratio in the cells of ASS66 mutant by HPLC and the fact that about half of all Pheo that could be detected in the ASS66 mutant by HPLC are photochemically active [16].

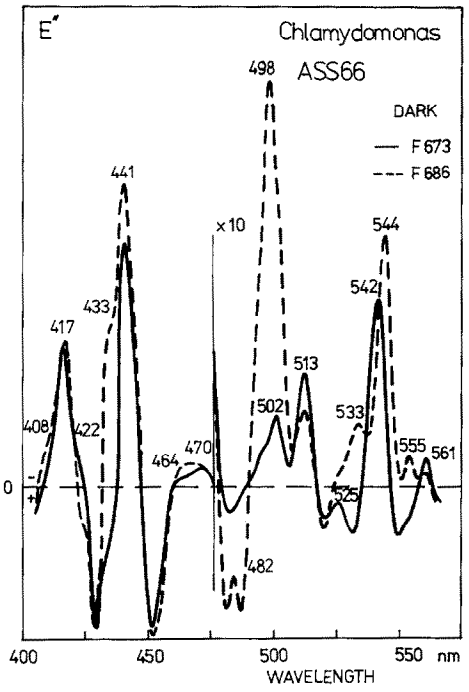

Fig.4. The second derivative of the excitation spectrum for 673 nm (——) and 686 nm (---) emission bands of dark-adapted *Chl. reinhardtii*, mutant ASS66, cells.

For more detailed spectral characterization of the RC and of the internal antennae of PS II in the photosynthetic membrane, we measured the second derivative of their fluorescence excitation spectra (fig.4). Such spectra indicate the participation of at least two carotenoids (four bands in the 440–500 nm region) and several additional bands in the 510–560 nm region, which are close to the absorbance of a reduced cytochrome. The difference between excitation spectra for 673 and 686 nm emission bands is mainly due to some changes in the carotenoid composition, the shift of the Pheo band from 545 to 542 nm and the disappearance of the bands at ~535 and 555 nm. The difference in Car composition for the internal antennae and RC of PS II is not surprising as they are known to differ in Car content after isolation

[8]. The origin of the shift of Pheo maxima in the excitation spectrum of 673 nm emission is not clear yet, but it is quite similar to the well known Pheo band shift in PS II absorption spectrum after quinone reduction [1]. The bands at 535 and 555 nm, which disappear in the excitation spectrum of 673 nm band in the dark (fig.4), appear in it again under illumination in the presence of dithionite. The position and sensitivity of these bands to reducing conditions proves their cytochrome origin [17], but additional measurements are necessary to confirm this possibility.

The results obtained show that the emission at 673 nm, which could be detected in the low-temperature fluorescence spectrum of green alga cells after incubation in the dark belongs to the RC2, detached from the internal antenna. The origin of such detachment is not clear yet, but it could be related to the RC activity or biogenesis.

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