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Origin of the 77 K variable fluorescence at 758 nm in the cyanobacterium *Spirulina platensis*

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Fluorescence emission spectra of cells and membranes of the cyanobacterium *Spirulina platensis* cooled in the dark to 77 K exhibit an intense Photosystem (PS) I band at 756–759 nm (F758). The presence of a 735 nm band in the excitation spectrum with the same half-band-width (32 nm) as F758 indicates that F758 originates from chlorophyll (Chl) *a* absorbing at 735 nm. The absorption of this chlorophyll form (Chl_{735}^{758}) in the red region is about 5%. Monochromatic illumination of the sample frozen in the dark causes an accumulation of P-700$^+$ which is accompanied by a bleaching of F758 with the rate proportional to the intensity of the exciting beam; the kinetics of F758 photobleaching and that of P-700 photooxidation are identical. Approx. 30–40% of the initial level of F758 is recovered with biphasic kinetics after 30 min dark adaptation at 77 K. The rate constants obtained for F758 recovery are similar to those of P-700$^+$ dark reduction at 77 K. The F758 band disappears completely in the presence of ferricyanide but is independent of the presence of dithionite or ascorbate. If the sample is frozen in strong light in the presence of dithionite, F758 remains at its maximal level. Redox titration shows that F758 intensity has the same midpoint potential as P-700. We conclude that the fluorescence quantum yield of F758 is proportional to the concentration of P-700. The light-induced F758 quenching is suggested to be due to the energy migration from Chl_{735}^{758} to the cation radical of P-700.

Introduction

At room temperature, Chl *a* fluorescence from green plants emanates mainly from PS II, the yield of which is influenced by photochemical and non-photochemical quenching mechanisms [1–3]. The PS I photochemistry oxidizes the primary electron acceptor of PS II via the electron transport chain and quenches the PS II variable fluorescence. Only a small part of the variable fluorescence associated with PS I photochemistry is observed in PS I particles at room temperature [4–9]. Different mechanisms for the origin of variable fluorescence of PS I have been suggested [4,7]. For digitonin-isolated PS I particles (Chl : P-700 is 80–140), the light-induced accumulation of P-700$^+$ enhances the fluorescence yield of PS I antenna Chl at $\lambda > 710$ nm [4,6,9]. In highly enriched PS I particles obtained by diethyl ether extraction of lyophilized samples (Chl : P-700 is 8–10), the formation of P-700$^+$ has been shown to quench fluorescence at 694 nm [7]. When PS I secondary electron acceptors are prereduced by dithionite, the illumination of particles increases the fluorescence yield as a result of light-induced reduction of primary acceptors [5–8]. The different types of observation may be due to the state of the antenna pigments and their interaction with P-700, which in turn may depend on the Chl : P-700 ratio and temperature.

It is not clear, however, why the redox transformations of PS I reaction centers are accompanied by small changes in the fluorescence yield, whereas the variable

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Abbreviations: PS, Photosystem; P-700 and P-700$^+$, primary electron donor of PS I and its cation radical; Chl, chlorophyll; Chl_{735}^{758}, chlorophyll *a* with absorption maximum at 735 nm and fluorescence maximum at 758 nm; F758, fluorescence band with maximum at 758 nm; F_i, F_{ss}, initial and steady state fluorescence levels after the illumination is switched on; $\Delta F = F_i - F_{ss}$, variable fluorescence; $A_0A_1F_XF_BF_A$, acceptors of PS I; PMS, phenazine methosulfate.

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fluorescence of PS II and Photosystem of photosynthetic bacteria exceeds 2–3-times the initial yield upon the trap closure. Such type of behavior of PS I variable fluorescence indicates a special organization of PS I reaction centers and the interaction of P-700 with the antenna Chl.

Variable fluorescence at low temperature is related mainly to the electron transport within the reaction center. At 77 K a prominent fluorescence band at 720–740 nm in chloroplasts and algae emitted by the PS I antenna predominates in the emission spectrum (for reviews see Ref. 10). In contrast to PS II and Photosystem of photosynthetic bacteria at 77 K, in green plants no $P\text{-}700^+$-dependent variable fluorescence at 730 nm was found at 77 K [11–15].

For the cyanobacterium *Pseudanabaena*, an unusual 77 K fluorescence band at 750 nm (F750) was observed by Duval et al. [16,17]. The light-induced decrease of the F750 was supposed to be associated with back-reactions reducing $P\text{-}700^+$ [17]. These authors also postulated that F750 is emitted by a Chl *a* form absorbing at 710 nm. To show the dependence of the long-wavelength variable fluorescence on the redox state of P-700, we made a comparative investigation of F758 and P-700 at 77 K in the cyanobacterium *Spirulina platensis*. We demonstrate that the light-induced decrease of F758 at 77 K is due to quenching by $P\text{-}700^+$, and the Chl *a* absorbing at 735 nm emits F758. The light-induced F758 quenching is partially reversible in the dark, while the rate constants of dark reversibility of F758 and of $P\text{-}700^+$ dark reduction are similar in magnitude. We postulate that F758 quenching results from energy migration from Chl_{735}^{758} to $P\text{-}700^+$.

Material and Methods

Material

Cultures of *Spirulina platensis* were grown in the Zarrouk medium at $25 \pm 2\,^\circ C$ under continuous illumination ($\simeq 20\ W\ m^{-2}$) as described earlier [18,19]. The cells were harvested at the late log phase (12 days). For some experiments, we have used batch cultures of other cyanobacteria (*Anacystis nidulans, Phormidium uncinatum, Nostoc muscorum*) and the red alga *Cyanidium caldarium* grown under continuous illumination in their respective media [20–22].

Preparation of Spirulina membrane fragments

Washed cells of *Spirulina* were resuspended in 25 mM Hepes-NaOH buffer (pH 7.5) containing 20 mM NaCl. They were disrupted in a French pressure cell at $5\,^\circ C$ and then centrifuged at $9000 \times g$ for 5 min; the supernatant was again centrifuged at $50000 \times g$ for 45 min. The pellet was resuspended in 2–3 ml of Hepes-NaOH buffer (pH 7.5) at a Chl *a* concentration of 3–4 mg/ml.

Chl a fluorescence measurements

Fluorescence emission and excitation spectra were measured at 77 K with a Perkin Elmer LS5 or Hitachi MPF-4 spectrofluorimeter fitted with a low temperature attachment. The emission spectra were corrected for the spectral sensitivity of the instrument. The spectral half-band width of both the excitation and emission monochromator slits did not exceed 5 nm. A Corning CS4-96 filter was used before the excitation beam and a CS2-58 red glass filter was used before the emission beam to reduce stray light. A set of neutral density filters was used to reduce the intensity of the exciting light for kinetic measurements. The samples were diluted with 60% glycerol (v/v) and slowly cooled to liquid nitrogen temperature in darkness. 1–2 mm cuvettes were used for measurements; the final Chl *a* concentration was 2–4 μM; Chl : P-700 ratio in membrane preparations was about 150.

Absorption and P-700 measurements

77 K absorption spectra and their second derivatives were measured using a Hitachi-557 spectrophotometer. The concentration of P-700 was determined from the chemically induced absorbance change at 700 nm using 3 mM ferricyanide. An extinction coefficient of 70 $mM^{-1}\ cm^{-1}$ was used to calculate the amount of P-700 [23]. The light-induced kinetics of P-700 photooxidation and kinetics of $P\text{-}700^+$ dark recovery at 77 K were measured using a light guide attachment; the intensity of the 702 nm measuring beam was 0.2–0.4 $mW\ m^{-2}$. Intensity of the actinic light at 625 nm was varied between 500 and 10 $mW\ m^{-2}$ using neutral filters. The red cut-off ($\lambda > 670$ nm) and interference (625 nm) filters were used to separate the measuring and actinic beams.

For some experiments the same instrument was used to measure the fluorescence kinetics in the same sample, and at the same wavelength and intensity of the measuring beam as for P-700 measurements. For this, the fluorescence was excited by the measuring beam ($\lambda = 702$ nm, 5 $mW\ m^{-2}$) and monitored at $\lambda > 730$ nm. The glass filters with $A = 3.5$ at 702 nm was put in front of the photomultiplier to measure fluorescence. After removing the filters, the kinetics of P-700 photooxidation by the 702 nm measuring beam was recorded.

Other measurements

The Chl concentration was estimated as described elsewhere [24]. Dithionite was added in room light to a final concentration of 20 mM with neutral red to 3 μM or PMS to 10 μM in 0.1 M glycine buffer adjusted to pH 9.5. Ferri- and ferrocyanide were used at 3 mM, in titration experiments. Light intensity was measured with a YS I Kettering model 65 radiometer or with a germanium photodiode. The exponential fitting of the

kinetic curves was carried out using an XT personal computer with the programs of R.I. Leetherbarrow (Enzffitter). Statistical weighting was used for two and three component exponential deconvolution.

Results

Emission and absorption spectra

Fig. 1A shows 77 K absorption spectra of *Spirulina* cells and membrane fragments. The absorption bands of phycocyanin, allophycocyanin and Chl *a* are typical of intact cells (curve 1), while in the membrane fragments the absorption due to phycocyanin and allophycocyanins is considerably reduced (curve 2). A long-wavelength hump at 730–740 nm is clearly seen in the absorption spectra of both the intact cells and membrane fragments. From the second derivative spectrum (Fig. 1, inset) and the fluoresence excitation spectra (see later) we estimated the position of this band to be 735 ± 2 nm with a half-bandwidth of 30–32 nm (560–590 cm^{-1}). In contrast to the 710 nm band with a half-bandwidth of 14–15 nm and to other short-wavelength bands (668, 678, 682, 697 nm) with a half-bandwidth of 7–10 nm, the 735 nm absorption band is quite broad. In addition, the presence of a narrow 718 nm band with a half-bandwidth of 7–8 nm is clearly seen both in the derivative spectrum and in the absorption spectra. Thus, the *Spirulina* membranes contain the special long wavelength Chl form absorbing at 735 nm.

Fig. 1B shows the 77 K fluorescence emission spectra of intact cells frozen in dark and excited with various intensities of 435 nm light. Apart from the typical emission bands of 685 and 695 nm originating from PS II and the large F730 emission band of PS I, the F758 band is quite conspicuous in this alga, as F750 is for *Pseudanabaena* [17]. At high light intensity, this F758 band gets bleached (curves 1–3). The amplitude of this band diminishes significantly, even when the cells are frozen in weak monochromatic exciting light (curve 4). The difference fluorescence spectrum (curve 5) shows a symmetrical band with a maximum near 758 nm and a half-bandwidth of 32–34 nm (560–590 cm^{-1}). Dark adaptation of cells for 10–15 s at room temperature was sufficient for the development of F758, when the sample was frozen in the dark; for membranes this adaptation period depended on the presence of external electron donors.

Thus, a Chl form absorbing at 735 nm seems to be responsible for 758 nm emission suggesting a Stokes shift of 23 nm (410 cm^{-1}). This assumption was comfired by the fluorescence excitation spectra. The 735 nm and 758 nm bands have mirror-symmetry and a Stokes shift which coincides with the value that can be calculated by the Stepanov's universal equation between the absorption and fluorescence spectra [25]. According to this equation, the Chl_{710} with a half-bandwidth of 15 nm must fluoresce at 724 nm.

Fluorescence and P-700 photobleaching

F758 has a variable yield component if samples are initially frozen in the dark. The extent of light-induced decrease of the fluorescence yield depends on the excitation intensity (Fig. 2A), which explains the dependence of the fluorescence spectra on the intensity of exciting beam (Fig. 1B). Absorption changes at 702 nm indicate that the dependence of P-700 photooxidation on the intensity of actinic light is similar to that of fluorescence changes (data not shown). The dependence of F758 steady state level (F_{ss}) and ΔA at 702 nm on the number of absorbed quanta indicates that F758 photobleaching is reversible.

The photobleaching kinetics of the F758 in *Spirulina*

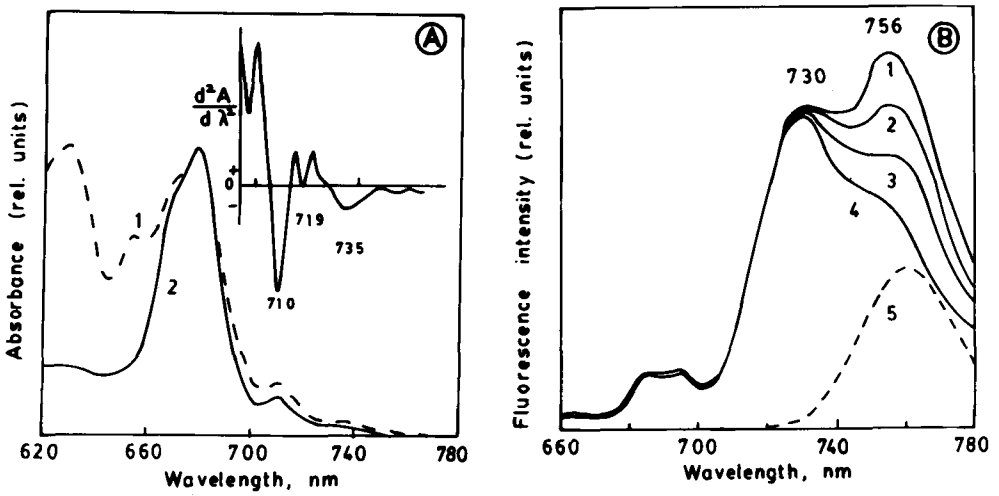

Fig. 1. 77 K absorption and fluorescence spectra of *Spirulina platensis* in 60% glycerol. (A) absorption spectra of whole cells (1) and membranes (2). Inset: the second derivative of absorption spectrum (2) in the red region. (B) fluorescence spectra of whole cells: $\lambda_{ex} = 435$ nm. Cells were frozen in the dark and spectra were measured at 5 (1), 20 (2) or 80 (3) mW m^{-2} intensity of exciting light or cells were frozen in monochromatic exciting light 20 mW m^{-2} (4); the spectra were normalized at 720 nm. (5) curve 1 minus curve 4.

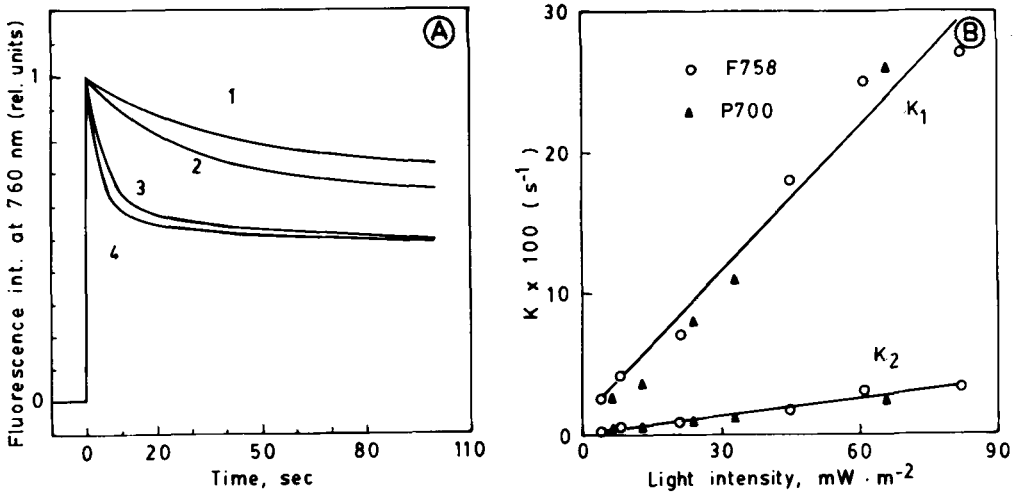

Fig. 2. Dependence of F758 and P-700 photobleaching on the intensity of the exciting (actinic) beam at 77 K for *Spirulina* membranes frozen in the dark. (A) Kinetics of fluorescence yield at 758 nm measured at different intensities of the exciting beam 5 (1), 10 (2), 60 (3) and 80 mW m^{-2} (4), $\lambda_{ex} = 435$ nm. Medium: 50 mM Tris-HCl buffer (pH 8), 10 mM sodium ascorbate, 10 μM PMS, 60% glycerol; Chl concentration 10 μg/ml. (B) Dependence of the apparent rate constants K_1 and K_2 for F758 photobleaching and P-700 photooxidation on the intensity of exciting (actinic) beam. The apparent rate constants were obtained by fitting of the kinetic curve by the sum of two exponents.

is bi-exponential, as that of F750 in *Pseudanabaena* [17]. The decay can be expressed as

$$F_t/F_i = A \cdot \exp(-K_1 t) + B \cdot \exp(-K_2 t) + C, \quad (1)$$

where A, B and C are constants, K_1 and K_2 are apparent kinetic constants; F_t and F_i are fluorescence intensities at different illumination times. We used the same equation for the fitting of P-700 photooxidation and $P\text{-}700^+$ dark reduction kinetic curves.

As can be seen from Fig. 2B, K_1 and K_2 of F758 bleaching and P-700 photooxidation show a nearly linear dependence on light intensity. In our experiments, the magnitude of K_1 (the fast component) varied from 0.025 to 0.3 s^{-1}, and the magnitude of K_2 (the slow component) varied from 0.0025 to 0.030 s^{-1}. The K_1/K_2 ratio remained about constant (7–10) for various samples used. However, the magnitude of the fast component (A) increased from 0.2 to 0.4 while the magnitude of the slow component (B) decreased from 0.25 to 0.11 with increasing intensity of exciting (actinic) beam (5 to 80 mW m^{-2}). If the light intensity was higher than 200 mW m^{-2}, the slow component was absent ($A/B > 10$). Thus, the fast decay component reflects the P-700 photobleaching that causes the quenching of F758. Since this F_{ss} level at 758 nm is dependent on light intensity (Fig. 2A), it is very probable that the slow component represents the rereduction of $P\text{-}700^+$ and the recovery of F758 during illumination at 77 K.

Since the light intensity in the low temperature attachments used in the present work cannot be easily measured accurately, it is important to compare the kinetics of F758 and P-700 photobleaching induced by exactly the same beam. In this experiment, measurements were made using a spectrophotometer (see Materials and Methods): the measuring beam at 702 nm oxidized P-700 and excited F758. Fig. 3 shows that the kinetics of P-700 photooxidation coincides with that of changes in F758.

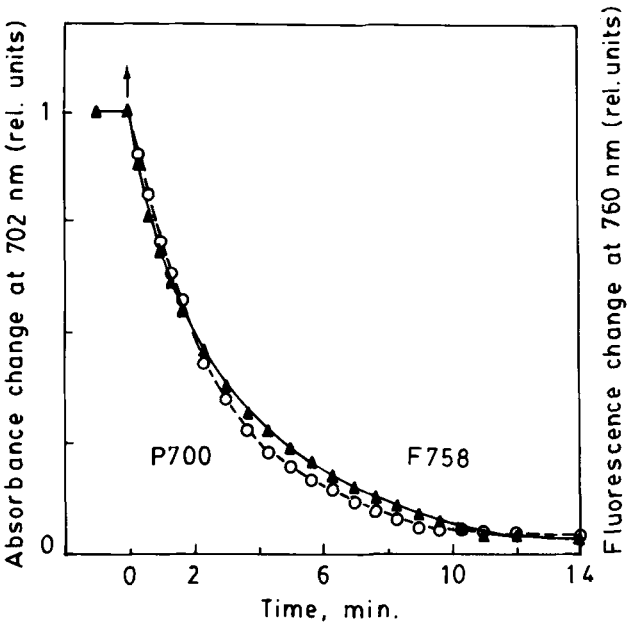

Fig. 3. The time-course of F758 and P-700 photobleaching measured at the same light intensity of the exciting (actinic) beam at 702 nm (see Materials and Methods). Membranes were frozen in the dark; $A = 0.15$ at 702 nm; light intensity was 5 mW m^{-2}. The reference sample contained 3 mM ferricyanide. Here and in Fig. 6 the signal of ΔF758 represents decreased and that of ΔA_{702} an increase of amplitudes. ↑, switching light on.

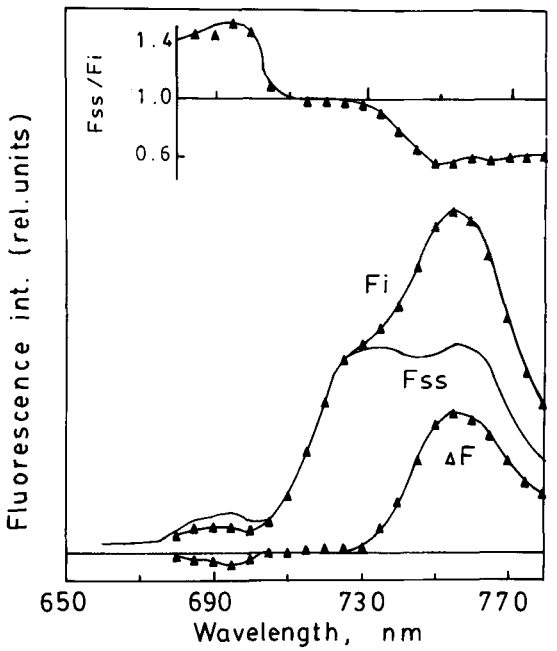

Fig. 4. 77 K fluorescence spectra of *Spirulina* cells frozen in the dark at the steady-state level (F_{ss}), at the initial level (F_i) and the spectrum of variable fluorescence (ΔF). F_i and ΔF spectra were calculated from kinetic measurements made with a 5 nm step; $\lambda_{ex} = 435$ nm; 20 mW m^{-2}. Inset: plot of the F_{ss}/F_i ratio versus λ_{em}; each point is the average of four to five kinetic measurements.

The fluorescence emission spectra of F_i and ΔF of *Spirulina* cells were obtained from the kinetics and F_{ss} spectra (Fig. 4). We recorded the amplitude of fluorescence transients ($a = F_{ss}/F_i$) in 5 nm steps in the range from 670 to 780 nm (inset). These data and F_{ss} spectrum were used to calculate the spectrum of initial level $F_i = F_{ss}/a$ and the spectrum of variable fluorescence $\Delta F = F_0 - F_{ss}$ (Fig. 4). The ΔF spectrum peaking at 757 nm was quite symmetric and had a half-bandwidth of 32–34 nm. The short-wavelength side of the spectrum is Gaussian in shape. This spectrum was very similar to that of F758 presented in Fig. 1B. The negative bands at 685 and 695 nm (curve ΔF) indicate the presence of PS II in isolated membranes.

Dark reversibility

Photooxidized P-700 is partially reduced at 77 K in the dark [26–28]. The partial dark reversibility of F758 is observed also after illumination at 77 K. This process was studied using two approaches. In the first case, the ΔF amplitude of samples cooled to 77 K in darkness and subsequently illuminated was registered with different dark intervals (Fig. 5A). After a 5 s dark period, approx. 5% of the initial ΔF signal was restored, and in 30 min, the reversibility increased up to 30–35% of ΔF. To estimate the rate constant of ΔF reversibility (K_r), repeated illumination was used to monitor the recovery of ΔF signal as a function of dark interval. The plot of $\ln(\Delta F/\Delta F_{max})$ versus time presented in Fig. 5B shows two components for fresh intact cells ($K_{r1} = 0.025$ s^{-1} and $K_{r2} = 0.0006$ s^{-1}), and for membranes kept at 230 K, $K_{r1} = 0.015$ s^{-1} and $K_{r2} = 0.0005$ s^{-1}.

In the second case, the fluorescence intensity at $\lambda > 730$ nm was registered using a very weak (0.3–0.5 mW m^{-2}) exciting beam at 702 nm after preillumination of membranes with actinic light ($380 < \lambda < 500$ nm, 10 W m^{-2}). Parallel measurements of P-700 restoration were made on the same sample (see Materials and Methods). Fig. 6 shows that the kinetics of the dark reversibility of photobleached F758 and photooxidized P-700 are essentially the same.

It is necessary to note that the ratio of the 'fast' and 'slow' components (K_1/K_2 and A/B) of F758 photobleaching (see Fig. 2) depends on the rate constants of dark reversibility: this ratio is high at low values of the dark reversibility constants (see Fig. 5), thereby ex-

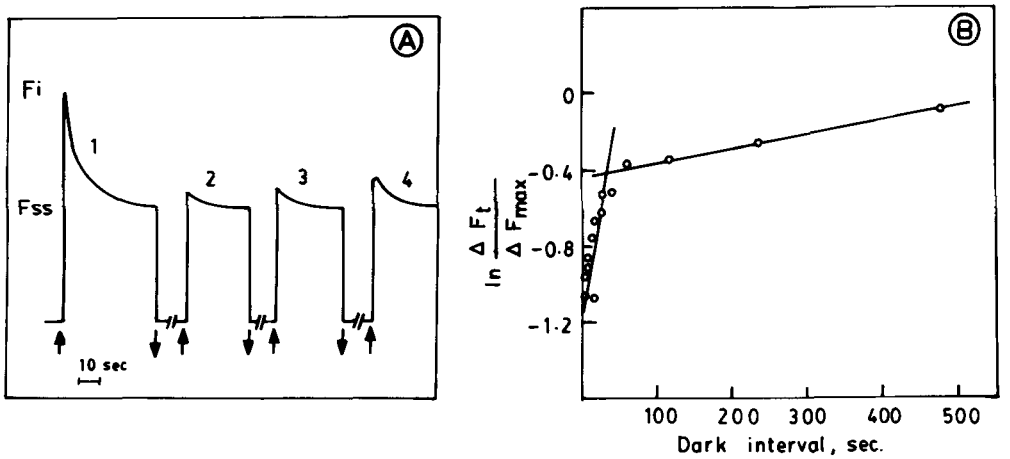

Fig. 5. Reversibility of F758 photobleaching of *Spirulina* cells at 77 K at different dark intervals. (A) F758 kinetics of the sample frozen in the dark (1) or the same sample after dark adaptation for 10 s (2), 1 min (3) or 30 min (4). (B) semilogarithmic plot of the variable part of F758 ($\Delta F_t/\Delta F_{max}$) versus duration of dark adaptation; ΔF_{max} is the variable fluorescence recovered after 30 min in the dark.

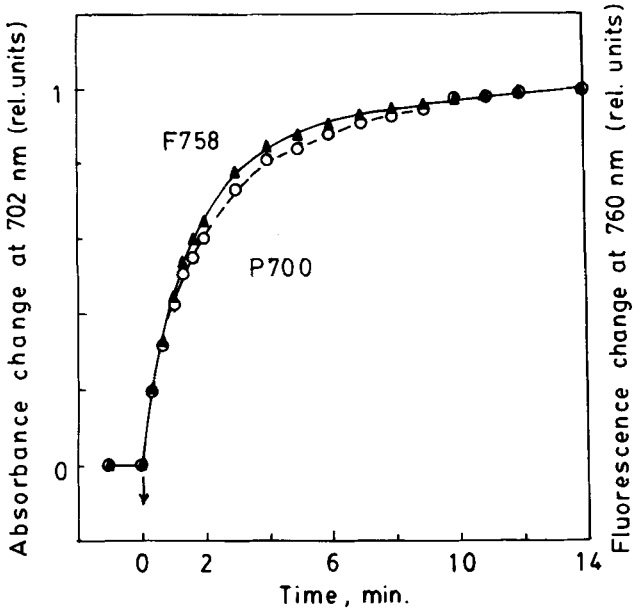

Fig. 6. Time-course of the reversibility of fluorescence at $\lambda > 730$ nm (F758) and ΔA at 702 nm (P-700) after illumination at 77 K. All measurements were made on the same sample. After cooling in the dark, membranes were illuminated with blue actinic light (1 mW m^{-2}) for 30 s. Intensity of the spectrophotometer beam at 702 nm was ≈ 0.2. mW m^{-2} (see Materials and Methods). The maximum amplitudes of ΔA and ΔF were normalized at 14 min. The reference sample contained 3 mM ferricyanide; $A_{702} = 0.15$. ↓ represents light off.

plaining the presence of the second component in F758.

Redox dependence of F758 and P-700

In dithionite-treated samples, the amplitude of F758 and ΔF did not change significantly. However, if the membranes treated with dithionite and 10 μM PMS were cooled slowly (10–15 min) in white light (50 W m^{-2}), F758 was approximately at the F_i level (Fig. 7A, curve 1) and no variable fluorescence was observed in low exciting light. If dithionite was replaced by ascorbate, the F758 intensity decreased dramatically (curve 2). The difference spectrum of 'curve 1 minus curve 2', after normalization at 720 nm (curve 4, Fig. 7A), is very similar to the ΔF spectrum calculated from kinetic measurements (see Fig. 4). The addition of 1 mM ferricyanide to *Spirulina* membranes completely eliminated F758 (curve 3, Fig. 7A): the intensity of F758 decreased at least 40–50-fold as compared with the F_i level (Fig. 7A, curve 4).

The fluorescence excitation spectra measured at 780 nm for these samples (Fig. 7B) clearly show that the intense broad absorption band at 735 nm emits at 758 nm. This band (735 nm) is absent in the excitation spectra of F780 in the presence of ferricyanide when F758 disappears. The excitation spectrum of F758 is very similar to that of F730 in the 350–710 nm region suggesting efficient energy transfer from PS I antenna Chl to Chl_{735}^{758} (data not shown). As the 735 nm band is well distinguished from the other absorption bands and F758 predominates at 780 nm (Fig. 7A, curve 1), the efficiency of the energy transfer from bulk antenna Chl to Chl_{735}^{758} was estimated by comparison of the absorption (Fig. 1A, curve 2) and fluorescence excitation spectra (Fig. 7B, curve 1) after normalization at 735 nm. For the sample frozen in the light with dithionite when F758 is stable this efficiency was found to be about 0.3. This Chl form absorption constituted approx. 5% of total Chl absorption in the red region.

The difference absorption spectrum (Fig. 8) indicates almost complete P-700 reduction (the $A_{678}/\Delta A_{702}$

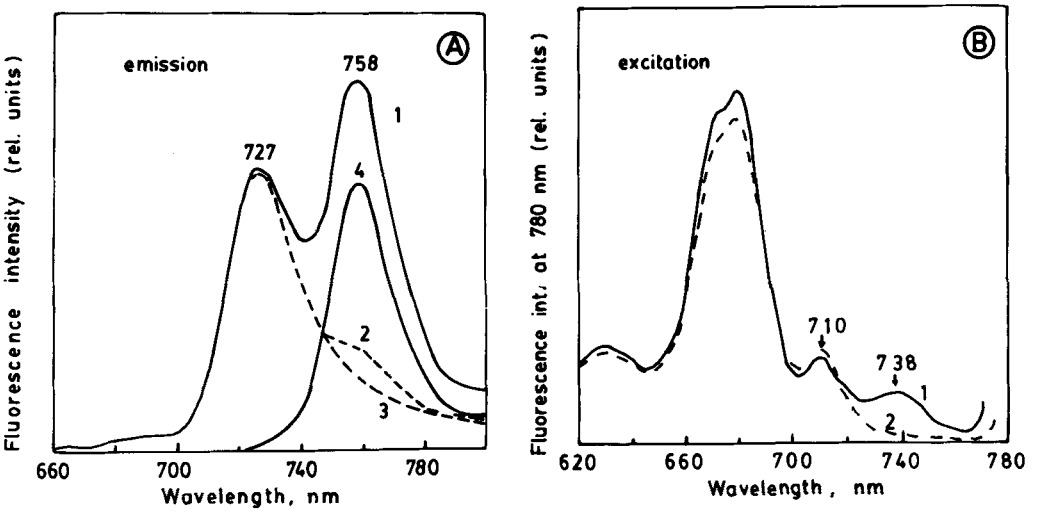

Fig. 7. The 77 K fluorescence emission (A) and excitation (B) spectra of the membranes frozen in 0.1 M glycine-NaOH buffer (pH 9.5) upon illumination with white light (10 W m^{-2}) (1) in the presence of 20 mM dithionite and 10 μM PMS, (2) 10 mM sodium ascorbate and 10 μM PMS, (3) or 1 mM ferricyanide. (4), spectrum 1 minus spectrum 3. For emission spectra $\lambda_{ex} = 620$ nm. (B) Excitation spectra were registered at $\lambda_{em} = 780$ nm; $A_{678} = 0.1$ (1) with dithionite plus PMS, (2) with ascorbate and PMS.

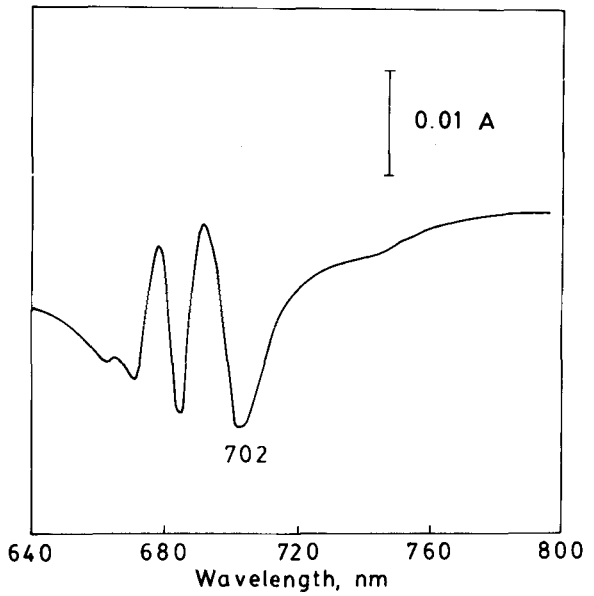

Fig. 8. The 77 K 'oxidized minus reduced' difference absorption spectrum of membranes frozen in 0.1 M glycine-NaOH buffer (pH 9.5) upon illumination with white light in the presence of 20 mM dithionite and 10 μM PMS (sample) or 1 mM ferricyanide (reference); other conditions as in Fig. 7. The maximum of ΔA_{700} corresponds to an $A_{678}/\Delta A$ ratio of approx. 90; $A_{678} = 1.2$.

ratio is 90–100), if the sample was frozen in the light in the presence of dithionite. Thus, we can conclude that the reduced iron-sulfur centers and probably A_1 (phylloquinone) do not quench F758. An insignificant decrease of absorption (6–8%) was observed in the 720–740 nm region in chemically induced P-700 spectra.

Fig. 9 demonstrates the redox titration curve of

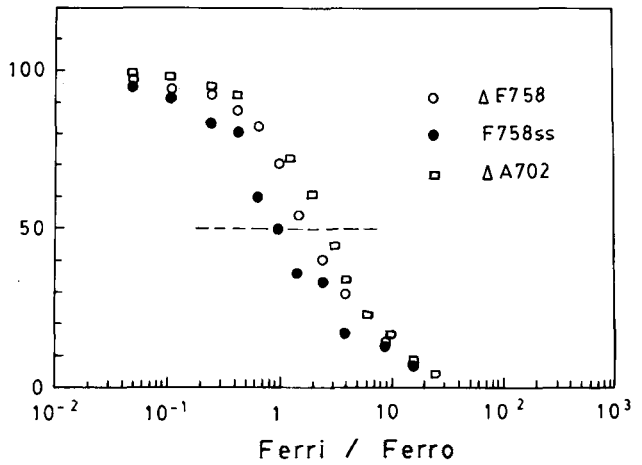

Fig. 9. Redox titration of the variable fluorescence (ΔF758) and steady-state intensity of F758 at 77 K, and P-700 absorption changes at 702 nm at room temperature. The extents for 0 and 100% yield were determined with membranes incubated with ferricyanide (3 mM) and ascorbate (10 mM) plus PMS (10 μM), respectively. Aliquots of membranes in 50 mM Hepes buffer (pH 7.5) were added to 1 ml of ferri-ferrocyanide mixture (3 mM) kept for 5 min in the dark and then cooled in the dark without glycerol. Each point is the average of three to four measurements of ΔF. Intensity of exciting beam at 435 nm was $\approx$ 50 mW m^{-2}.

P-700 absorbance changes at room temperature and that of the F758 amplitude at 77 K measured without glycerol. The F758 redox titration was performed for both ΔF and F_{ss} components of F758. The real intensity of F758 in the steady state level was calculated by subtraction of the F730 component at 758 nm measured with 1 mM ferricyanide from experimental F_{ss}. Both ΔF and F_{ss} show the similar mid-point potential (0.43 to 0.45 V). Since $F_i = \Delta F + F_{ss}$, it is also clear that F_i has the same redox potential. The titration curve for ΔF758 at 77 K coincides with that of P-700 measured at 20 °C (Fig. 9); some difference between them is due to the measurements being at different temperatures [29].

F758 in other organisms

The F758 quenched by the light at 77 K was also observed in the fluorescence spectra of *Nostoc muscorum* and *Phormidium uncinatum*, but the amplitude of F758 was much less in these organisms than that in *Spirulina*. It is possible that the F758 intensity depends on growth and nutritional conditions. However, this band was absent in the phycocyanin-containing red alga *Cyanidium* and green plants. Thus, F758 may be emitted by a special Chl *a* form associated with the PS I reaction center of several cyanobacteria.

Discussion

F758 emission

All difference fluorescence spectra (Figs. 1, 4, 7) show that the real position of the F758 band is near 758–759 nm, and the position of maximum at 756 nm (see Fig. 1) and, probably, the peak at 750 nm in *Pseudanabaena* registered by Duval et al. [16,17] are due to superposition with F730. The shape of this band is similar to that of other low temperature emission bands reported as F718 (*Dunaliella*), F726 (*Chlorella*), F730 (*Anabaena*) and F738 (beans) [30]. The excitation spectra (Fig. 7B) and theoretical dependence between the absorption and fluorescence spectra clearly demonstrate that F758 in *Spirulina* is emitted by a specific Chl form with absorption maximum of Q_y transition at 735–736 nm (Chl_{735}^{758}). This form is characteristic of many cyanobacteria, but is absent in higher plants. A long-wavelength-absorbing pigment (marked as P750) was found earlier in *Anacystis* cells which was ascribed to the observed 77 K fluorescence shoulder at 760 nm [31]. However, this pigment seems to be located in the cell wall of the cyanobacterium, not in photosynthetic membrane [32].

The multicomponent decay of F758 and P-700 photobleaching

The decay of F758 and P-700 photooxidation depends on the light intensity (Fig. 2) and the reversibility

rate. At high light intensity, photobleaching proceeds more rapidly than re-reduction of P-700 $^+$, and the kinetics of photobleaching are thus mono-exponential. At low light intensity, the photobleaching and recovery occur with comparable rates, and the kinetics are bi-exponential due to a higher value of the slow component. Since there are two components in F758 and P-700 dark recovery (Figs. 5 and 6), one may expect three-exponential kinetics of photobleaching. Indeed, regular distribution of residues after bi-exponential curve computer-fitting shows that at low light intensity (5–30 mW m^{-2}) at least three exponents are present.

Influence of the redox state of the PS I reaction center on F758

We did not observe any influence of the redox state of the iron-sulfur centers F_BF_A on F758, which is in accord with the results obtained by Duval et al. [17]. They ascribed a low fluorescence yield (F_L) in the state P-700$A_0A_1^-F_xF_BF_A$ (dithionite + light at 230 K, and subsequent cooling to 77 K). Since the redox state of P-700 was not measured by these authors they did not consider the possibility that P-700 was oxidized. We have found that in this state of the reaction center (obtained under similar conditions) P-700 was completely reduced, and the fluorescence yield was stable. In our experiments in the presence of dithionite F758 intensity was high and close to the F_i level (Fig. 7A). This means that the acceptors in the reduced state do not quench F758.

The results obtained show that F_i and F_{ss} levels of F758 depend only on the redox state of P-700. The remarkable similarity between the kinetics of F758 and P-700 photobleaching (Fig. 3), between the kinetics of dark reversibility of F758 and P-700$^+$ (Fig. 6), between the redox potential dependence of $F758^{ox}/F758^{red}$ and $[P\text{-}700^+]/[P\text{-}700^{red}]$ (Fig. 9) leads us to conclude that F758 intensity is proportional to the concentration of P-700. Since the absorption band at 735 nm does not change (< 6–8%), when F758 disappears (Fig. 8), we can conclude that the quantum yield of F758 (ϕ) is proportional to the concentration of P-700, i.e., $\phi_{758} = M\ [P\text{-}700^{red}] = M([P\text{-}700]\text{–}[P\text{-}700^+])$, where $[P\text{-}700] = [P\text{-}700^{red}] + [P\text{-}700^+]$.

Mechanism of F758 photobleaching

To explain the relation between F758 and the redox state of P-700, Duval et al. [17] suggested that the bleaching of F758 may result from recombination between reduced acceptors and P-700$^+$, i.e., F758 may be delayed fluorescence. However, the following experimental results cannot be explained by this recombination hypothesis: (1) Comparison of the fluorescence excitation and absorption spectra (Figs. 1 and 7) showed that the relative quantum yield of F758 is maximal at excitation in the 730–740 nm range, where P-700 absorption is minimal. (2) The fluorescence quantum yield of Chl_{735}^{758} is comparable with that of Chl_{705}^{730} (0.2–0.3), while the PS I delayed fluorescence yield is very low and can be detected only at an extremely low redox potential [33]. We have found that, in the sample frozen with dithionite in the light (Fig. 7), the F758 has mono-exponential fluorescence life-time equal to 1.2 ns (data not shown).

The results presented in this paper can be interpreted in two possible ways: (1) F758 exists only together with the reduced P-700, which is possible if Chl_{735}^{758} is a component of PS I reaction center and there is a 'strong' resonance interaction between P-700 and Chl_{735}^{758}. When P-700 is oxidized, the properties of Chl_{735}^{758} may change and as a result the rate constant of heat dissipation may increase. This assumption is in agreement with our proposal that P-700 is not a 'discrete dimer' but it resides within a cluster of 6–10 Chl molecules, and that P-700 photooxidation leads to a change in the resonance interaction between P-700$^+$ and its nearest Chl molecules [34–36]. The size of this cluster is not constant: the light-induced CD spectra of P-700 measured with isolated PS I chlorophyll-protein complex of *Spirulina platensis* show an additional band at 710 nm [34]. In any case, it is very difficult to explain the large increase of the heat dissipation constant without alternatives. (2) F758 is quenched by P-700$^+$. Earlier Ikegami suggested that P-700$^+$ may quench the fluorescence at 694 nm in PS I particles containing only 8–10 Chl per P-700 [7]. Such a mechanism is more probable and does not exclude the 'strong' resonance interaction between P-700 and Chl_{735}^{758}. The F758 quenching by P-700$^+$ may result from either decrease of the energy migration from antenna Chl to Chl_{735}^{758}, or energy migration from Chl_{735}^{758} to P-700$^+$. The overlap integral between the absorption band of P-700$^+$ and fluorescence band of F758 is sufficiently large, as P-700$^+$ absorption extends from 700 to 850 nm with an extinction coefficient of approx. 10 mM cm^{-1} [23]. This overlap integral and the distance of 30–50 Å are sufficient for effective energy migration from Chl_{735}^{758} to P-700$^+$, which may cause the quenching of F758 as well as the fluorescence of short-wavelength bands. It should be noted that the overlap integral between the absorption band of reduced P-700 and the fluorescence band of Chl_{735}^{758} is negligible and the efficiency of back energy migration from Chl_{735}^{758} to P-700 is lower than that of direct energy migration. Indeed, the intensity of the 735 nm band in the action spectra of ΔF750 [16,17] is considerably lower than in the fluorescence excitation spectra of F758 (Fig. 7).

Thus, the 77 K variable fluorescence of PS I in cyanobacteria originates from different processes than the variable fluoresence of PS II and PS I at room temperature.

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