Biochimica et Biophysica Acta 848 (1986) 352–358
Elsevier

BBA 41931

77 K fluorescence quenching induced by reduction of Photosystem I primary electron acceptors in a cyanobacterium

Jean-Claude Duval [a], Jean-Claude Thomas [a] and Yves Choquet [b]

[a] *Laboratoire des Biomembranes et Surfaces Cellulaires Végétales, Ecole Normale Supérieure, U.A. C.N.R.S 04311, 24 rue Lhomond, F-75231 Paris Cedex 05 and* [b] *Service de Biophysique, Département Biologie, CEN Saclay, F-91191 Gif-sur-Yvette (France)*

(Received June 14th, 1985)

Key words: Chlorophyll fluorescence; Photosystem I; Fluorescence decay; (Cyanobacterium)

A *Pseudanabaena* strain isolated in our laboratory exhibited a large amount of a 710 nm chlorophyll form, associated with Photosystem I, the chromophore of which was found to be parallel to the membrane plane as shown by linear dichroism measurements. Whole cells, thylakoid vesicles and Photosystem I particles obtained after digitonin treatment presented a particular 77 K fluorescent component at 750 nm (F_{750}): a high initial level of fluorescence (F_0) was obtained when samples were frozen in the dark; illumination induced a decrease to a low level (F_L). Kinetic analysis showed that this decrease was biphasic, with a first phase 3-times faster than the second one. F_0/F_L ratio was about 3. Action spectra demonstrate the origin and properties of chlorophyll 710. By poising Photosystem I electron acceptors at different oxidoreduction states, and from experiments with ferricyanide, we conclude that the fluorescence at 750 nm originates from a chlorophyll form absorbing at 710 nm in a close relation to P-700 and that its kinetics can be used to evaluate Photosystem I primary electron acceptor pools.

Introduction

Fluorescence emission spectra recorded at 77 K from photosynthetic organisms exhibit several components. In green plants, the 705–740 nm emission range is generally attributed to PS I [1]. This photosystem is arranged into a peripheral antenna, emitting fluorescence at about 730–740 nm, and a core with the photochemical centre (P700), which fluoresces at 720–725 nm [2]. Fluorescence originates mainly from the antenna in plants and from the core in green algae [3]. In whole cells, fluorescence intensity is modulated by several parameters such as energy distribution [4], pH gradient [5], ATP level [6] and the redox state of the PS I reaction centre or electron acceptors [7,8].

On the reductant side of PS I, electron spin resonance and absorption studies have revealed several steps in electron transfer involving two primary acceptors (A_0 and A_1) and three iron-sulphur proteins (X, A, B) as secondary acceptors (for a review, see Ref. 9). The redox state of these components can be modified by chemical agents (ferricyanide, ascorbate, dithionite) and by freezing in the dark or under illumination. Recent work [10,11] suggests that A_0 is probably a chlorophyll *a* and A1 a quinone, and that the secondary acceptors act in parallel ways.

Abbreviations: Chl *a n*, chlorophyll form absorbing at *n* nm; DCIP, 2,2-dichlorophenol indophenol; F_n, fluorescence recorded at *n* nm; F_L, F_0, F_V, fluorescence at 750 nm recorded in the light, in darkness and variable part of this fluorescence ($F_0 - F_L$); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; LD, linear dichroism; P-700, Photosystem I reaction centre; PS I (II), Photosystem I (II).

Cyanobacteria are particularly suitable for PS I studies because of their relatively low chlorophyll/P-700 ratio (about 200) which remains relatively constant under different growth conditions [12]. Mechanical and detergent fractionation of thylakoids have demonstrated that PS I and PS II are structurally independent subunits [13]: phycobiliproteins are connected only with PS II complexes and PS I possesses its own antenna; 715–725 nm fluorescence originates from PS I [14]. Electron acceptors are very similar to those of green plants [15] although the iron-sulphur proteins may be connected in a different way. Although devoid of Chl *b*, PS I is organized as in green plants and immunological cross-reactivity has been found between protein complexes [16].

In a preliminary report [17], we have shown that a strain of *Pseudanabaena*, isolated in this laboratory, has a fluorescence kinetic decay at 750 nm (F_{750}) upon illumination at 77 K. In this paper, we specify the relationships between the F_{750} and the PS I oxidoreduction state. We show that the F_{750} level is related to the redox state of P-700 and PS I electron acceptors. We also postulate that F_{750} originates from Chl 710 and is sensitive, through P-700, to the redox state of the primary electron acceptors of PS I.

Material and Methods

Biological material

Pseudanabaena sp. M2, isolated in our laboratory, was grown autotrophically in 'Z' medium as previously described [18]. Cells were harvested by mild centrifugation and resuspended in 10 mM Hepes-NaOH buffer (pH 7.4)/2 mM $MgCl_2$/0.2% bovine serum albumin. They were disrupted in a French-pressure cell at 0.28 Pa; crude extract was then centrifuged at $1000 \times g$ for 10 min and the pellet was discarded; the supernatant was centrifuged at $20000 \times g$ for 60 min; the pellet constitutes what is hereafter named 'thylakoid vesicles'.

Active Photosystem I particles were obtained by digitonin treatment as in Ref. 19 except that 10 mM Hepes-NaOH buffer (pH 7.4) was used instead of Tricine buffer.

Thylakoid vesicles or PS I particles were then resuspended in 50 mM glycine-NaOH buffer (pH 9)/10 mM sodium ascorbate with 20 mM glucose and 100 units/ml glucose oxidase to maintain anaerobic conditions [8] (as verified with a Clark-type YSI oxygen electrode).

For fluorescence measurements, 50 μl of the suspension (3 μg chlorophyll) was layered in the dark on an AP 20 Millipore prefilter.

Spectroscopic measurements

The P700 concentration was assayed by measuring photooxidations detected with a DW 2 Aminco Chance spectrophotometer in the dual-beam mode [19] at 697 nm (reference at 730 nm). For this purpose, preparations were suspended in 50 mM glycine-NaOH buffer (pH 9)/10 mM sodium ascorbate.

77 K fluorescence emission spectra were recorded with a home-made apparatus as previously described [20]. To calculate the initial F_{750} emission level (F_0 level, see text), a Tektronix 5103 N oscilloscope was added in parallel to the plotter to record fluorescence kinetics at a given emission wavelength.

Absorption spectra in liquid nitrogen were obtained as in Ref. 20 with a 1000 Hz modulated beam. This apparatus was modified to measure F_{750} kinetics as follow: a set of filters (Wratten W 50 + M.T.O. J. 662 a) which allowed only wavelengths greater than 745 nm to pass, was put in front of the photomultiplier. A shutter (Compur, opening time 1 ms) was used to control the actinic beam. Results were recorded either with a Tektronic TEK 31 calculator or with a Tandy TRS-80 microcomputer. It was verified that a resolution time of 0.3 s was sufficient to obtain complete kinetics with an actinic beam of 2.5 $pE \cdot m^2 \cdot s^{-1}$. When required, the F_L level was induced by white light ($9\ \mu E \cdot m^{-2} \cdot s^{-1}$).

Action spectra were obtained with the same apparatus using a 2500 W xenon lamp and a Jobin Yvon HRS-1 monochromator. Light intensity was adjusted to be isoquantic at each wavelength (1 $pE \cdot m^{-2} \cdot s^{-1}$) and effectiveness was expressed as F_V/F_L level ($F_V = F_0 - F_L$) for each point. Preincubation at 230 K was performed in a Dewar containing 66% glycerol (w/v) cooled by a Flexydry (FTS-System) probe. When necessary, samples were illuminated by a 150 W quartz iodine lamp ($400\ \mu E \cdot m^{-2}\ s^{-1}$).

Linear dichroism measurements were performed as in Ref. 3, on thylakoid vesicles.

Results

General organization of PS I in Pseudanabaena

We used three kinds of preparation: whole cells, thylakoid vesicles isolated by differential centrifugation after French pressure cell breaking and PS I particles obtained from a sucrose density gradient following digitonin treatment. Chl/P700 ratios were 250 for whole cells, 200 for thylakoid vesicles and 110 for PS I particles.

Pigment composition of whole cells is illustrated by 77 K absorption spectra (Fig. 1): peaks at 630 nm and 652 nm originated from phycocyanin and allophycocyanin respectively. Besides a main peak at 680 nm, chlorophyll absorption in the red range exhibited a minor peak at 710 nm.

Pseudanabaena thylakoid vesicles were a good material for linear dichroism (LD) experiments, probably because of their relatively large size and absence of chlorophyll other than Chl *a*. The absorption spectrum at 5 K (Fig. 2) showed that phycobiliproteins were lost during fractionation procedure. The LD spectrum revealed a difference in absorption of light antiparallel and parallel, respectively, to the direction of compaction. The ratio of these two spectra emphasizes the similar orientation of the chromophores of the three chlorophyll forms (Fig. 2), Chl *a*-688, Chl *a*-697 and Chl *a* 710. These chromophores are subparallel to the membrane plane. As previously described [3], Chl *a*-688 is related to PS I core antenna and Chl *a*-697 is homologous to P-700. It can be seen that the accuracy of the ratio reflects a great homogeneity in the sample both for chromophore and membrane orientations.

77 K absorption spectrum of PS I particles exhibited an enrichment in far-red chlorophyll forms, especially in the 695–720 nm range (Fig. 1).

77 K fluorescence emission spectra of whole cells frozen in the light revealed, under 430 nm excitation beam, four peaks (Fig. 3): F_{660} and F_{687} from phycobiliproteins, F_{695} attributed to PS II and F_{725} from PS I; a shoulder near 750 nm was clearly distinguishable on the spectra.

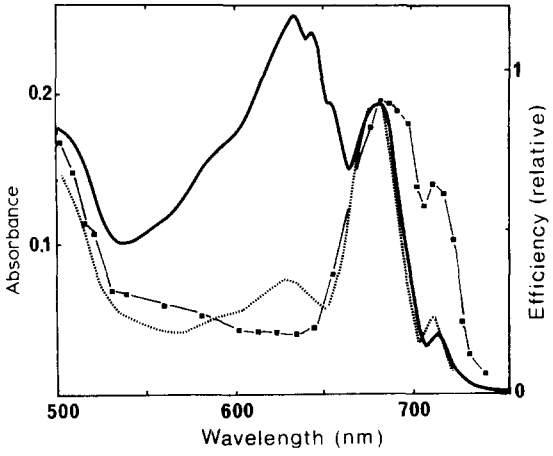

Fig. 1. 77 K absorption spectra of whole cells (———) and PS I particles (·····). Action spectra of F_{750} of whole cells expressed as $(F_0 - F_L)/F_L$ (■———■). (F_0, initial level of 750 fluorescence: F_L, 750 nm fluorescence intensity after strong illumination at 77 K). Medium: 50 mM glycine-NaOH buffer (pH 9), 10 mM sodium ascorbate, 20 mM glucose, 100 units/ml glucose oxidase; 3 μg chlorophyll layered on prefilter.

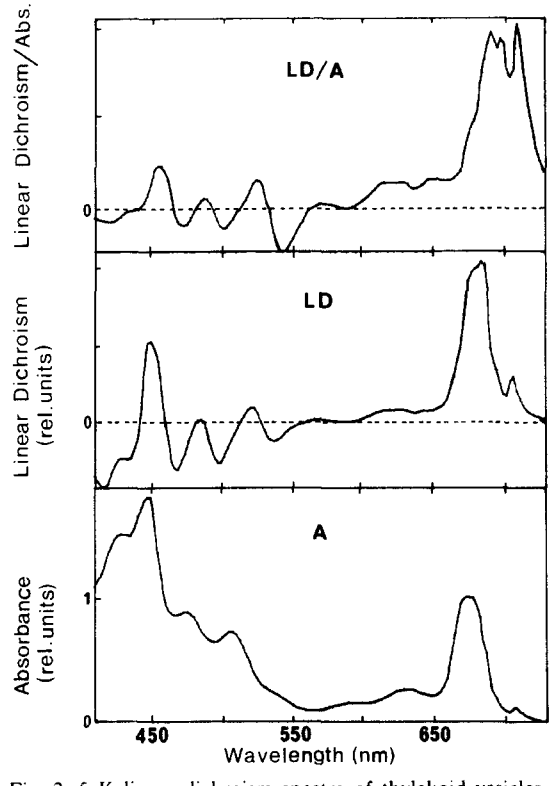

Fig. 2. 5 K linear dichroism spectra of thylakoid vesicles. *A*, absorption spectra; LD, linear dichroism; LD/*A*, ratio of these.

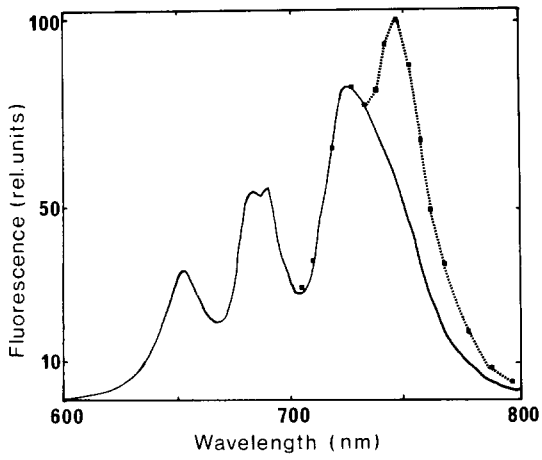

Fig. 3. 77 K emission fluorescence spectra. Sample frozen in the light (F_L state) (———). Fluorescence emission in the F_0 state recalculated from kinetic experiments on samples frozen in the dark (■·····■); normalization at 725 nm with the first curve and adjusted by $F_L \cdot (1 + (F_0 - F_L)/F_L)$. Conditions same as for Fig. 1.

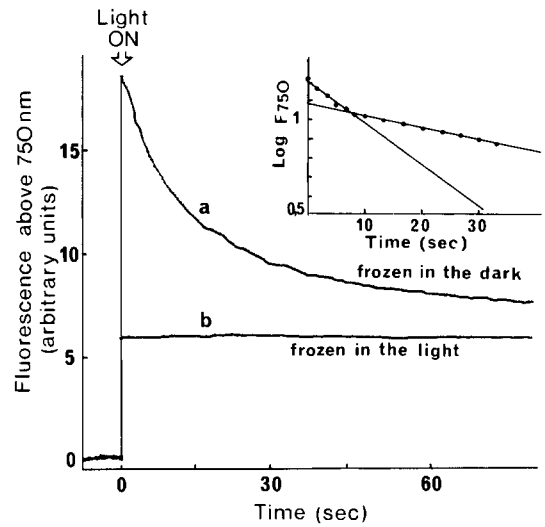

Fig. 4. 77 K kinetics of fluorescence at 750 nm (excitation beam at 685 nm). a, Sample frozen in the dark. b, sample frozen in the light. Inset: semilogarithmic plot of curve a. Conditions same as for Fig. 1.

Therefore, PS I in *Pseudanabaena* M2 presents, in addition to the general features of this photosystem, a particular chlorophyll form, namely Chl *a*-710, which is oriented like P-700. Moreover, a significant level of fluorescence near 750 nm (F_{750}) appears in emission spectra. We focused on this point, as the amount of F_{750} appeared to vary from one experiment to another, depending on the sample preparation.

Main characteristics of F_{750} in whole cells

We analyzed kinetics of 77 K fluorescence emission for whole cells. When samples are frozen in liquid nitrogen in the dark, a large fluorescence decrease occurred at 750 nm upon illumination of cells with blue light (Fig. 4). We defined the initial level as F_0 and the illuminated steady state as F_L; variable fluorescence, F_V, was evaluated as F_0–F_L. We then recorded fluorescence kinetics for various emission wavelengths in the 700–800 nm range with a 5 nm step. Assuming that F_L levels correspond to emission spectra recorded after a long excitation time, we were able to calculate the emission spectrum corresponding to F_0 state; for this purpose, we multiplied the relative spectrum values by $1 + (F_0 - F_L)/F_L$ (Fig. 3). No fluorescence decrease was observed for emission below 725 nm, and the greatest ratio $F_V/F_L = 2$ was found at 750 nm. So we named this phenomenon F_{750}. For further experiments, we have chosen a filter combination which selected the 750 nm emission.

In order to establish the action spectrum of F_{750} (Fig. 1), we plotted F_V/F_L ratios recorded with isoquantic excitation beam (1 $pE \cdot m^{-2} \cdot s^{-1}$); the complete F_L level was induced by illuminating the sample with strong white light (9 $\mu E \cdot m^{-2} \cdot s^{-1}$). Compared with absorption spectra, it appeared that phycobiliproteins are ineffective, whereas far-red-absorbing chlorophyll forms, like Chl *a* 688, Chl *a*-697 and Chl *a*-710, are very efficient in inducing an F_{750} decrease (Fig. 1). Nitrogen depleted cells, which have lost phycobiliproteins [18], thylakoid vesicles and PS I particles exhibited similar action spectra in the 700–800 nm range (data not shown).

The F_L level is stable for several hours at 77 K in the dark. When samples were thawed in the light or in the dark and then frozen again in the dark, F_0 was restored; this cycle could be run several times without significant loss of F_V or shape modification of the decay; if preilluminated samples were frozen in the dark, a full F_{750} decrease was recorded; on the other hand, with samples preincubated in the dark and frozen in the

light, an F_L level was immediately obtained.

The semi-logarithmic plot of the decay revealed that F_{750} kinetics were composed of two successive exponential phases: the first with a rate of $-14.4 \cdot 10^{-3}$ units/s. and the second with a rate of $-3.7 \cdot 10^{-3}$ units/s (Fig. 4, inset). These values vary from one experiment to another, but the ratio between phase I and phase II is always greater than 3. These results imply that F_{750} is related to two different pools.

F_{750} properties of thylakoid vesicles and PS I particles

Chemical agents do not enter whole cells because of cyanobacterial envelopes; thus, the following experiments were done with thylakoid vesicles or PS I particles. Measured under the same conditions as for whole cells, F_V/F_L decreased slightly in these preparations and reached 0.8. This ratio was increased by a long incubation time in the dark at room temperature or by adding 10 mM sodium ascorbate in the medium before freezing. Under these conditions, F_V/F_L rose to 1.8 for thylakoid vesicles and 1.2 for PS I particles. So, the redox state of samples is an important factor in F_{750} amplitude. To specify this point,

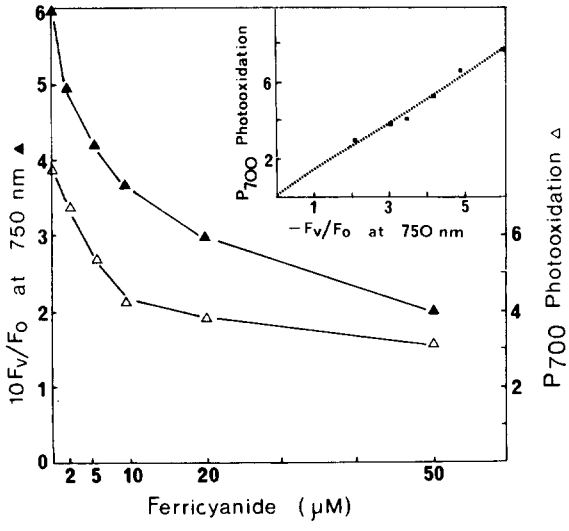

Fig. 5. Effect of ferricyanide on P-700 (△———△) and F_{750} kinetics (▲———▲) of thylakoid vesicles. Inset: Plot of P-700 versus F_{750} kinetics. P-700 evaluated by photobleaching at 697 nm versus 730 nm in dual-beam-mode spectrophotometer; F_{750} kinetics measured as in Fig. 4.

P-700 photooxidation and F_{750} kinetics were measured on identical aliquots which were incubated in the dark with various potassium ferricyanide concentrations. 1 mM ferricyanide fully oxidized P-700 and induced an F_L level. In the 10–100 μM range, a progressive decrease in the F_0 level was parallel to a decline in the P-700 photooxidisable pool. A constant ratio of about 1 connects this pool to F_{750} variable fluorescence (Fig. 5, inset).

Several authors have described experimental conditions in which PS I and its electron acceptors are set in a given redox state [9–11,19]. We attempted to evaluate a possible link between F_{750} and the redox state of a particular electron acceptor. We used anaerobic conditions in order to avoid oxidation by dithionite when this was used. All experiments were conducted in the presence of 10 mM sodium ascorbate and 10 μM DCIP. We assayed in the following situations:

(I) Reference sample, incubated in the dark: all electron acceptors were in the oxidized state. The freezing of samples in the dark induced a F_0 level but when they were frozen in the light, a F_L level was recorded.

(II) 40 mM dithionite was added to the medium: if samples were preincubated in the dark, same results as above were obtained. Then samples in the F_L state were thawed for 20 min in the dark at room temperature and frozen again, in the dark. In this case electron acceptors A and B were reduced and X was in the oxidized state [11]; an initial F_0 was observed.

(III) 40 mM dithionite was added and samples were illuminated 10 min with strong white light at 0°C. In this situation, X, A and B were in the reduced state [19]. The same results as in (II) were obtained.

(IV) 40 mM dithionite was added and samples were cooled to 230 K in 66% glycerol in the dark: the same results as in (II) were recorded. When they were illuminated at 230 K and then cooled in the dark or in the light, an F_L level was reached.

Finally, it appeared that when samples were frozen in the dark under conditions I, II and III, an F_0 level was always recorded; experiment IV induced only an F_L level, even when samples were kept for several minutes at 230 K in the dark before cooling in liquid nitrogen. As discussed later, this implies that there is a close relation

between the redox state of the primary acceptors A_0 and A_1, and the F_{750} level [19].

Hence, *Pseudanabaena* ML appears to be a particular cyanobacterium which possesses a high amount of long-wavelength chlorophyll-absorbing forms, especially Chl *a*-710. This pigment, together with Chl *a*-697 and Chl *a*-688, is particularly active in reducing fluorescence in the 750 nm region, at 77 K. We shall discuss now the relationships between F_{750} and the redox state of the components of PS I centre.

Discussion

A fluorescence peak near 750 nm was previously reported in the cyanobacterium *Anacystis nidulans* [21] but was shown to be related to cellular envelopes and not to thylakoid fragments.

Chl a-710 and F_{750}

Chlorophyll forms absorbing near 710 nm are common among cyanobacteria [22], as revealed by fourth-derivative absorption spectra. In PS I pigment-protein complexes isolated from *Synechococcus lividus* after polyacrylamide gel electrophoresis, Chl *a*-710 was found to absorb 10% of the red range incident light, as measured by deconvolution of absorption spectra [23]. 77 K absorption spectra of PS I complexes from *Oscillatoria limosa* also revealed a low but distinctive peak at 710 nm [14]. *Pseudanabaena* M2 is thus primarily characterized by a large amount of Chl *a*-710.

Linear dichroism experiments showed that chromophores of Chl *a*-688 Chl *a*-697 and Chl *a*-710 have the same orientation, parallel to the membrane plane. Electrophoresis of thylakoids from *O. limosa* [14] have shown that Chl *a*-710 is essentially associated with the heaviest complexes of PS I (about 260 kDa); the main peak of 77 K emission in fluorescence spectra is near 735 nm for the complexes, as in green plants. Preliminary electrophoresis of PS I complexes from *Pseudanabaena* gave similar results (not shown). Analysis of another species, *Oscillatoria splendida*, which is devoid of Chl *a*-710, have shown that in this case, PS I complexes exhibited a peak near 725 nm, as in green algae. It was supposed that in green plants, F_{735} originates in a chlorophyll form specific to these plants [3]. This molecule is located in the peripheral antenna of PS I, absorbs in the 705–720 nm range and has the same orientation as Chl *a*-685 and P-700. The situation of Chl *a*-710 appears to be similar. However, if F_{750} is related to Chl *a*-710, ferricyanide experiments demonstrate a close relationship between P-700 oxidoreduction state and this molecule. We may assume that F_{750} originates from Chl *a*-710 and that this molecule is located near P-700, in the PS I core.

Origin of the quenching

Fluorescence yield of PS I particles from green plants is modulated by the redox state of the centre [7,8]. By the combined use of chemical agents, temperature and light, it it possible to poise the redox state of PS I electron acceptors [8,11]. Under conditions II F_{750} properties were not modified. It has been verified by several authors, through EPR measurements, that in these conditions, PS I centres are in the state P-700, A_0, A_1, X, A^-, B^- [9–11,24]. In experiments III, X is presumed to be reduced [25]. The results are the same as in II: hence we may conclude that there is no correlation between F_{750} kinetics and the redox state of the iron-sulphur proteins X, A and B. Under conditions IV, PS I centres are thought to be in the state P-700, A_0, A_1^-, X^-, A^-, B^- [25]; however, the reduction of A_0 and A_1 may be partial [11,24]. In this case, we always obtained an F_L level.

Hence, we have three kinds of conditions which define the F_L level: freezing of samples in the light, reducing intermediary acceptor A1, and chemical oxidation of P700 by ferricyanide. When PS I is illuminated at a cryogenic temperature, the charge separation between $P\text{-}700^+$ and A^- is stable [9]; when secondary acceptors A and B are prereduced (conditions II), several back-reactions take place between $P\text{-}700^+$ and A_0^-, A_1^- and X^- [9,11,24]. From ferricyanide experiments, we conclude that $P\text{-}700^+$ alone (all the electron acceptors being in the oxidized state) is sufficient to determine the F_L level; conditions IV alone (intermediary acceptor A_1 and perhaps A_0 prereduced) brought about the F_L level. All these facts mean that in the dark, at temperatures higher than (and equal to) 0°C, a back-reaction takes place between $P\text{-}700^+$ and A_1^-, before freezing: $P\text{-}700^+$ is reduced and an F_0 level is seen upon illumination at

77 K. At 230 K, this back-reaction; is suppressed and P-700$^+$ remains in the oxidized state, even when samples are frozen in the dark. In all cases, thawing in the dark induces a reduction of P-700$^+$.

F_{750} kinetics is then an illustration of back-reactions reducing P-700$^+$ at various temperatures. We have shown that there are two phases in the kinetics, with different half-time decay: this probably indicates that there are two kinds of back-reaction or two reductant pools (Fig. 4). The time of decay is out of line with the half-times measured by laser flash spectroscopy [11], which are in the 920–1000 μs range, but we use a beam which is very weak when compared with laser intensity. At this time, we have reasons to identify the two phases of F_{750} decay with the two primary electron acceptors and it appears that this characteristic could lead to new work in the study of the primary electron transport processes in PS I. EPR experiments are needed to complement this approach, and improvements in electrophoresis of PS I particles allow more precise models for the organization of chlorophyll-protein complexes to be considered [26]. Using mild detergents which would preserve F_{750} kinetics and by fine analysis of pigment protein complexes of PS I containing Chl *a*-710 we hope to pinpoint the origin of the F_{750}.

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