Biochimica et Biophysica Acta, 1102 (1992) 167–176

BBABIO 43693

Energy-dependent quenching of chlorophyll *a* fluorescence: effect of pH on stationary fluorescence and picosecond-relaxation kinetics in thylakoid membranes and Photosystem II preparations

A. Krieger [a], I. Moya [b] and E. Weis [c]

[a] *Institute of Ecological Plant Physiology, University of Düsseldorf, Düsseldorf (Germany),* [b] *LURE, Université Paris XI, Orsay (France) and* [c] *Institute of Botany, University of Münster, Münster (Germany)*

(Received 22 January 1992)

Key words: Chlorophyll *a* fluorescence lifetime; Proton gradient; Fluorescence quenching; Photosystem II; Photosynthesis; Charge recombination

Quenching of chlorophyll *a* fluorescence by low pH ('high-energy quenching') has been characterized by stationary fluorescence and time-resolved fluorescence decay in thylakoid membranes and photosystem PS II preparations. Fluorescence is quenched when the pH on the lumen-side of PS II decreases below 5.5. It is likely to involve an one-proton transition (apparent $pK = 4.5–4.8$) and quenching is accompanied by an inactivation of the photosynthetic oxygen evolution. The overall quenching is due to a decline in the two nanosecond fluorescence decay components ($\tau = 1.6$ ns and 5 ns at pH 6.5), which mainly contribute to the stationary fluorescence in the presence of the reduced acceptor Q_A. Two picosecond components ($\tau = 30$ ps and 500 ps at pH 6.5) are little affected by low pH and the stationary 'F_0'-fluorescence (with oxidized Q_A) remains constant. When artificial e^--donors were added at low pH, the fluorescence recovered, suggesting that quenching is caused by inhibition of e^- donation from the water-splitting side. There is no indication that the transfer of excitation energy from the antennae to centers or the trapping process itself is disturbed by internal acidification. We propose that, at centers with an inactive donor side, excitation-energy is 'quenched' by charge recombination between Q_A^- and P-680$^+$. Under physiological conditions quenching induced by a high ΔpH may serve to dissipate excess excitation-energy and to keep Q_A oxidized, even at high irradiance.

Introduction

pH-dependent control of the photosynthetic electron transport between PS II and PS I occurs in plants when photosynthesis is limited by the carbon metabolism and a high proton gradient builds up across the thylakoid membrane. PS I is then controlled by a shortage of electron donation from the plastoquinone pool (photosynthetic control). As a consequence, the PS I reaction center pigment, P700, tends to accumulate in its inactive oxidized form [1].

Recently it has been proposed that a high proton gradient also exerts control on PS II, by which the activity of this photosystem is adjusted to the electron consuming processes of carbon metabolism (Refs. 1–4; for a review see Ref. 5). pH-dependent control of PS II is accompanied by quenching of Chl *a* fluorescence ('high energy quenching', see Ref. 5). Yet, its molecular basis is still unknown. It has been suggested that PS II is controlled by a pH-dependent interconversion between a photochemically active and an inactive state; in centers of inactive PS II non-photochemical decay processes at centers are enhanced and compete efficiently with regular photochemical charge separation [1,2,5]. In an alternative concept it has been proposed that quenching at PS II centers is caused by a futile electron cycle around PS II [6–8], where cyt *b*-559 or other mediators are assumed to react with the oxidized donor side. Also, the possibility has been discussed, that excitation energy is dissipated by charge recombination processes within the center, and that recombination is induced by a donor side limitation of PS II [9]. Schreiber and Neubauer [9] have pointed out that a

Correspondence to: E. Weis, Institut für Botanik, Universität Münster, Schlossgarten 3, D-4400 Münster, Germany.

Abbreviations: F_0, initial (minimal) fluorescence level; F_m, maximal fluorescence level; F_v, variable fluorescence ($F_m - F_0$); PS, photosystem; tyr_z, intermediate electron donor in PS II; P-680 reaction center pigment in PS II; Pheo, pheophytin; Q_A, primary quinone in PS II; Chl, chlorophyll; DPC, diphenylcarbazide; Mops, 3-[*N*-morpholino]-propanesulfonic acid; Mes, 2-[*N*-morpholino]ethanesulfonic acid; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

donor side limitation is a requirement for any cyclic or charge recombination process at PS II centers. Inactivation of the donor side of PS II has been shown to occur at pH < 5.5 [10–13], and this inactivation is induced by release of Ca bound close to the water splitting side [13,14]. Ca release, as induced by a high ΔpH, may actually be a physiological process which induces reversible inactivation of PS II and 'high energy quenching' [11].

In contrast to models of 'reaction center quenching', it has been suggested that a high Δ pH exerts an effect by increasing non-radiative decay processes in the pigment matrix of PS II, which efficiently compete with photochemical processes at centers [15]. Recently, Horton and co-workers have proposed that quenching in the antennae is caused by aggregation of light-harvesting complexes of PS II [16,48].

In the energized state the well characterized enzymatic carotenoid cycle [17] is shifted towards zeaxanthin formation and a possible role of this carotenoid species in energy-dependent quenching has been discussed [18]. Yet, it is not clear whether zeaxanthin formation is directly involved in the quenching process or whether it occurs in parallel. Zeaxanthin formation has been proposed to amplify a preexisting quenching mechanism (see Ref. 19).

In this study, we have investigated the effect of pH on stationary fluorescence and time-resolved fluorescence decay. Time-resolved fluorescence spectroscopy can be used to investigate the early stages of energy gathering and conversion in different states of PS II. Two simplified experimental systems have been used in this study: (1) isolated thylakoid membranes, in which a proton gradient is created in the dark by ATP-hydrolysis via the activated thylakoid ATPase; and (2) isolated PS II particles suspended at different pH values. We attribute ΔpH-dependent quenching and inactivation of PS II to charge recombination between Q_A^- and P-680$^+$, induced by a donor side limitation of PS II. Quenching in the pigment matrices of PS II is unlikely to occur in our experimental system. We discuss the significance of the model derived from our data to in vivo quenching.

Materials and Methods

ΔpH by hydrolysis of ATP

Intact chloroplasts were isolated from spinach (*Spinacia oleracea* cv Monatol) as described by Laasch [20]. The chloroplasts were incubated in a medium containing 0.3 M sorbitol, 50 mM KCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA, 0.5 mM KH_2PO_4, 15 mM Hepes (pH 7.6), 1 mM Na_2CO_3 was added as electron acceptor. To activate the thylakoid ATPase, chloroplasts were illuminated 3–5 min with white light (500–1000 μmol quanta m^{-2} s^{-1}) and then osmotically shocked in the light by adding a medium containing 7 mM $MgCl_2$ and 15 mM Hepes (pH 7.6). After 30 s the same amount of a medium containing 0.6 M sorbitol, 7 mM $MgCl_2$, 100 mM KCl, 15 mM Hepes (pH 7.6) and 30 μM ATP was added in the dark. The final chlorophyll concentration was 10–20 μg Chl/ml. Broken chloroplasts were uncoupled by 10 mM NH_4Cl or 2 μM nigericine. The transthylakoidal pH gradient was calculated from 9-aminoacridine fluorescence [21].

pH-dependent quenching in PS II particles

PS II particles (BBY) were prepared from spinach according to Ref. 22 and modified as in Ref. 23. The preparations contained no detectable contamination with PS I, as tested by gel electrophoresis and fluorescence spectroscopy at 77 K. pH-dependent quenching was obtained by incubating the particles in a medium containing 0.3 M sorbitol, 50 mM KCl, 5 mM $MgCl_2$ and 30 mM of different buffer substances: glycylglycine (pH 4.0–4.5), succinic acid (pH 4.5–5.0), Mes (pH 5.0–6.5), Mops (pH 6.5–7.0). The particles were incubated for 15 min in the different media before starting a measurement.

PS II activity was measured with a Clark-type oxygen-electrode, using 1 mM 1,5-dimethylbenzoquinone as acceptor at saturating light intensities ($I = 3000$ μmol quanta m^{-2} s^{-1}). Usually, the maximal rate of oxygen evolution in BBY-preparations was about 350 μmol O_2/mg Chl per h. Stationary fluorescence was measured with a pulse modulation fluorometer (PAM 101, Walz). The F_m level of fluorescence (with reduced Q_A^-) was recorded during short (1–2 s) saturating light pulses. Actinic light (190 μmol quanta m^{-2} s^{-1}) from a halogen lamp was filtered through Calflex C, K 65 (Balzers) and RG 630 (Schott) filters. Illumination was started with a Compur-photoshutter. Fluorescence induction was measured following direct fluorescence emission with a photomultiplier (EMI 9558B) protected by the filters RG 9 (Schott) and 742 nm interference filter (Balzers). The signal was recorded by a transient recorder (Vuko VKS 22-16).

Chlorophyll fluorescence-lifetime measurements were carried out and analyzed as described by Moya and coworkers [24]. The source of light consists of a mode-locked and cavity-dumped dye laser system (dye Rhodamine 6 G) synchronously pumped by a mode-locked Ar^+ laser. This system provides pulses of 10–15 ps duration at a repetition rate up to 4 MHz (0.8 MHz in the present measurements). The excitation wavelength was usually set at 630 nm. Fluorescence was measured at 90° to the excitation beam in a 2 × 2 mm cuvette situated at the place of the entrance slit of a monochromator (Jobin & Yvon M25). The band width was 3 nm and the analysis wavelength was 681 nm. In order to ensure the best elimination of the excitation light, a red filter was introduced behind the monochro-

mator (Schott RG 665, 3 mm), which was removed when measuring the scattered light. The detector was a micro-channel plate photomultiplier tube (Hamamatsu R1564U with S20 spectral response) kept at −30°C by a Peltier cooler in order to reduce the dark counts to about 5–10 counts/s. As a consequence no background was recorded in the time window used (20 to 40 ns). This time window is necessary to follow the complete decay processes in the ns range. Under these conditions, the full-width at half maximum of the instrumental response function was approx. 60 ps, when scattered light was examined instead of fluorescence. The counting rate was set at 5000 counts/s for both flash and fluorescence. The decays were accumulated over 1024 channels in a multichannel analyzer (Tracor Northern 1750) with 10^4 to $3 \cdot 10^4$ counts at the peak channel. Each measurement was repeated several times until reproducibility of the results was reached. Deconvolution of the decays into a sum of exponential was carried out on line by means of a least-squares programme using the Marquardt search algorithm for the non linear parameters. Quality of the fits were judged by the reduced χ^2 and the distribution of the weighted residuals.

The single-photon counting apparatus and deconvolution programme has been checked by measuring and analyzing the fluorescence decay of oxazine in methanol [25]. It was found, with this dye, that a single exponential decay with a lifetime of 785 ps was enough to fit the data and to obtain a reduced χ^2 of about 1. Deconvolution on the basis of four or five components, with lifetimes and amplitudes similar to those found under our experimental conditions, was tested in Ref. 25, using simulated decay curves in the presence of a gaussian noise.

Fluorescence decay at F_0 and intermediary levels were measured with a flow system. Intermediary fluorescence levels were produced by preillumination – to reduce Q_A – and by allowing the sample to flow at different flow rates. During time-resolved measurements the level of stationary fluorescence was measured simultaneously with a PAM-fluorimeter.

Results

To investigate the influence of a ΔpH on chlorophyll fluorescence, we used an artificial experimental system, in which a high ΔpH across the thylakoid membrane was created by hydrolysis of ATP (see Figs. 1 and 2). Provided the thylakoid ATPase is preactivated and ATP is added, ATP hydrolysis starts and protons are pumped into the thylakoid lumen via the cF_0 protein of the ATPase. Under such conditions a proton gradient is built up in the dark [26]. We preactivated the ATPase by illuminating intact chloroplasts. When the chloroplasts were ruptured in the light the

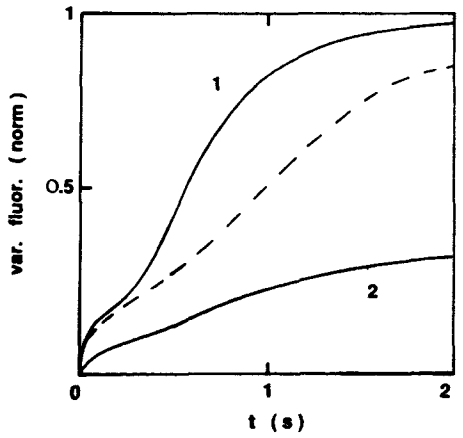


Fig. 1. Induction of variable fluorescence (total fluorescence minus the initial, F_0, fluorescence) in the absence (curve 1) or presence (curve 2) of a ΔpH. The initial (F_0) fluorescence at the beginning of illumination was set as zero, the maximal amplitude of variable fluorescence in uncoupled samples (absence of a ΔpH, curve 1) was set as one. Dashed line: curve 2 normalized on the maximal amplitude of curve 1. In uncoupled samples, the ratio F_m / F_0 was about 4. In presence of a ΔpH ('high energy state', curve 2) the maximal amplitude of the variable fluorescence was about 0.35. A ΔpH was pre-established in the dark by ATP hydrolysis, as described in the text. Uncoupled samples were pretreated, as for curve 2, but 2 s before starting the measurement, 2 μM nigericine has been added.

activated state of the ATPase was preserved in the dark and a ΔpH driven by ATP hydrolysis was stable over a period of 20 to 30 min. The inner thylakoidal pH could be as low as 4.5 (estimated by means of 9-aminoacridine fluorescence quenching and corrected considering Ref. 27). The ATP-induced ΔpH was not affected by inhibitors of the electron transport, such as DCMU, but was highly sensitive to uncouplers (data not shown). In the presence of an ATP-driven ΔpH the maximal level of chlorophyll fluorescence, F_m, (fluorescence level in saturating light), was lowered by 30–70% depending on the preparation , but the fluorescence completely recovered within a few seconds after the addition of an uncoupler.

The light-induced fluorescence increase from F_0 level (Q_A oxidized) to the F_m level in the presence of a ΔpH ('high energy state') and after addition of an uncoupler is shown in Fig. 1. There was no detectable change in the F_0 level of fluorescence, however, the maximal level of the variable fluorescence (F_m–F_0) was quenched by 66% in the high energy state. Also the increase from F_0 to F_m was slowed down (see dashed line). In both energized and uncoupled thylakoids, the slope of the F_0 to F_m rise increased with the intensity of the actinic light, but the F_m level, relative to F_0, was not affected by light (not shown).

pH-dependent quenching is actually related to the absolute H^+ concentration in the thylakoid lumen, rather than to the ΔpH [5,28]. In Fig. 2, the depen-

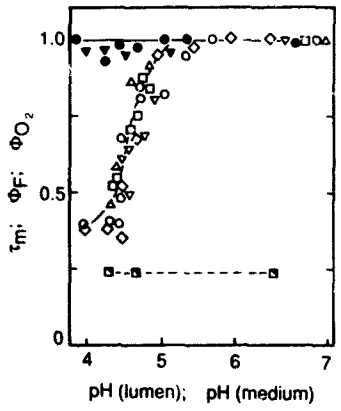


Fig. 2. Dependency of photoreduction of Q_A, oxygen evolution, stationary fluorescence and life time of fluorescence decay on the pH in the thylakoid lumen (isolated thylakoids) or on the pH of the suspension medium (PS II particles). □, Φ_P = rel. quantum yield of photoreduction of Q_A of thylakoids (calculated from the growth of the complementary area above fluorescence induction as in Fig. 1) vs. pH in the thylakoid lumen: a ΔpH was induced by hydrolysis of ATP, the pH in the medium was 7.6; △, Φ_{O_2}, maximal rate of oxygen evolution of thylakoids in saturating light (in presence of 1,5-dimethylbenzoquinone) vs. the pH in the lumen. The ΔpH was induced by light. At pH 7.0 (lumen) a rate of 680 μmol O_2 mg Chl^{-1} h^{-1} has been measured. ▽, Φ_f, rel. yield of fluorescence in thylakoids during a saturating light pulse vs. pH in the thylakoid lumen, ΔpH induced by hydrolysis of ATP. ◇, Φ_F, rel. yield of fluorescence in PS II particles during a saturating light pulse vs. pH in the medium. ◇, average lifetime of fluorescence decay τ_m, in PS II particles in the F_m state vs. the pH in the media. ◹, lifetime of fluorescence decay, τ_m, in PS II particles in the F_0 state vs. the pH in the medium. ▼ Φ_F (in saturating light) of PS II particles in presence of the donor DPC (1 mM) vs. the pH in the medium. ●, Φ_F (in saturating light) of PS II particles in presence of 5 mM ascorbate vs. the pH in the medium.

dency of the maximal fluorescence (in a saturating light pulse F_m) on the pH in the lumen is shown. Fluorescence was normalized by setting the maximal value at pH 6.5 as 1. Different values for ΔpH have been obtained by addition of small amounts of the weak uncoupler NH_4Cl. Fluorescence decreased at pH < 5.5 in the lumen. Basically, the same pH response of fluorescence was seen when a ΔpH was built up in the light in the presence of an electron acceptor (see Fig. 6 in Ref. 1).

We also isolated PS II particles from chloroplasts and suspended them in media with pH values between 4.0 and 7.0. Both donor and acceptor side were then exposed to the pH indicated. Nevertheless, the pH-response of particles was similar to that obtained from thylakoids where only the donor side was exposed to various pH values. Best fit of the pH-response of fluorescence was obtained by the assumption of a 1 H^+ transition. The apparent pK value of the variable fluorescence could vary in different preparations between 4.5 and 4.8, while only little variation in the slope of the titration curve was seen.

The pH response of the average lifetime with closed centers, τ_m, obtained from time-resolved fluorescence decay, followed that of the stationary fluorescence, i.e., τ_m was directly proportional to the maximal stationary fluorescence, F_m. In the F_0 state (when Q_A was oxidized) no change in τ_m could be seen, suggesting, that the F_0 level of fluorescence is not affected by variation in pH This seems to contradict observations with intact systems, where 'energy-dependent' quenching is often accompanied by a significant decline in the dark-level level of fluorescence [2,15,19].

Addition of artificial electron donors to PS II, like ascorbate or DPC, completely reversed the F_m quenching in PS II particles at low pH (Fig. 2). Reversal of

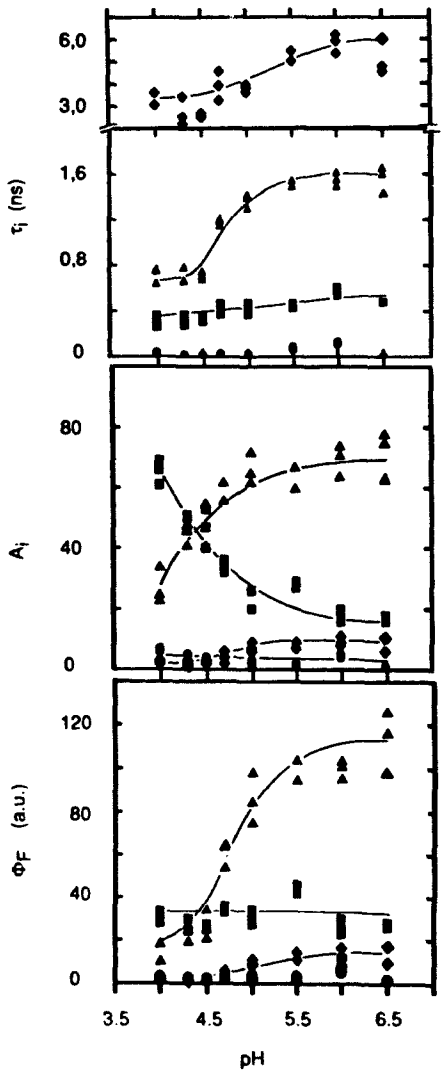


Fig. 3. Dependency of the lifetime (τ_i), the relative amplitude (A_i) and the relative quantum yield (Φ_F) of the four deconvoluted decay components in PS II particles (measured in the F_m state) on the pH in the medium ● C_1, ■ C_2, ▲ C_3, ♦ C_4.

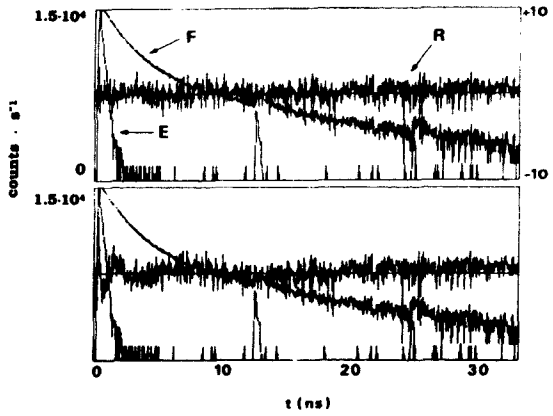


Fig. 4. Excitation profile and fluorescence decay at 681 nm at F_m. E: excitation, F: fluorescence decay, R: distribution of weighted residuals, scale + 10 to − 10. Lower curve, deconvolution with three kinetic components, $\chi^2 = 1.30$; upper curve, deconvolution with four kinetic components, $\chi^2 = 1.03$.

pH-dependent quenching by ascorbate has already been demonstrated [29].

The photosynthetic oxygen evolution of PS II, measured at saturating light intensities in presence of 1.5-dimethylbenzoquinone decreased in parallel with fluorescence quenching (Fig. 2), i.e., the capacity of PS II to evolve oxygen is affected. This agrees well with previous suggestions from analysis of steady-state photosynthesis, that energy-dependent quenching of fluorescence is related to an increase in the fraction of photochemically inactive PS II [1,2].

Fig. 3 shows the pH-response of the different decay components of time-resolved fluorescence of PS II particles in the F_m state, obtained by deconvolution of the overall fluorescence decay. We assume that, in the saturating light used, most centers were closed. In several studies, the sum of three decay components is supposed to be sufficient to fit the overall decay [30,31]. The data presented here could be adequately described only by the sum of four decay components. The upper limit of the time window used for the analysis was set to 30 or 40 ns. This allowed detection of long-living nanosecond components ($\tau > 2$ ns). Fig. 4 shows the deconvolution of fluorescence decay and recording of χ^2 values, using three and four decay components, respectively.

The four components (C_1–C_4) were characterized by their specific lifetimes and relative amplitudes. At pH 6.5, the individual lifetimes, τ_i, were identified as $\tau_1 = 30$ ps, $\tau_2 = 450$ ps, $\tau_3 = 1.7$ ns and $\tau_4 = 5$ ns, and at pH 4.0 as $\tau_1 = 30$ ps, $\tau_2 = 380$ ps, $\tau_3 = 650$ ps and $\tau_4 = 2.5$ ns. As shown in Fig. 3, τ_3 and τ_4 were lowered by 50% at pH values < 5.5, while τ_1 and τ_2 did not vary significantly.

Relative amplitudes (A_i, pre-exponential factor), of the different components are also shown in Fig. 3. A_3 decreased from 65% at pH > 5.0 to 25% at pH 4.0. Also A_4 decreased at low pH values, whereas A_2 seemed to have increased and A_1 remained relatively constant. To interpret these data, it is necessary to know that, for each pH value, the total sum of amplitudes has been set to 100%, irrespective of the amount of emitted fluorescence. For example, the absolute

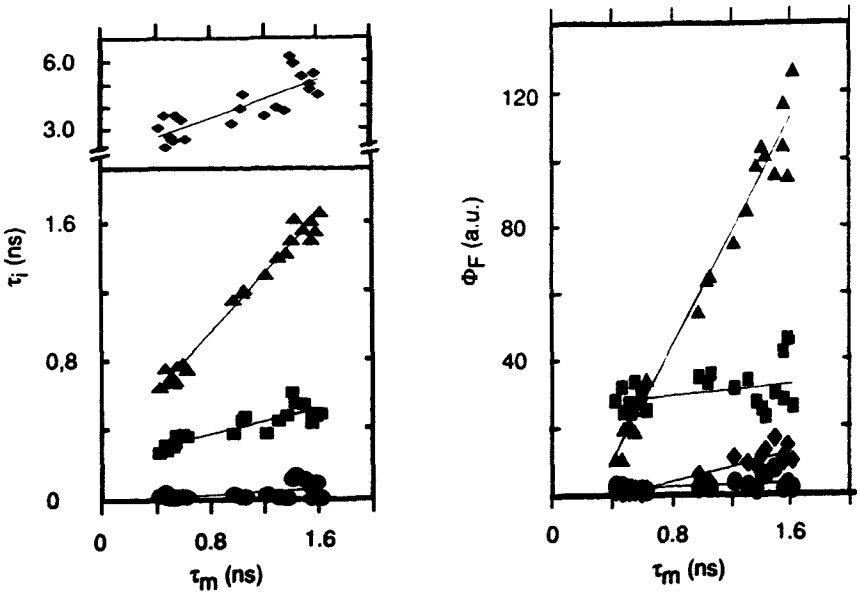


Fig. 5. Relationship between the avarage lifetime, τ_m, and the specific lifetimes (left side) and the relative quantum yields (right side) of the four decay components in PS II particles. The pH was varied. Data from Fig. 3, symbols as in Fig. 3.

TABLE I

Variable part of the stationary fluorescence, F_v (maximal fluorescence in a saturating light pulse minus F_0) and fluorescence decay components at pH 6.5 and 4.2 in isolated PS II particles: the influence of artificial donors.

Symbols as explained in the text: τ = ns, F = arbitrary units. F_v at pH 6.5 was set as 1.

pH	Artificial donors	F_v (norm.)	τ_m	τ_1	τ_2	τ_3	τ_4	Φ_1	Φ_2	Φ_3	Φ_4
6.5	–	1	1.6	0.02	0.57	1.5	4.2	0.4	32	117	19
	5 mM ascorbate	1.2	1.97	0.03	0.74	1.8	5.5	0.6	15	122	61
4.2	–	0.25	0.55	0.01	0.36	0.8	2.3	0.2	30	62	7
	5 mM ascorbate	0.96	1.94	0.03	0.80	1.9	6.2	0.6	23	112	60
	30 mM $K_4[Fe(CN)_6]$	0.75									
	10 mM KI	0.94									
	1 mM DPC	1									

emission of C_2 did not really change with pH, but its relative contribution to the total emission increased (65% at pH 4.0, compared to 25% at pH 6.5).

The specific contribution of each component to the total fluorescence at each pH is given by the yield $\Phi_i = A_i * \tau_i$ (Fig. 3). Φ_3 decreased from 73% at pH 6.5 to about 9% at pH 4.2, while Φ_1 and Φ_2 remained relatively constant. Φ_4 varied parallel to Φ_3, but the overall contribution of this component to the total emission was low ($\Phi_4 < 9\%$ at pH 6.5). The data shown in Fig. 3 have been used to plot the average lifetime, τ_m vs. τ_i and Φ_i (where τ_m varies in parallel with F_m, see above). A proportional change of τ_3 and Φ_3 with τ_m was seen, while the other components remained relatively constant (Fig. 5). Summarizing, the data indicate, that the pH-dependent decline in τ_m (and in stationary fluorescence) is mainly due to a decrease in the lifetime and amplitude of C_3.

As already shown in Fig. 2, upon addition of artificial electron donors at low pH, fluorescence reappeared. Table I shows, that even donors with relatively high redoxpotentials like $K_4[Fe(CN)_6]$ ($E_m = +360$ mV), DPC ($E_m = +440$ mV) and KI ($E_m = +560$ mV) were efficient. After addition of ascorbate at low pH, the fluorescence parameters τ_i and Φ_i were restored to their original values (pH 6.5, absence of artificial donors). τ_4 and Φ_4 even increased compared to their original values. The component C_4 may reflect charge recombination between $P\text{-}680^+$ and $Pheo^-$ in the reaction center [32–34], and this recombination could be stimulated by artificial electron donors. A further characterization of the C_4 component will be the subject of a following paper.

Time-resolved fluorescence has also been analyzed in isolated thylakoids in the presence of an ATP-induced ΔpH (Fig. 6). The ΔpH was varied by altering

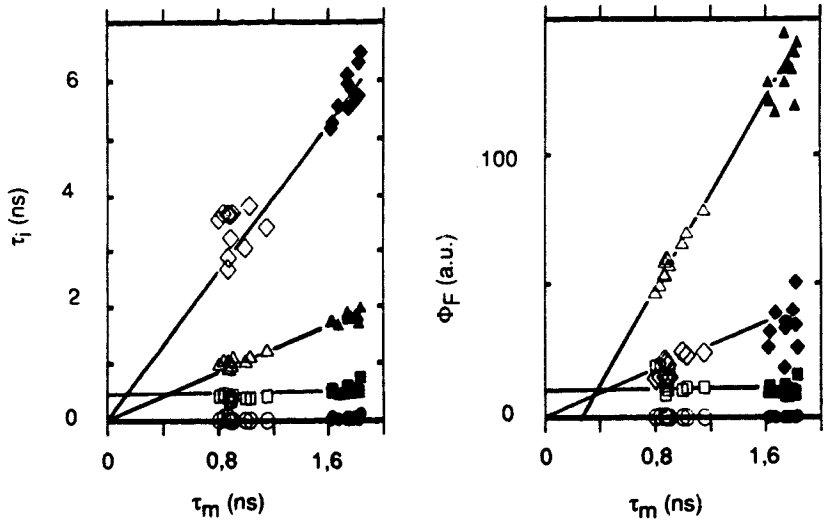


Fig. 6. Relationship between the avarage lifetime, τ_m, and the specific lifetimes (left side) and the relative quantum yields (right side) of the four decay components in thylakoids. Open symbols: different ΔpH values as induced by hydrolysis of ATP. Closed symbols: samples measured in presence of NH_4Cl. Different decay components denoted as in Fig. 3.

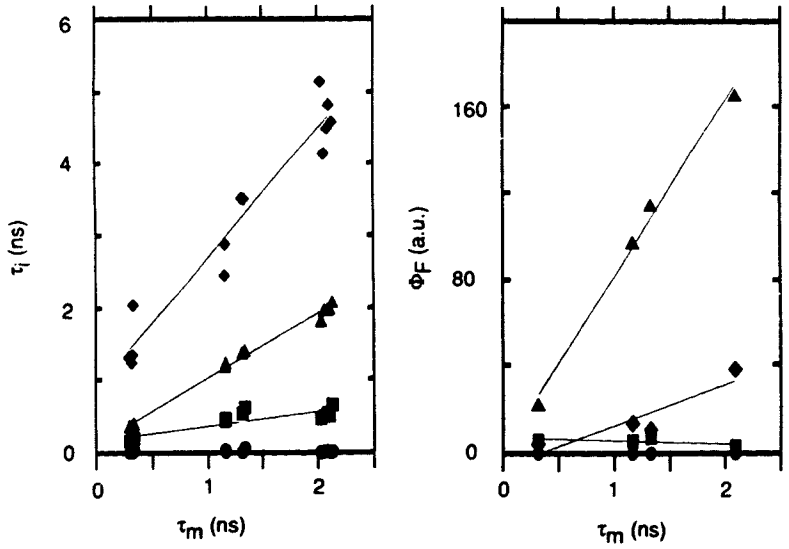


Fig. 7. The relationship between the avarage lifetime, τ_m, of the specific lifetimes (left side) and the quantum yields (right side) of the four decay components in thylakoids in the presence of an uncoupler (NH_4Cl). The intensity of the background light was varied to obtain different states between F_0 and F_m (gradual reduction of Q_A). Symbols as in Fig. 3.

the activation state of the ATPase (by different preillumination times). The lowest pH value in the lumen, as estimated by 9-aminoacridine fluorescence, was 4.3. In the uncoupled state (10 mM NH_4Cl) the individual lifetimes of the components C_1–C_4 in the F_m state were: $\tau_1 = 30$ ps, $\tau_2 = 500$ ps, $\tau_3 = 1.9$ ns and $\tau_4 = 6$ ns. Keuper and Sauer [35] observed similar values with thylakoid membranes, with the exception of the 6 ns component, which was not described by these authors. Changes in fluorescence parameter, as seen when an ATP-dependent ΔpH was formed, were similar to those observed by suspending PS II particles at low pH: τ_3 and Φ_3 (and τ_4 and Φ_4) declined with τ_m while few or no changes of the components C_2 and C_1 were seen (Fig. 6).

The pattern of changes in lifetime and quantum yield of decay components as seen here in saturating light (F_m state) by decreasing the pH below 5.5 (Fig. 6), was similar to that usually observed going at moderate pH (6.5) from the F_m state (closed centers) to the F_0 state (open centers). F_0, and intermediate levels of fluorescence, were measured with a flow system, where the time of preillumination (before the sample entered the measuring chamber) was varied (see Fig. 7 and similar results in Ref. 32). In both cases, the slope of τ_i and Φ_i vs. τ_m was nearly identical, except that, in the case of pH-dependent quenching, the intersection point with the x-axis was slightly different (compare Figs. 6 and 7). Obviously, there is a residual level of the component C_3 which seemed to be insensitive to low pH values.

The data shown in Table II demonstrate that, in the F_0 state (oxidized Q_A), high ΔpH across the thylakoid membrane had no detectable effect on values for τ_i and Φ_i. It strongly supports the suggestion that changes in the pH do not affect fluorescence in the F_0 state (see also Figs. 1 and 2).

Discussion

The analysis of chlorophyll fluorescence decay kinetics of intact photosynthetic organisms can be statistically well defined by at least four exponential decay components [25]. More recent studies, based on isolated membranes enriched in certain complexes or well-characterized mutants, suggest at least two fast components for PS I and three components for PS II [36,37]. Although we may expect some contribution of

TABLE II

Fluorescence decay components in presence or absence (+ NH_4Cl) of a ΔpH in thylakoids in the F_0 state.

Symbols as explained in the text: τ = ns, Φ = arbitrary units.

	τ_m	τ_1	τ_2	τ_3	τ_4	Φ_1	Φ_2	Φ_3	Φ_4
Control	0.30	0.03	0.18	0.40	1.3	0.4	10	20	6
ΔpH	0.31	0.03	0.20	0.45	1.4	0.4	10	20	5

PS I emission in thylakoids it was not separated from the picosecond components, C_1 and C_2. The wavelength of detection (681 nm) was selected to optimize PS II emission and, thus, contribution of PS I was expected to be very low. In BBY preparations we did not expect significant contribution of PS I, anyway.

The 0.8–1.6 ns component (C_3), responsible for most of the appearance of variable fluorescence upon closure of centers, may reflect the sum of decay processes in the antennae, provided the photochemical trapping process at centers is blocked by Q_A^-. In our preparations, we consistently found a slow component, C_4 ($\tau_i = 5$–10 ns); however, its contribution to the overall emission was low. Perturbations of the lipid–protein interaction within the thylakoid membrane could generate lifetime components in the 4–5 ns range by artificially disconnected chlorophyll proteins [38]. In intact systems luminescence components may also appear, under conditions where recombination between P-680$^+$ and Pheo$^-$ is stimulated [34].

When traps were opened to various degrees (between the F_m and F_0 levels) lifetime and yield of the ns-decay components decreased in parallel with average lifetime, τ_m of fluorescence (Fig. 7, see Refs. 25, 35). The individual lifetimes of the ns-decay components decrease with an increased fraction of open centers, probably as a consequence of energy exchange between connected PS II antennae. In a system with connected antennae, the effective size of antennae, and the probability that excitation energy is located in the antennae, decreases when the fraction of 'quenching' centers increases [25]. The actual lifetime of each decay component depends on the effective size of antennae connected to a 'quenching' center [39]. We assume a certain degree of energy exchange even in BBY preparations, which may consist of membrane fragments rather than of isolated PS II units. The assumption that PS II units are connected and energy exchange occurs in BBY preparations is further supported by the observation that fluorescence induction curves in the presence of DCMU were usually sigmoidal (not shown).

Similar to quenching by 'opening' centers, a specific decline in the two ns components C_3 and C_4 (with C_1 and C_2 remaining almost constant) is also seen by lowering the pH (Figs. 3, 5, 6). In both cases, going from the non-quenched state (F_m) to the quenched state, lifetime and yield of C_3 and C_4 decrease linearly with the average lifetime (compare Figs. 5, 6, 7). In this respect, pH-dependent quenching resembles 'photochemical' quenching by Q_A and the question arises whether this similarity points to a similar quenching mechanism.

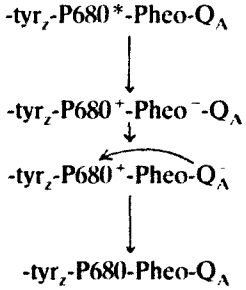
An important criterion to interpret the data is insensitivity of the F_0 fluorescence. In the F_0 state, when excited states from the antennae are trapped and deactivated by a fast photochemical process at the reaction centers, the fluorescence mainly consists of two picosecond components, C_1 and C_2. Low pH had no significant effect on these components, and also, the F_0 level of stationary fluorescence remained unaffected (see Table II). An insensitivity of F_0 on pH in BBY preparations has already been reported by Crofts and Horton [40]. It can best be explained by the assumption that, at least in the experimental system we used, neither the energy transfer from the antenna to centers, nor the trapping efficiency of PS II centers is affected by acidification, nor is the effective absorption cross-section altered. Also, other quenching mechanisms which would effectively compete with photochemical quenching of centers as, for example, 'antennae quenching' (see Ref. 41), are unlikely to occur, as they would affect the F_0 fluorescence. As 'energy-dependent' quenching is caused by internal acidification rather than by the transmembrane proton gradient [5,28], it is expected to affect, primarily, the donor side of PS II. The reversal of fluorescence quenching at low pH values by artificial electron donors (Fig. 2; Table I, see also Ref. 40) actually indicates a donor side limitation of PS II: electrons from artificial donors may replace electrons from the water-splitting side and, thus, restore photoaccumulation of Q_A^- and 'variable' fluorescence at low pH values.

Inhibition of the water-splitting system at low pH was shown by several groups [12–14,42,43] and Schreiber and Neubauer [6] already discussed the possibility that energy-dependent quenching is induced by a donor side limitation of PS II. Ono and Inoue [13,14] provided evidence that inactivation at low pH is caused by a release of calcium from the water-splitting complex (see also Ref. 44). The apparent pK value of inactivation described by these authors is similar to that shown in Fig. 2 for fluorescence quenching and inactivation of oxygen evolution. In the Ca-depleted state, the formation of higher S states is inhibited [14,45]. As a consequence, a positive charge is stabilized on the donor side and the reduction of P-680$^+$ by tyr$_z$ is inhibited [46]. Actually, Krieger and Weis [11] proposed that reversible Ca-release by internal acidification is a primary step to induce 'high-energy quenching'.

The question remains to be answered as to how excitation energy absorbed by centers with an inactive donor side is 'de-excited'. As a possible explanation Schreiber and Neubauer [9] proposed the recombination of the primary radical pair, P-680$^+$ Pheo$^-$ to the triplet state, ^{3}P-680 · Pheo. However, at least in open centers (Q_A oxidized) this sort of recombination is unlikely to occur as the fast charge stabilizing electron transfer from Pheo$^-$ to Q_A may efficiently compete with P-680$^+$ · Pheo$^-$ recombination, irrespective of whether the donor side is active or not. In closed

centers (Q_A reduced) primary charge separation, P-680$^+$· Pheo$^-$ has been assumed to be suppressed, anyway [30,47]. Also, triplet state formation associated to P-680$^+$· Pheo$^-$ recombination is known to cause potentially damaging oxidative side-reactions and additional mechanisms would be required to quench these states safely. Finally, the long-living nanosecond component, shown in this study (C_4), possibly reflecting P-680$^+$· Pheo$^-$ recombination [34] does not increase, but even decreases, upon acidification (Fig. 3).

We propose an alternative recombination model, in which it is assumed that, in both the active (oxygen-evolving) state and the inactive, 'high energy state' of PS II, energy is trapped by the radical pair, P-680$^+$· Pheo$^-$ formation, followed by the regular stabilizing electron transfer from Pheo$^-$ to Q_A. In active centers, recombination is then suppressed by rapid (20 ns) reduction of P-680$^+$ by tyr_z. In centers with an inactive donor side, however, P-680$^+$ is stabilized and the formation of the state P-680$^+$· Pheo · Q_A^- may then be followed by an electron transfer from Q_A^- to P-680$^+$, either directly or via an unknown route. There is actual experimental evidence for a lengthening of the P-680$^+$ reduction time from the nanosecond to the microsecond range upon acidification [12,42,43] or Ca depletion [46], and the microsecond relaxation of P-680$^+$ has been suggested to indicate recombination of this radical with Q_A^-. This may become the dominant reduction reaction when electron transfer from tyr_z to the center is inhibited as proposed for a Ca-depleted state [46]. Recently, it has been shown that inactivation by low pH [14] or by internal acidification [11] is actually induced by Ca depletion. Recombination in the low pH state may also be favored by an increase in the redox potential of Q_A by 150 mV [11]. For some reasons, in the low pH state the free energy change related to recombination of P-680$^+$· Q_A^- may not be sufficient to create excited states of the reaction center and, thus, energy would be lost as heat. A scheme of the route of de-excitation in the 'high energy state' proposed in this model is shown in the following:



As in this model, excitation energy in inactive (non-oxygen evolving, non-fluorescent) centers is trapped by a regular charge separation process (as in oxygen evolving centers). The similarity in the pattern of fluorescence changes (compare Figs. 5–7) may actually reflect a similarity in the trapping process in both active and inactive centers. In inactive centers, even at high irradiance, the acceptor Q_A would be kept in the oxidized state by a recombination process. This recombination process is induced by a blockage of electron donation from tyr_z. No additional quenching processes, either in the center or in the pigment matrix, are required to explain the data shown here. By this mechanism excitation energy not required for photosynthesis, could be safely dissipated at the center.

Yet the question remains to be answered whether the P-680$^+$· Q_A^- recombination model also applies to high energy quenching in vivo. We are aware of an apparent discrepancy between the insensitivity of F_0 reported here (Table II) and data from intact systems, where an energy-dependent decline in the dark-level fluorescence was frequently observed (e.g., Refs. 2, 15, 19). For intact systems, alternative mechanisms have been proposed, which include quenching processes in the pigment matrix (e.g., Refs. 15, 16, 18). Very recently, Horton and co-workers [48] proposed a mechanism by which excess excitation energy is dissipated by quenching at the level of antennae pigments caused by a structural rearrangement (aggregation) of light-harvesting complexes. As discussed above, antennae quenching is not consistent with the data of fluorescence lifetime shown here. Perhaps, for some reason, pH-dependent light harvesting complex aggregation does not appear in thylakoid membranes under the particular conditions used in our study. We cannot exclude the possibility that in the intact system different mechanisms are operating in concert and amplify each other. Also, a preexisting mechanism could be modified by additional mechanisms such as the zeaxanthin cycle [18]. To clarify the problem, we obviously need a more rigorous analysis of the quenching process in intact systems.

Acknowledgements

The authors are most grateful to U. Mukherjee for reading and correcting the manuscript. Part of the work was supported by the Deutsche Forschungsgemeinschaft. This forms part of the PhD thesis of A.K.

References

1 Weis, E. and Lechtenberg, D. (1989) Phil. Trans. R. Soc. London B 323, 253–268.
2 Weis, E. and Berry, J.A. (1987) Biochim. Biophys. Acta 894, 198–208.
3 Krause, G.H., Laasch, H. and Weis, E. (1988) Plant Physiol. Biochem. 26, 445–452.
4 Horton, P. and Hague, A. (1988) Biochim. Biophys. Acta 932, 107–115.

5 Krause, G.H. and Weis, E. (1991) Annu Rev. Plant Physiol. Plant Mol. Biol. 42, 313–349.
6 Schreiber, U. and Neubauer, Ch. (1987) Z. Naturforsch. Teil C 42, 1255–1264.
7 Noctor, G. and Horton P. (1990) Biochim. Biophys. Acta 934, 135–143.
8 Oxborough, K. and Horton, P. (1988) Biochim. Biophys. Acta 934, 135–143.
9 Schreiber, U. and Neubauer, C. (1990) Photosynth. Res. 25, 279–293.
10 Krieger, A. and Weis, E. (1990) in Current Research in Photosynthesis (Baltscheffsky, ed.), Vol. I.2, pp. 563–566, Kluwer, Dordrecht.
11 Krieger, A. and Weis, E. (1992) Photosynthetica 27, 1, in press.
12 Schlodder, E. and Meyer, B. (1987) Biochim. Biophys. Acta 890, 23–31.
13 Ono, T. and Inoue, I. (1988) FEBS Lett. 227, 147–152.
14 Ono, T. and Inoue I. (1989) Biochim. Biophys. Acta 973, 443–449.
15 Genty, B., Harbinson, J., Briantais, J.-M. and Baker, N.R. (1990) Photosynth. Res. 25, 249–257.
16 Ruban, A.V., Rees, D., Noctor, G.D., Young, A., Horton, P. (1991) Biochim. Biophys. Acta 1059, 355–360.
17 Yamamoto, H.Y. (1979) Pure Appl. Chem. 51, 639–648.
18 Demmig-Adams, B. (1990) Biochim. Biophys. Acta 1020, 1–24.
19 Rees, D., Noctor, G.D. and Horton, P. (1990) Photosynth. Res. 25, 199–211.
20 Lausch, H. (1987) Planta 171, 220–226.
21 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.
22 Berthold, D.A., Babcock, G.T. and Yokum, C.F. (1981) FEBS Lett. 134, 231–234.
23 Völker, M., Ono, T. and Renger, G. (1985) Biochim. Biophys. Acta 806, 25–34.
24 Moya, I., Hodges, M. and Barbet, J.-C. (1986) FEBS Lett. 198, 256–262.
25 Hodges, M. and Moya, I. (1986) Biochim. Biophys. Acta 849, 193–203.
26 Avron, M. and Schreiber, U. (1979) Biochim. Biophys. Acta 546, 436–447.
27 Strotmann, H. and Lohse, D. (1988) FEBS Lett. 229, 308–312.
28 Briantais, J.-M., Vernotte, C., Picaud M. and Krause, G.H. (1979) Biochim. Biophys. Acta 548, 128–138.
29 Crofts, J. and Horton, P. (1990) in Current Research in Photosynthesis, (Baltscheffsky, ed.), Vol. I.2, pp. 394–397, Kluwer, Dordrecht.
30 Schatz, G.H., Brock, H. and Holzwarth, A.R. (1987) Proc. Natl. Acad. Sci. USA 84, 8414–8418.
31 Hodges, M. and Moya, I. (1987) Biochim. Biophys. Acta 892, 42–47.
32 Klimov, V.V., Shuvalov, V.A. and Heber, U. (1985) Biochim. Biophys. Acta 809, 345–350.
33 Schlodder, E. and Brettel, K. (1990) in Current Research in Photosynthesis, (Baltscheffsky, M., ed.), Vol. I.2, pp. 447–450, Kluwer, Dordrecht.
34 Shuvalov, V.A., Klimov, V.V., Dolan, E., Parson, W.W. and Ke, B. (1980) FEBS Lett. 118, 279–282.
35 Keuper, H.J.K. and Sauer, K. (1989) Photosynth. Res. 20, 85–103.
36 Hodges, M. and Moya, I. (1987) Biochim. Biophys. Acta 893, 480–489.
37 Holzwarth, A.R. (1990) in The Chlorophylls CRC Handbook, (Scheer, H., ed.), pp. 1125–1151, CRC Press, Boca Raton.
38 Bassi, R., Silvestri, M., Dainese, P., Moya, I. and Giacometti, G.M. (1992) Photochem. Photobiol 9, 335–354.
39 Joliot, A. and Joliot, P. (1964) CR Acad. Sci. 258, 4622–4625.
40 Crofts, J. and Horton, P. (1991) Biochim. Biophys. Acta 1058, 187–193.
41 Boussac, A., Hodges, M., Briantais, J.-M., and Moya, I. (1989) Photosynth. Res. 20, 173–189.
42 Meyer, B., Schlodder, E., Dekker, J.P. and Witt, H.T. (1989) Biochim. Biophys. Acta 974, 36–45.
43 Pulles, M.P.J., Van Gorkom, H.J. and Verschoor, G.A.M. (1976) Biochim. Biophys. Acta 440, 98–106.
44 Homann, P.H. (1988) Biochim. Biophys. Acta 934, 1–13.
45 Boussac, A. and Rutherford, A.W. (1988) FEBS Lett. 236, 432–436.
46 Boussac, A., Sétif, P. and Rutherford, A.W. (1992) Biochemistry 31, 1224–1233.
47 Schatz, G.H., Brock, H. and Holzwarth, A.R. (1988) Biophys. J. 54, 8414–8418.
48 Horton, P., Ruban, A.V., Rees, D., Pascal, A.A., Noctor, G. and Young, A.J. (1991) FEBS Lett. 292, 1–4