Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1709 (2005) 138 – 149
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

The size of the population of weakly coupled chlorophyll pigments involved in thylakoid photoinhibition determined by steady-state fluorescence spectroscopy

Stefano Santabarbara[a,b,*], Robert C. Jennings[a]

[a] *Istituto di Biofisica del CNR, Sezione di Milano, Dipartimento di Biologia, Universita' degli Studi di Milano, via Celoria 26, 20133 Milano, Italy*
[b] *School of Biological Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK*

Received 20 April 2005; received in revised form 24 May 2005; accepted 3 June 2005
Available online 27 June 2005

Abstract

On the basis of experiments with singlet quenchers and in agreement with previous data, it is suggested that a population of energetically weakly coupled chlorophylls may play a central role in photoinhibition in vivo and in vitro. In the present study, we have used steady state fluorescence techniques to gain direct evidence for these uncoupled chlorophylls. Due to the presence of their emission maxima, near 650 nm and more prominently in the 670–675 nm interval both chlorophylls *b* and *a* seem to be involved. A straightforward mathematical model is developed to describe the data which allows us to conclude that the uncoupled/weakly coupled population size is in the range of 1–3 molecules per photosystem.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Photoinhibition; Non-photochemical quenching; Fluorescence emission; Thylakoid; Uncoupled chlorophyll

1. Introduction

Plants in their natural environments are often exposed to high photon fluences which exceed one absorbed photon per photosystem per millisecond. This leads to saturation of Photosystem II electron transport due to the relatively slow kinetics of plastoquinone oxidation by the cytochrome b_6f complex, which is of the order of 0.05–0.1 ms^{-1} [1]. Under these conditions the excessive excitation may induce photodamage which decreases PSII photochemical activity, a phenomenon known as photoinhibition [2–4]. Photoinhibition is a complex process which involves oxidative damage to both the electron donor [5–7] and acceptor sides of PSII [8–10] and which seems to be primarily caused by singlet oxygen [11–14]. This reactive oxygen species is generated by an interaction between the ground state electrons of triplet oxygen with the triplet state of Chl, formed by intersystem crossing from either the first excited singlet state of Chl antenna or from the primary charge separated state involving P_{680} and pheophytin (the recombination triplet). While the rate constant for intersystem crossing in protein-bound Chls is quite high and competitive with that for the other trivial decay processes [15–17], the antenna triplet population is maintained at very low levels by carotenoid-induced quenching [17]. On the other hand, it is often suggested that the recombination triplet may be significantly populated under high light conditions and furthermore that this may give rise to photoinhibition [18–21]. This has led to the suggestion that the non-photochemical quenching (NPQ) of chlorophyll fluorescence may be one of the major protective strategies against photoinhibition by increased thermal dissipation in PSII antenna. Although the mechanism of NPQ has not been fully elucidated yet, the involvement of acidification of the lumenal pH, the

Abbreviations: Chl, chlorophyll; NPQ, non-photochemical quenching; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; DBMIB, 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone; DNB, m-dinitrobenzene; PSII, photosystem II; PSI, photosystem I; F_m, maximal fluorescence yield

* Corresponding author. School of Biological Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK. Fax: +44 20 8983 5489.

E-mail address: s.santabarbara@qmul.ac.uk (S. Santabarbara).

0005-2728/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2005.06.001

xanthophyll cycle pigments, in particular zeaxanthin (reviewed in e.g. [22]), and the PsbS protein in the developing of rapidly reversible NPQ has been demonstrated (recent review, e.g., [23]). However, there is evidence in the literature that this straightforward idea may not be exactly correct. Thus, Sinclair et al. [24] observed that the degree of photoinhibition in thylakoids was largely insensitive to both the PSII antenna size and the so-called high energy quenching (qE) of PSII fluorescence. It was also demonstrated that while PSII photoinhibition displayed a linear light dosage/response plot in isolated thylakoids [25–28] as well as in intact unicellular green algae [29,30] it was only slightly decreased by excited singlet state quenchers of antenna fluorescence [25–30]. In addition, quenching of the PSII excited state population by singlet antenna quenchers resulted in a very limited protection against D1 degradation [28]. Common to the interpretation of all these results is the suggestion that a small population of uncoupled antenna Chls may be present which could have a high triplet yield and hence give rise to photoinhibition. Evidence for the presence of such a population has been also obtained by analysis of the sensitivity of fluorescence emission to singlet excited state quenchers both by means of steady-state [27] and time-resolved [31] fluorescence spectroscopy. In addition, the detailed action spectrum of photoinactivation has been determined for isolated thylakoids [27,30]. This shows a blue shift of about 2 nm compared to the PSII photon absorption spectrum and it was suggested that the weakly coupled chlorophyll population may be associated with an heterogeneous population of damaged or incorrectly assembled Chl–protein complexes [27,30]. Comparison of action spectra in the *Chlorina* f_2 and the wild-type barley thylakoids [30] seemed to indicate the involvement of both inner- and outer-antenna complexes.

With respect to the above-mentioned suggestion of uncoupled chlorophylls giving rise to triplet populations, a recent systematic analysis of chlorophyll triplet states in thylakoids, not poisoned to reducing condition, has revealed the presence of several, hitherto undiscovered, long living triplet states associated with PSII. These triplet states are not associated with the P_{680} recombination triplet, on the basis of zero field splitting parameters [32]. They are detected under conditions in which the reaction centres are expected to be reduced, but not under the severely reducing condition necessary to detect the P_{680} recombination triplet. It has therefore been proposed that these triplets may be active in the photoinhibition. It seems likely that the triplet states observed in thylakoids are populated in Chl–protein complexes in which the chlorophyll–carotenoid coupling is imperfect and which may well correspond to those observed in the photoinhibition action spectra [27,30], and in the small fraction of Chl molecules involved in rapid oxygen uptake as described by Boichenko et al. [33]. This is also in agreement with singlet oxygen detection [34] in etiolated leaves which also display high levels of Chl triplets.

In the present study, we present further evidence for the existence of a small uncoupled/weakly coupled Chl population involved in photoinactivation by a refined analysis of steady-state fluorescence emission quenching which permits a partial characterisation of their fluorescence emission characteristics. The data are analysed in terms of a straightforward mathematical model, which considers both weak coupling and complete uncoupling and it is concluded that this population is in the range of 1–3 chlorophyll molecules per Photosystem II in freshly prepared chloroplasts from freshly harvested spinach leaves.

2. Material and methods

2.1. Sample preparation

Thylakoids were prepared from freshly harvested spinach leaves grown in a home built growth-chamber under controlled condition, including a 8:16 day–night light period at photon flux density of 150 μE m^{-2} s^{-1}. The leaves were harvested during the dark period after about 6 h and incubated in a cold room at 4 °C for half an hour before thylakoid isolation. The leaves were blended in an isotonic buffer (0.4 M Sucrose, 30 mM Tricine, 10 mM NaCl and 5 mM, $MgCl_2$, pH 7.8) and filtered through eight layer of cheesecloth. The material was then centrifuged at 1500×*g* for 5 min, the pellet resuspended in a hypotonic, sucrose free, buffer (30 mM Tricine, 10 mM NaCl and 5 mM, $MgCl_2$, pH 7.8), diluted to isotonicity and centrifuged again at 1500×*g* for 5 min. The pellet was then resuspended in a small volume of isotonic buffer at an approximate Chl concentration of 500 μg/ml [35]. Unstacked thylakoids membranes were prepared in the same way in media free of magnesium ions and in the presence of 1 mM EDTA.

2.2. Detergent treatment

Thylakoid membranes were incubated in medium containing 0.1 M sucrose, 30 mM Tricine, 10 mM NaCl, 5 mM, $MgCl_2$, pH 7.8 and 0.00125% w/v Triton X-100 under constant stirring for 15 min at 4 °C. The membranes were then centrifuged at 1500×*g* for 5 min. The supernatant was discarded and the pellet washed with about four times the initial incubation volume in the same medium without the non-ionic detergent Triton X-100, and then once again centrifuged at 1500×*g* for 5 min. The pellet was then resuspended and used for the analysis.

PSII enriched membranes were obtained as described by Berthold et al. [36], as modified by Dunahay et al. [37]. PSI-200 particles were prepared by *n*-octyl-glucopyranoside solubilisation as described by Croce et al. [38].

2.3. Chemicals

DCMU, DNB and DBMIB were added from a stock alcoholic (methanol or ethanol) solution so that the final v/v ratio was lower than 1%.

2.4. Photoinhibition treatment

Light from a 900-W Xenon arc lamp (Applied Photophysics Ltd.) was filtered through a Calflex C and a 10-cm water filter in order to remove UV contributions. The beam was focused on a 1-cm optical pathlength cuvette to minimise inhomogeneity of the sample treatment. The flux of the light used for the photoinhibitory treatment at the level of the sample was 30 mW cm^{-2}. The sample concentration was 4 μg/ml, and the temperature was kept at 4 °C. Thylakoids were illuminated in the presence of 10 μM DCMU if not differently stated. The decrease of maximal PSII photochemistry was estimated as the relative decrease of the F_v/F_m ratio. The photoinhibition associated quenching constant K_I was calculated from the maximal fluorescence level F_m as described by Santabarbara et al. [25,27].

2.5. Fluorescence spectroscopy

Steady state fluorescence emission spectra were detected in a home-built fluorimeter equipped with an EG & G OMAIII (model 1460) with an intensified diode array detector mounted on a HR320 Jobin-Ivon spectrometer. The wavelength scale was calibrated using a neon spectral calibration source (Cathodeon). Excitation wavelengths were selected by a Heath monochromator and two Corning CS 4-96 broad-band pass filters and, where not differently stated, was fixed at 435 nm. The light path was 1 cm. In order to have an adequate signal to noise ratio, spectra were accumulated to around 10^7 counts at the emission maxima. All spectra were measured at a chlorophyll concentration of 4 μg/ml and the temperature was 4 °C. Spectra were corrected for the detector sensitivity as previously described [39].

Pigments were extracted using 80% v/v acetone–water solution and their concentration was determined according to Lichtenthaler [40].

3. Results

3.1. Theoretical

In the present study, we investigate whether there are spectroscopically distinguishable populations of chlorophyll molecules which might be associated with energetically uncoupled or weakly coupled pigments and which would therefore display a reduced sensitivity to excited singlet state quenchers in isolated thylakoids. To avoid possible confusion we want to point out that the terms weakly coupled and uncoupled Chls are not referring to strong or weak excitonic coupling but are used to indicate pigments where singlet energy transfer is either absent or considerably slower than in pigments constituting the bulk antenna. To this end, we will consider a two-level system in which one level is the strongly coupled matrix of antenna molecules and the other is a population of either weakly coupled or uncoupled Chls, and derive a straightforward expression which describes its fluorescence in the presence of a singlet quencher (Fig. 1). This model can be easily extended to the case of a few molecules of uncoupled/weakly coupled chlorophyll molecules bound to a damaged or incorrectly assembled Chl–protein complex as suggested by the action spectra of photoinactivation [26,30].

In the case of PSII with closed reaction centres (F_m fluorescence level), it is well known that the simple Stern–Volmer representation is quite accurate, i.e., $F_m = k_f/(k_f + k_d + Pk_q)$, where k_f is the fluorescence rate, k_d is the thermal dissipation rate which includes intersystem crossing to the triplet, k_q is the quenching rate induced by a singlet

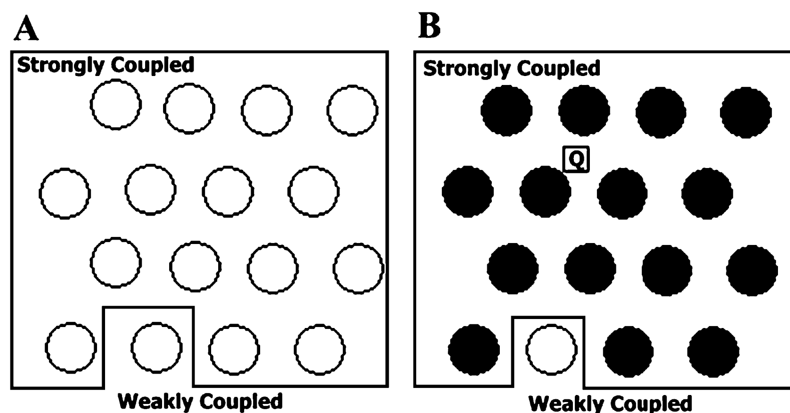

Fig. 1. Schematic representation of the principle of the quenching experiments. A: Matrix of pigments in the photosynthetic antenna in the absence of singlet quenchers (white dots). Weakly and strongly coupled pigments are undistinguishable. B: In the presence of a singlet quencher the strongly coupled pigments are quenched (black dots) but the weakly coupled ones are not (white dots). Weakly coupled pigments can now be discriminated.

quencher and P is the probability that a chlorophyll is associated with a quencher [41–44]. This approximation is valid as energy transfer between the antenna chlorophylls is roughly three orders of magnitude faster than the trivial decay processes [45–48]. If, however, there is a small population of Chls weakly coupled from singlet energy transfer, the situation is more complex as the slow energy transfer into and out of these weakly coupled pigments from the antenna must be considered. A straightforward way of doing this is by means of a statistical model approach in which the fluorescence is described by the sum of four fluorescence population terms which account for:

(a) direct absorption and emission of the strong coupled population (F_1);
(b) emission of the strongly coupled population when absorption is in the weakly coupled molecules (F_2);
(c) direct absorption and emission from the weakly coupled/uncoupled population (F_3);
(d) emission by the weakly coupled population when absorption is by the strongly coupled antenna (F_4).

Thus, the total fluorescence yield is given as:

$$\Phi f = F_1 + F_2 + F_3 + F_4 \quad (1)$$

Each fluorescence term can be expressed in the form of a series expansion as follows:

$$\begin{cases} F_1 = (1-N)(1-\alpha)\Psi_F + (1-N)\alpha\alpha'(1-\alpha)\Psi_F + \ldots \\ F_2 = N(1-\alpha)\alpha'\Psi_F + N\alpha\alpha'^2(1-\alpha)\Psi_F + \ldots \\ F_3 = N(1-\alpha')\Psi'_F + N\alpha\alpha'(1-\alpha')\Psi'_F + \ldots \\ F_4 = (1-N)\alpha(1-\alpha')\Psi'_F + (1-N)\alpha^2\alpha'(1-\alpha')\Psi'_F + \ldots \end{cases} \quad (2)$$

where N is the probability that a Chl is weakly coupled/uncoupled to the antenna; α is the probability that excitation energy is transferred from the antenna to the weakly coupled population and is given by $\alpha = k_t/(k_t+k_f+k_d+Pk_q)$ with k_t representing the rate of transfer from the antenna to the weakly coupled population, α' is the probability of energy transfer from the weakly coupled molecules to the antenna and is given by $\alpha' = k'_t/(k'_t+k_f+k_d)$. It should be noted that the relative values of k_t, k'_t depend on the degeneracy of the antenna matrix and weakly coupled populations and hence on the relative sizes of the $(1-N)$ and N population. Ψ_F is the fluorescence yield of the strongly coupled antenna matrix and is given by $\Psi_F = k_f/(k_f+k_d+Pk_q)$. Ψ'_F is the fluorescence yield of the weakly coupled population and is given by $\Psi'_F = k_f/(k_f+k_d)$. It should be noted that as P is thought to be a very small number, i.e., only a few quencher molecules per photosystem lead to strong fluorescence quenching [42], we do not consider the interaction of quencher with the small fraction of weakly coupled chlorophylls in the Ψ'_F and α' terms. It is assumed that the fluorescence lifetime of uncoupled chlorophylls $(k_f+k_d)^{-1}$ is equal to that of coupled antenna chlorophylls at F_m, which is approximately correct as both are in the 2–3 ns range.

The four series of Eq. (2) may each be summed giving:

$$\begin{cases} F_1 = \dfrac{(1-N)(1-\alpha)\Psi_F}{(1-\alpha\alpha')} \\ F_2 = \dfrac{N\alpha'(1-\alpha')\Psi_F}{(1-\alpha\alpha')} \\ F_3 = \dfrac{N(1-\alpha')\Psi'_F}{(1-\alpha\alpha')} \\ F_4 = \dfrac{(1-N)\alpha(1-\alpha')\Psi'_F}{(1-\alpha\alpha')} \end{cases} . \quad (3)$$

In the extreme case of complete uncoupling (α, $\alpha'=0$), only the direct absorption terms (F_1 and F_3) remain. It should be pointed out that the terms F_1, F_2, F_4 all display equal sensitivity to quencher, due to rapid energy transfer in the strongly coupled antenna, while the F_3 term displays reduced sensitivity. Thus, in a quenching experiment, it is the signal associated with direct absorption by the weakly coupled molecules (F_3) which will be detected.

The same consideration can be readily extended to the case of a Chl–protein complex which binds pigments which are weakly coupled in the singlet state, as long as singlet energy transfer in the antenna matrix is faster than the equilibration time in the damaged complex. In this case, it will be the whole Chl–protein complex, as a unit, which will be detected in the quenching experiment. However, when just a fraction of the pigments in the complex are weakly coupled, or uncoupled, and given that the equilibration amongst the coupled pigments is faster than the energy transfer to and from the uncoupled pigments, and of the same order of magnitude as equilibration in the well-coupled antenna matrix, then the case simply reduces to the one considered for a single, weakly coupled or uncoupled chlorophyll molecule.

3.2. Experimental

In the experiments with thylakoids described below it is necessary to realise that fluorescence from both PSII and PSI is measured. In this context, two points should be considered: (i) the mean lifetime of PSII at F_m is in the 2–3 ns time range (e.g. [49–51]) while that of PSI is in the 90–120 ps range (e.g. [52–54]); (ii) about 80–90% of the PSI fluorescence at room temperature is associated with the long wavelength emission forms with maximal emission near 720–725 nm. For PSII, maximal emission is near 683 nm. Thus, it is expected that roughly 99% of the fluorescence at 683 nm in thylakoids is of PSII origin and, as will be demonstrated below, the PSI component further decreases at shorter wavelengths.

3.2.1. Photoprotection by decreasing excited state population

Photoinhibition in isolated thylakoids and leaves is characterised by a decreased F_v/F_m ratio, indicative of a decreased maximum quantum efficiency of PSII. As the F_0

parameter remains substantially constant the decreased F_v/F_m ratio is given be an F_v decrease associated with irreversible quenching of F_m. As the Stern–Volmer equation is applicable to PSII (see Theoretical) it is therefore possible to estimate a rate parameter associated with the irreversible F_m quenching due to photoinhibition (K_I) as described by Santabarbara et al. [25]. In that study, it was demonstrated that reduction of the excited state population by the singlet quencher DBMIB had only a rather small protective effect on photoinhibition, estimated to be approximately 25–30% of that expected on the basis of the linear light dose/photoinhibition response plot. In Fig. 2, we present results of similar photoinhibition experiments in which in addition to DBMIB, data are presented also for another exogenously added singlet quencher DNB as well as for the "spillover" induced singlet quenching of PSII fluorescence due to energy transfer to PSI when cations are removed from the thylakoid suspension medium. It is apparent that photoinhibition displays an almost identical insensitivity to all three singlet quenchers which in the K_I versus the excited state population ([S^*]) plot is well approximated by a single straight line. The slope of this line indicates that photoprotection does not exceed 25–30% of the excited state population reduction due to singlet quenching. We will refer to this in the present study as the residual photoprotection due to singlet excited state quenching. This low sensibility of photoinhibition to reduction in the excited state population, when compared with the linear light dose/response plot [25–27,30], suggests that photoinhibition may be caused by chlorophylls which are uncoupled or poorly coupled to the antenna pigment matrix. In the remaining part of this study, we present spectroscopic evidence supporting this view.

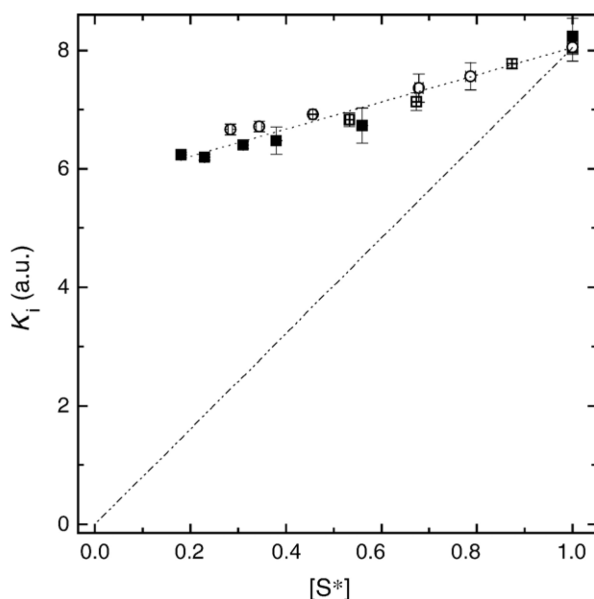

Fig. 2. The effect of reducing the exited state population in Photosystem II antenna [S*] on the photoinhibition induced rate process K_I as modified by DBMIB titration (open circles), DNB titration (full squares) and Mg^{2+} titration (open squares). The dotted line is a linear fit through the experimental points and the dash-dotted line shows the predicted behaviour as expected from the light dose–response 'reciprocity' rule.

The results presented in Fig. 2 together with previous reports from this and other laboratories [24–30] suggest that the role attributed to the NPQ and qE associated singlet excited quenching might need to be reconsidered. However because artificial quinones (and energy *spillover* to PSII) are utilised instead of the naturally occurring qE we wish to discuss whether artificial quinone can efficiently mimicking qE.

Recent studies have indicated that a charge transfer complex involving a zeaxanthin molecule and a Chl could represent the quenching centre involved in qE [55]. As this is the same mechanism by which artificial quinones quench Chl fluorescence [43], we are of the opinion that the chemical singlet quenchers are a good model system for analysing NPQ effects.

A second aspect which should be discussed is whether the localisation of the quencher in the photosystem is of any relevance. While artificial quenchers are thought to distribute randomly within the photosystem compartment (i.e., inner and outer antenna complexes) it has been suggested that qE centres develop mainly in the outer antenna complex. We have previously investigated [28] this aspect for the case in which the excited state equilibration in the antenna is much faster than the trapping by the reaction centre (trap-limited case) or in that in which the trapping is only partially limited by excitation diffusion in the antenna (transfer-to-trap limited case), which may be the more accurate representation of PSII [56–58]. From this analysis, it was clear that the exact location of the quencher within the antenna matrix is of little importance and thus the use of artificial singlet quenchers is a reasonable way to investigate the effects of NPQ. In addition, we would also point out that if qE were to act by lowering the excited state population in the antenna, and thus reduce the probability of a excited state reaching the reaction centre Chl, the precise mechanism by which the singlet excited state quenching is obtained is probably not particularly important, as long as the singlet excited state level is lowered.

3.2.2. *The effect of singlet excited state quenching on the steady-state fluorescence emission spectra of thylakoids*

In Fig. 3, data are presented for steady state emission spectra measured in the absence and presence of two different singlet quenchers, DBMIB and DNB together with the difference spectra. The latter spectra were calculated (quenched minus unquenched) after normalisation to unity of quenched and unquenched spectra at their maximum emission (683.5 nm).

The steady state fluorescence emission spectra of stacked and unstacked thylakoid membranes are presented in Fig. 4 for preferential excitation in the Chl *a* (435 nm) Soret absorption band. The fluorescence emission at the maximum is almost halved in unstacked membranes due to

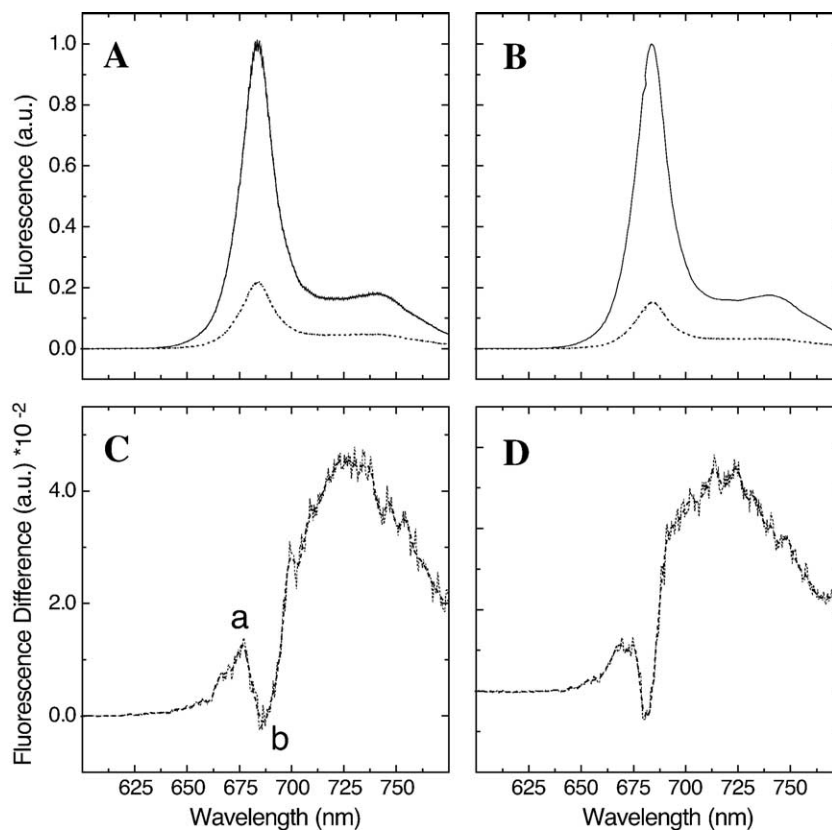

Fig. 3. Fluorescence emission spectra of spinach thylakoids at F_m in the absence (solid line) and presence (dotted line) of singlet quenchers. A, C: Quenched and unquenched spectra using DBMIB (3 μM, A) or DNB (2 mM, C). B, D: Quenched minus unquenched difference spectra after normalisation of each spectrum to its maximum value. Dashed lines represent smoothed spectra. The number of counts in the peak channel for unquenched spectra was 1.7×10^7 for DBMIB experiments and 6.4×10^6 for DNB experiments. Excitation wavelength: 435 nm.

energy *spillover* to PSI. The difference, quenched minus unquenched, spectra, calculated as for the exogenous singlet quenchers, are also presented (Fig. 4).

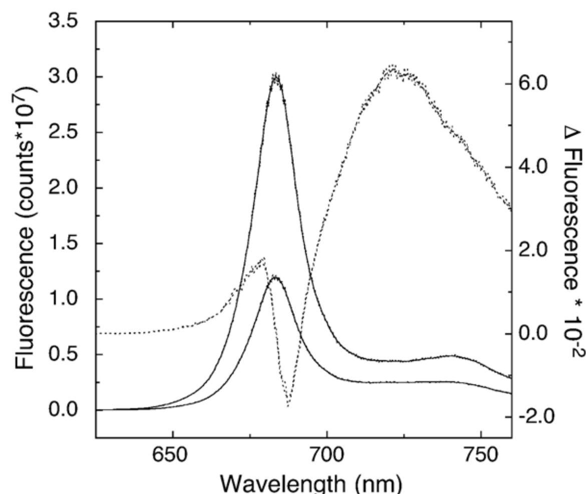

Fig. 4. Fluorescence emission spectra of stacked (dashed lines) and unstacked (dash-dotted lines) spinach thylakoids at F_m. Quenched minus unquenched difference spectrum after normalisation of each spectrum to its maximum value (dotted line). Excitation wavelength: 435 nm.

All difference spectra are dominated by a long wavelength structure which peaks near 723 nm. This structure is clearly associated with the long wavelength spectral forms of PSI ([38] and Fig. 5A) and is due to this photosystem being less sensitive to singlet quenchers than PSII by virtue of its short excited state lifetime [52–54]. In addition, a smaller, rather broad, difference spectrum feature is evident on the short wavelength side of the PSII emission maximum. The broadness of this structure may be due to some spectral heterogeneity with maxima near 673–675 and 666–668 evident in smoothed spectra. In order to check that this difference spectrum structure is not also associated with PSI emission we have performed two types of measurements. Firstly, we have compared the emission spectra of isolated PSII particles with that of isolated PSI-200 (Fig. 5A). It is evident from these fluorescence spectra that PSI emission is relatively much less intense than PSII emission over the entire wavelength interval below 683 nm and therefore cannot be responsible for the quenching difference spectrum feature in the shorter wavelength region. In addition, we have taken advantage of the relatively greater chlorophyll *b* content of PSII with respect to PSI to determine a PSI *minus* PSII emission difference spectrum in thylakoids. In this case, excitation was with

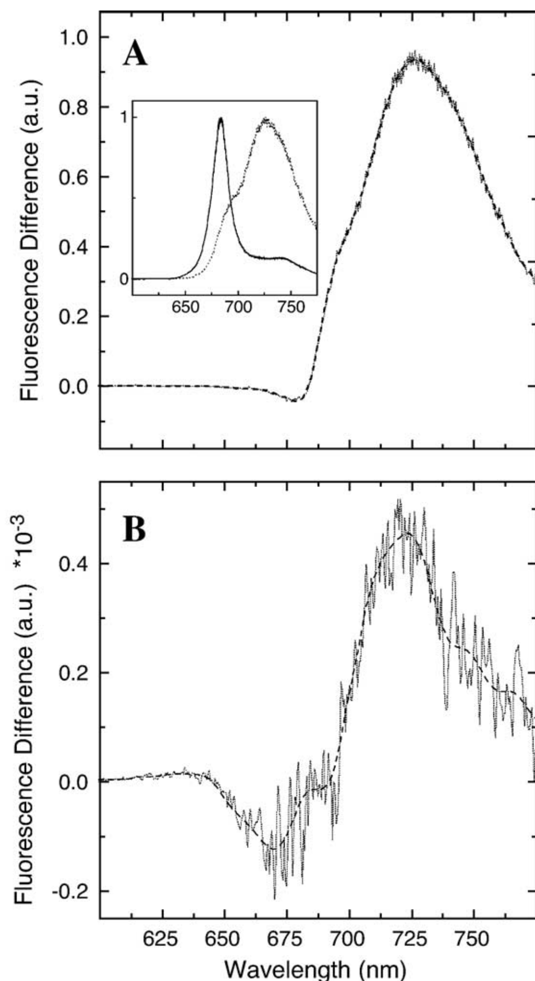

Fig. 5. PSI minus PSII emission difference spectra. A: PSI-200 minus PSII membrane (BBY) after normalisation to the PSII emission maximum (dashed line). The fluorescence emission spectra of PSII (solid line) and PSI (dotted line) normalised at their maxima are presented in the figure insert. B: Fluorescence emission difference spectrum of thylakoids with 435 and 475 nm excitation and calculated as 435 nm minus 475 nm (dotted line) after normalisation at the PSII maximum. The smoothed spectrum (dashed line) is also shown.

either 475 nm (chlorophyll b) or 435 nm (chlorophyll a) and after normalisation to the emission peak the 435 nm minus 475 nm difference spectrum was calculated (Fig. 5B). This spectrum clearly shows the PSI emission at long wavelengths and furthermore indicates, as expected, that this PSI emission with respect to PSII, decreases progressively towards the blue, thus confirming the above conclusion that the 670–675 nm quenching feature is not due to PSI.

It is generally known that the absorption and fluorescence of uncoupled "monomerised" chlorophylls are blue shifted with respect to most of the protein-bound spectral forms. We have therefore examined the effects of singlet quenchers on the fluorescence spectra of thylakoids incubated with a low concentration of the detergent Triton X-100, known to "monomerise" protein bound chlorophylls. In Fig. 6 the quencher difference spectrum for detergent incubated thylakoids is directly compared with that in control, detergent untreated, thylakoids. We emphasise the fact that the intensity of the difference spectrum feature is over 50-fold greater in detergent treated thylakoids with respect to the controls. It is evident that apart from this large and expected difference in intensity, due to the detergent-induced uncoupling of a significant amount of chlorophyll, the two spectra are remarkably similar. We therefore interpret the difference spectrum structure seen in the quenching spectra in thylakoids in terms of a small population of weakly or completely uncoupled chlorophyll molecules.

The quenching experiments described above were all performed by excitation at 435 nm, which means under conditions of preferential excitation into the chlorophyll a Soret band. If the difference spectrum structure near 675 nm were in fact due to chlorophyll a molecules which are uncoupled or weakly coupled to the antenna matrix they should be less populated when excitation is preferentially into the chlorophyll b band. To this end, we have compared the DBMIB and DNB quenching spectra with 435 nm and 475 nm excitation wavelengths. From Fig. 7, it can be seen that while the 670–675 nm difference spectrum structure is still present upon 475 nm excitation, it is significantly less pronounced and apparently somewhat blue shifted towards the 650-nm region, characteristic of chlorophyll b emission [59].

In order to gain information on the population size of the weakly coupled/uncoupled chlorophylls, which we will refer to as the N population, it is necessary to quantitate the difference spectrum structure in relevant units. Owing to the presence of PSI, an accurate determination is not

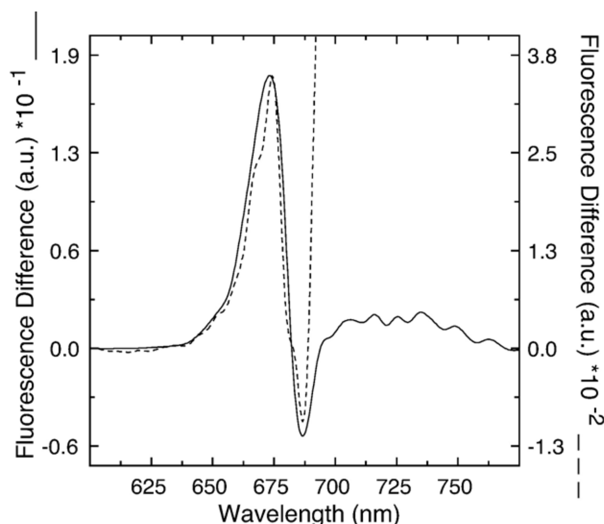

Fig. 6. Quenched (DBMIB 1.6 μM) minus unquenched difference spectra for thylakoids (a) incubated at a low concentration of Triton X-100 (0.00125% w/v, solid line), and (b) incubated without detergent (dashed line).

possible, however, an approximate estimation may be obtained from the maximum and minimum values indicated by the letters “a” and “b” in Fig. 3B. In this way, we define a difference spectrum parameter (ΔF; Eq. (4)) which is expressed in terms of the maximal emission of the unquenched sample (F_{683}) normalised to unity:

$$\Delta F = \left(\frac{F_{\lambda}^{q}}{F_{683}^{q}} - \frac{F_{\lambda}}{F_{683}}\right)\frac{F_{683}^{q}}{F_{683}} \quad (4)$$

where F_{λ}, F_{λ}^{q} are the unquenched and quenched spectra, respectively; F_{683}, F_{683}^{q} are the fluorescence values of unquenched and quenched samples at 683 nm. For quenching by different concentrations of DBMIB in the 2- to 8-fold range, we find ΔF values between 0.002 and 0.003 (Fig. 8). It is possible to relate these experimentally determined values of ΔF to the fluorescence emission of the N population. However, as will become clear in Discussion, where this point is further analysed, these are minimum values of the fractional size of the N population.

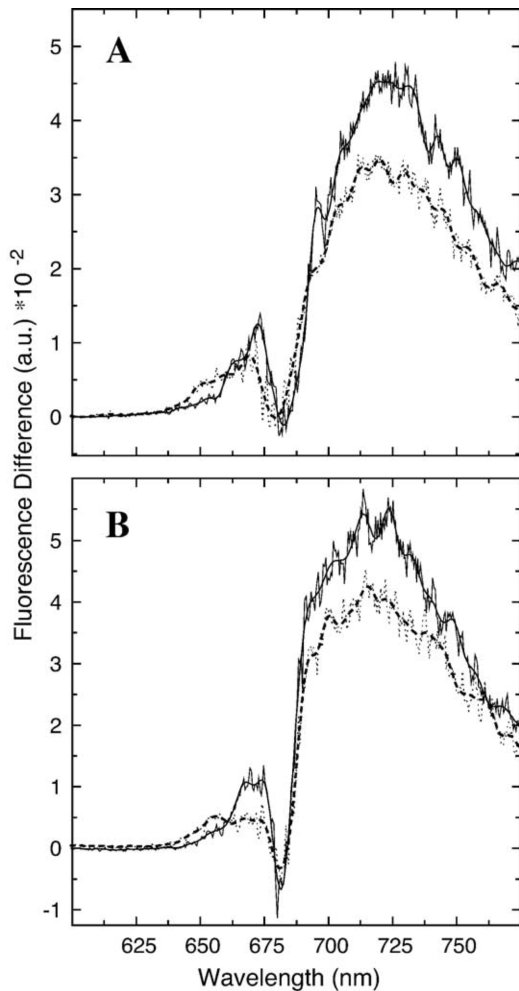

Fig. 7. Quenched minus unquenched difference spectra at F_m for spinach thylakoids excited either at 435 nm (solid line) or at 475 nm (dashed line). A: DBMIB (3 μM) B: DNB (2 mM).

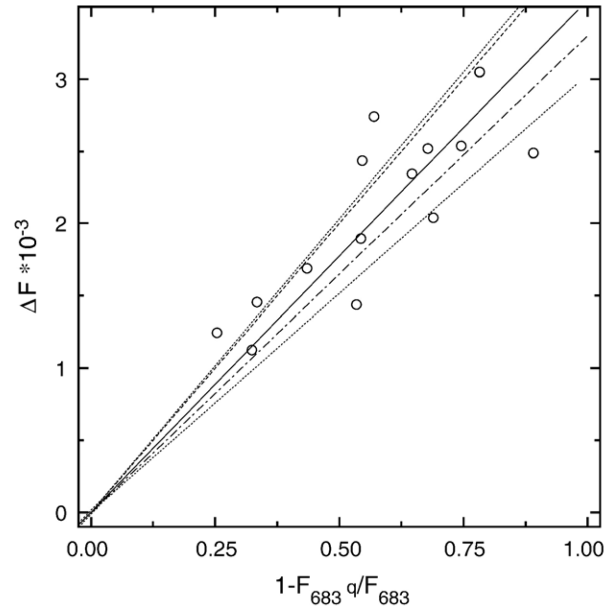

Fig. 8. Plot of ΔF versus $1-(F_{683}^{q}/F_{683})$. F_{683}^{q} and F_{683} are the intensities at the peak position of the fluorescence emission spectra for the quenched and unquenched samples respectively. The solid line represents the linear extrapolation of data points and the intercept at infinitely high quenching is an estimate of the fluorescence of the N population. The extrapolation to infinite quenching yields a value 0.0036. Also shown are the confidence intervals ($P=0.01$) for the linear regression fit (dotted lines). The dashed line is a numerical simulation for a completely uncoupled Chl population ($N=0.004$, $\alpha=0$). The dash-dotted line is the numerical simulation for a weakly coupled population ($N=0.005$, $\alpha=0.26$).

4. Discussion

The results presented in Fig. 2 of this paper, in agreement with previous studies [25–30], show that photoinhibition of PSII in isolated thylakoids displays a rather limited sensitivity to decreasing the antenna singlet excited state population by addition of a wide variety of singlet quenchers. For all singlet quenchers used, the residual photoprotection is of the order of 25–30% of that which is expected on the basis of the linear light dose–response behaviour. This is in agreement with previous data for chloroplasts from a variety of species, studied in vitro [25,27–29] as well for the in vivo studies on the green alga *Chlamydomonas reinhardtii* [29,30]. These observations can be explained by the suggestion that photoinhibition might be associated with a small population of weakly coupled or uncoupled chlorophylls. Based on the photoinactivation action spectra [27,30], it has been suggested that these chlorophylls may be bound to an inhomogeneous population of damaged or incorrectly assembled chlorophyll–protein complexes.

In the present paper, we present further data on these uncoupled/weakly coupled chlorophylls. It is demonstrated that a small population of chlorophylls which fluoresce in the 668–670 nm and 670–675 nm range are less sensitive to different singlet quenchers than are the chlorophylls of the main antenna matrix, giving rise to a difference spectrum features at these wavelengths. We exclude the possibility that these spectral features are associated either with PSI, which is also less sensitive than PSII to singlet quenchers due to its very short excited state lifetime [52–54], or some uncoupled chlorophyll–protein complexes in their native state, by analysis of the emission spectra of the isolated complexes (data not shown). It is furthermore demonstrated that when chlorophylls are uncoupled by incubation of thylakoids with a low concentration of the detergent Triton X-100, the singlet quencher difference spectrum structure is very similar to that of thylakoids not treated with detergent. The band-shape of the excited singlet quenching associated difference structure is rather asymmetric and extends towards the region of Chl *b* emission [59]. Preferential excitation of the Chl *b* in the Soret region results in a relative increase of the shoulder in the difference spectra at about 665 nm. This is a signature of imperfect thermal equilibration within this population of molecules. Indeed, for a population which is in "perfect equilibrium" with the matrix in which it is embedded the shape of the emission is independent of the excitation wavelength [56,60,61]. These data therefore strongly suggest that the 668–670 nm and 670–675 nm difference spectra structures represent the emission of the small population of the weakly coupled or even completely uncoupled chlorophyll *a*/*b* molecules proposed to be involved in photoinactivation.

Experiments are also presented on the sensitivity of the steady state fluorescence emission of thylakoids to excited state quenching in order to derive an estimate of the size of this uncoupled chlorophyll population. These chlorophylls are described by the F_3 and F_4 terms in Eq. (3). Based on the fluorescence spectroscopy data alone it is not easy to decide whether the difference spectrum feature is due to chlorophylls which are completely uncoupled from the antenna matrix or chlorophylls which are only weakly coupled to it. We will now discuss the two different situations in the frame of the model described in the 'theoretical' section. The most straightforward situation is that of complete uncoupling. In this case the coupling terms (α, α') are zero and the PSII fluorescence yield is given by F_1 (antenna matrix) plus F_3 (uncoupled pigments). In this context, we wish to underline the above-mentioned residual sensitivity of PSII photoinhibition to singlet quenchers ([25,27,30] and Fig. 2). If photoinhibition in thylakoids were due to a completely uncoupled chlorophyll population we would expect there to be no detectable sensitivity to singlet quenchers (Eq. (3)), if direct interaction between the quencher molecule and the uncoupled Chl population is neglected. On the other hand, for a small population of weakly coupled chlorophylls, i.e., when α,α' have non-zero values which are also much less than 1 (if α, $\alpha' \cong 1$ then the coupling would be strong), these chlorophyll molecules are populated both by slow energy transfer from the antenna (F_4) and by direct absorption (F_3). As the F_4 term is sensitive to singlet quenchers in exactly the same way as the antenna matrix terms (F_1, F_2), the N population fluorescence will display some quencher sensitivity, the degree of which depends on the relative weighting for the two terms F_3 and F_4. For $F_3 >> F_4$, the quencher sensitivity is slight, because the emission is almost completely due to direct absorption by uncoupled Chl, and vice versa. The residual sensitivity of thylakoid PSII photoinhibition to different singlet quenchers (chemical quenchers or energy spillover to photosystem I) is about 25%–30% of that expected on the basis of dose/response behaviour (Fig. 2). Numerical simulations indicate that the F_3/F_4 ratio is expected to be about 2 in this case. It is useful to realise that this ratio has a simple analytical meaning (Eq. (5)) which comes directly from Eq. (3):

$$\frac{F_3}{F_4} = \frac{N}{(1-N)\alpha} \quad . \tag{5}$$

We will now address the question of the size of the N population. As mentioned in Results, it is not possible to accurately determine the size of the quencher difference spectrum structure (ΔF) due to the presence of PSI and also due to the low signal to noise ratio for the very small difference spectra structures. However, an approximate value may be obtained (a minus b in Fig. 3B) and these values will be used to gain an estimate of the fluorescence of the N population and hence of its fractional size by means of the model described above. In these calculations, we assume that only the strongly coupled population $(1-N)$ emits at the PSII maximum near 683 nm while the N population emits only in the 670–675nm interval. In Fig. 8 the measured ΔF values are given for different DBMIB concentrations which yield an overall PSII fluorescence quenching in the 2- to 8-fold range. It may be readily shown from Eq. (3) that for completely uncoupled chlorophylls the ΔF versus PSII quenching $(1-F_1^q/F_1)$ plot yields a straight line, where F_1^q is the fluorescence of the quenched PSII antenna, and that the intercept on the ΔF axis at infinitely high quenching ($F_1^q=0$) equals F_3 which is the total fluorescence of the N population. On the other hand, when a weakly coupled population is considered the ΔF versus PSII quenching $[1-(F_1+F_2)^q/(F_1+F_2)]$ plot is closely approximated by a straight line and the $(F_1+F_2)^q=0$ intercept also yields F_3 which in this case represents that part of the fluorescence of the population N due to direct absorption and does not include the term (F_4) due to energy transfer from the antenna matrix. A numerical simulation of these two situations is represented in Fig. 8, where the dashed line represents the completely uncoupled case for an N value of 0.004 and the dash dotted line represents the weakly coupled assumption for N equal to 0.005. From

these considerations, it is clear that in the absence of information on whether the N population is completely or partially uncoupled this intercept value should be understood as a minimum value for its fluorescence.

In Fig. 8, we also show the linear approximation of the ΔF data (full line) and find that the intercept value at infinitely high quenching is $\Delta F \approx 0.004$. This is an approximate value for the intrinsic fluorescence of the population N if it is completely uncoupled. Assuming that the N chlorophylls have a similar fluorescence yield to that of coupled PSII antenna chlorophylls (at F_m), this gives an approximate population value for N of 0.4%, i.e., about 1 chlorophyll per PSII. However, if we drop the complete uncoupling assumption and assume weak coupling of the N population with the antenna matrix, in accordance with the photoinhibition data discussed above, then the analytical situation is more complex as the F_3 term obtained by linear extrapolation to infinite quenching now contains the coupling term $(1-\alpha')$, which is also a function of N, and it is therefore not possible on the basis of the fluorescence data alone, to obtain an estimate of this parameter. This, however, may be achieved if we consider the about 30% residual sensitivity of photoinhibition to singlet quenchers (Fig. 2). As discussed above this yields an F_3/F_4 ratio close to 2. Thus from Eq. (5) it is possible to obtain a numerical value for the coupling term α for different assumed $N/(1-N)$ and from this determine $(1-\alpha')$ via the simple expressions which define α and α' (see Eq. (2)). For $N/(1-N)$ ratios in the range 0.005–0.11 (i.e. for N=0.05–10%), the $(1-\alpha')$ term has values between 0.63 and 0.65. From F_3 (Eq. (3)) this yields N=1.4%, i.e., about 3 molecules per PSII. Thus, we conclude that the approximate range of values for the N population is between 1 and 3 molecules per PSII unit, depending on the degree of weak coupling/uncoupling to the antenna matrix. In calculating these values, we have assumed that the fluorescence yield for the N population equals that of the antenna matrix. If the fluorescence yield of N were to be somewhat greater, these numbers would then be a small overestimate.

The values for the N population size which we have estimated are somewhat smaller than those which come out of the time resolved analysis of Vasil'ev et al. [31] for spinach thylakoids. These authors associate the N population with a 3.6-ns decay component which has an amplitude of 1.5% at F_0, giving a relative yield of 14%. At F_m, this is expected to be approximately 3%, whereas we find an N population fluorescence value of 0.4%, about eight times less. This difference could be due to the fact that Vasil'ev et al. [31] used market spinach for the preparation of thylakoids which were then subsequently frozen and stored at − 80°C prior to use. In our case experiments were performed with thylakoids prepared from freshly harvested leaves.

In this study, evidence is presented which indicates that isolated spinach thylakoids, which have not been subjected to pigment solubilising treatments such as detergent incubation, contain a small population of chlorophylls which are uncoupled or only weakly coupled to the antenna matrix in the singlet state. If these pigments are in fact involved in photoinhibition, then they should also have a triplet population which is significantly higher than that of the antenna matrix. The triplet population is expected to be determined basically by two factors, i.e., the triplet yield and the triplet quenching rate either directly or indirectly by carotenoids. The triplet yield for weakly coupled/uncoupled chlorophylls is not expected to be very different from that of the antenna matrix pigments as the intrinsic lifetime $(k_f+k_d)^{-1}$ of both pigment populations is similar, i.e., between 2 and 3 ns. Taking the rate value suggested by Kramer and Mathis [17] for intersystem conversion of 0.1 ns^{-1} one calculates a 'intrinsic' triplet yield of about 20%. This high 'intrinsic' triplet yield however does not normally lead to a high triplet population due to quenching by carotenoids [17,42]. However, in the case of pigments which are uncoupled or weakly coupled in the singlet state, it is expected that they will also be uncoupled in the triplet state. This is because triplet transfer proceeds via the Dexter electron exchange mechanism which, while involving a similar orientation term as the well known Förster mechanism for singlet transfer, is possible only when there is direct electronic orbital overlap between the donor and acceptor molecules, i.e., within the so-called Van der Waals contact distance [62]. On the other hand Förster singlet transfer may proceed with high efficiency over a distance of tens of Å [63–65]. We would therefore expect that the N population of weakly coupled/uncoupled chlorophylls, which have a maximum fluorescence between 670 and 675 nm, to have a high triplet population.

In a recent investigation of the Chl triplet state in thylakoids, three populations of Chl triplets associated with Photosystem II have been detected. None of this bears the magnetic signature of the previously characterised $^3P_{680}$ recombination triplet, which has been proposed to participate in photoinactivation [32]. However, one of the triplet populations detected, having resonance maxima at 766/989 MHz, and showing an unusually short lifetime (50–150 μs), compared to the Chl in organic solvents (~1–3 ms), is likely to originate from the reaction centre, and represent the $^3P_{680}$ recombination triplet when Q_A is singly reduced [66]. The other two triplets detected have been assigned to antenna Chl–Protein complexes [32]. It is likely that the triplets observed in thylakoids represent the small population of damaged, or incorrectly assembled, protein complexes that are characterised in the present study. The triplet originating from the reaction centre can, in principle, explain the approximately 30% photoprotection induced by singlet excited state quenching ([25,27–30] and Fig. 2). However, it should be mentioned that because of the shorter lifetime of this population, the recombination triplet is expected to have a lower probability of interacting with molecular oxygen and generate the reactive singlet species.

Acknowledgements

This work was in part supported by the Italian Ministry for University and Research (MURST) under the projects FIRB RBAU01E3CX "Meccanismi molecolari della Fotosintesi", the U.K. Biotechnology and Biological Sciences Research Council (BBSRC) (CO0350, CO7809 and B18658) and the European Union TMR programme (Contract No. FMRX-CT98-0214) to S.S.

References

[1] H.T. Witt, Energy conversion in the functional membranes of photosynthesis. Analysis of light pulse and electric pulse methods. The central role of the electric field, Biochim. Biophys. Acta, Bioenerg. 505 (1979) 355–427.
[2] O. Prásil, N. Adir, I. Ohad, Dynamics of Photosystem II: mechanism of photoinhibition and recovery processes in: J. Barbers (Ed.), The Photosystems: Structure, Function and Molecular Biology, Elsevier Science Publishers B.V., Amsterdam, 1992, pp. 295–348.
[3] E.-M. Aro, I. Virgin, B. Andersson, Photoinhibition of Photosystem II: inactivation, protein damage and repair, Biochim. Biophys. Acta, Bioenerg. 1143 (1993) 113–134.
[4] W.S. Chow, Photoprotection and photoinhibition damage, in: E.E. Bittar (Ed.), Advanced Molecular and Cell Biology, vol. 10, JAI Press, Greenwich, 1994, pp. 150–196.
[5] L.W. Jones, B. Kok, Photoinhibition of chloroplast reactions: I. Kinetic and action spectra, Plant Physiol. 41 (1966) 1037–1043.
[6] I. Ohad, H. Koike, H.S. Shochat, Y. Inoue, Changes in the properties of RCII during the initial stages of photoinhibition as revealed by thermoluminescence measurements, Biochim. Biophys. Acta, Bioenerg. 993 (1988) 288–298.
[7] I. Setlik, S.I. Allakhverdiev, L. Nebdal, E. Setlikova, V.V. Klimov, Three types of Photosystem II photoinhibition: I. Damaging processes on the acceptor side, Photosynth. Res. 23 (1990) 39–48.
[8] S.I. Allakhverdiev, E. Setlikova, V.V. Klimov, I. Setlik, In photoinhibited Photosystem II particles pheophytin reduction remains unimpaired, FEBS Lett. 226 (1987) 186–190.
[9] C. Jegerschold, I. Virgin, S. Styring, Light-dependent degradation of the D1 protein in Photosystem II is accelerated after inhibition of the water splitting reaction, Biochemistry 29 (1990) 6179–6186.
[10] I. Vass, S. Styring, T. Hundal, A. Koivuniemi, E.-M. Aro, B. Andersson, Reversible and irreversible intermediates during photoinhibition: stable reduced Q_A species promote chlorophyll triplet formation, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 1408–1412.
[11] A.N. Macpherson, A. Telfer, J. Barber, T.G. Truscott, Direct detection of singlet oxygen from isolated Photosystem II reaction centres, Biochim. Biophys. Acta, Bioenerg. 1143 (1993) 301–309.
[12] E. Hideg, C. Spetea, I. Vass, Singlet oxygen and free radical production during acceptor- and donor-side induced photoinhibition, Biochim. Biophys. Acta, Bioenerg. 1186 (1994) 143–152.
[13] N.P. Mishra, D.F. Ghanotakis, Exposure of a Photosystem II complex to chemically generated singlet oxygen results in D1 fragments similar to the ones observed during aerobic photoinhibition, Biochim. Biophys. Acta, Bioenerg. 1187 (1994) 296–300.
[14] M. Miyao, Involvement of active oxygen species in the degradation of the D1 protein under strong light illumination in isolated subcomplexes of Photosystem II, Biochemistry 33 (1994) 9722–9730.
[15] J. Breton, N.E. Geacintov, C.E. Swenberg, Quenching of fluorescence by triplet excited states in chloroplasts, Biochim. Biophys. Acta 548 (1979) 616–635.
[16] A. Sonneveld, H. Rademaker, L.M.N. Duysens, Chlorophyll *a* fluorescence as a monitor of nanosecond reduction of the photooxidized primary donor $P680^+$ of Photosystem II, Biochim. Biophys. Acta 548 (1979) 536–551.
[17] H. Kramer, P. Mathis, Quantum yield and rate of formation of the carotenoid triplet state in photosynthetic structure, Biochim. Biophys. Acta 593 (1980) 319–329.
[18] A. Telfer, J. Barber, M.C.W. Evans, Oxidation-reduction potential dependence of reaction centre triplet formation in the isolated D1/D2/cytochrome b-559 Photosystem II complex, FEBS Lett. 232 (1988) 209–213.
[19] F.J.E. vanMieghem, W. Nietschke, P. Mathis, A.W. Rutherford, The influence of the quinone–iron electron acceptor complex on the reaction centre photochemistry of Photosystem II, Biochim. Biophys. Acta, Bioenerg. 977 (1989) 207–214.
[20] J.R. Durrant, L.B. Giorgi, J. Barber, D.R. Klug, G. Porter, Characterisation of the triplet state in isolated Photosystem II reaction centres: oxygen quenching as a mechanism for photodamage, Biochim. Biophys. Acta, Bioenerg. 1017 (1990) 167–175.
[21] I. Vass, S. Styring, Characterisation of chlorophyll triplet promoting states in Photosystem II sequentially induced during photoinhibition, Biochemistry 31 (1992) 5957–5963.
[22] P. Horton, A.V. Ruban, R.G. Walters, Regulation of light harvesting in green plants, Annu. Rev. Plant Physiol. Mol. Biol. 47 (1996) 665–684.
[23] N.E. Holt, G.R. Fleming, K.K. Niyogi, Toward an understanding of the mechanism of nonphotochemical quenching in green plants, Biochemistry 43 (2004) 8281–8289.
[24] J. Sinclair, Y.-I. Park, W.S. Chow, J.M. Anderson, Target theory and photoinactivation of Photosystem II, Photosynth. Res. 50 (1996) 33–40.
[25] S. Santabarbara, F.M. Garlaschi, G. Zucchelli, R.C. Jennings, The effect of excited state population in Photosystem II on the photoinhibition-induced changes in chlorophyll fluorescence parameters, Biochim. Biophys. Acta, Bioenerg. 1409 (1999) 165–170.
[26] E. Tyystjarvi, N. King, M. Hakala, E.-M. Aro, Artificial quenchers of chlorophyll fluorescence do not protect against photoinhibition, J. Photochem. Photobiol., B Biol. 48 (1999) 142–147.
[27] S. Santabarbara, K.V. Neverov, F.M. Garlaschi, G. Zucchelli, R.C. Jennings, Involvement of uncoupled antenna chlorophyll in photoinhibition in thylakoids, FEBS Lett. 491 (2001) 109–113.
[28] S. Santabarbara, R. Barbato, F.M. Garlaschi, G. Zucchelli, R.C. Jennings, The quenching of Photosystem II fluorescence does not protect the D1 protein against light-induced degradation in thylakoids, FEBS Lett. 505 (2001) 159–162.
[29] S. Santabarbara, I. Cazzalini, R. Barbato, D. Tarantino, F.M. Garlaschi, G. Zucchelli, R.C. Jennings, Is non photochemical quenching protective? Proceeding of the XII International Conference on Photosynthesis, Brisbane CSIRO Publishing, Collingwood, Australia, 2001.
[30] S. Santabarbara, I. Cazzalini, A. Rivadossi, F.M. Garlaschi, G. Zucchelli, R.C. Jennings, Photoinhibition in vivo and in vitro involves weakly coupled chlorophyll–protein complexes, Photochem. Photobiol. 75 (2002) 613–618.
[31] S. Vasil'ev, S. Wiebe, D. Bruce, Non-photochemical quenching of chlorophyll fluorescence in photosynthesis. 5-hydroxy-1,4-naphtoquinone in spinach thylakoids as a model for antenna based quenching mechanism, Biochim. Biophys. Acta, Bioenerg. 1363 (1998) 147–156.
[32] S. Santabarbara, E. Bordignon, D. Carbonera, R.C. Jennings, Chlorophyll triplet states associated with Photosystem II of thylakoids, Biochemistry 41 (2002) 8184–8194.
[33] V.A. Boichencko, Action spectra and functional antenna size of photosystem I and II in relation to thylakoids membrane organisation and pigment composition, Photosynth. Res. 58 (1998) 163–174.
[34] V. Caspi, S. Malkin, J.B. Marder, Oxygen uptake photosensitized by disorganized chlorophyll in model systems and thylakoids of greening barley, Photochem. Photobiol. 71 (2000) 441–446.

[35] R.C. Jennings, F.M. Garlaschi, P.D. Gerola, R. Etzion-Katz, G. Forti, Proton induced grana formation in chloroplast. Distribution of chlorophyll–protein complexes and Photosystem II, Biochim. Biophys. Acta 638 (1981) 100–107.
[36] D.A. Berthold, G.T. Babcock, C.F. Yocum, A highly resolved oxygen evolving Photosystem II preparation from spinach thylakoids membranes, FEBS Lett. 134 (1981) 231–234.
[37] T.G. Dunahay, L.A. Staehelin, M. Seibert, P.D. Ogilvie, S.P. Berg, Structural, biochemical and biophysical characterisation of four oxygen-evolving Photosystem II preparations from spinach, Biochim. Biophys. Acta 764 (1984) 179–193.
[38] R. Croce, G. Zucchelli, F.M. Garlaschi, R. Bassi, R.C. Jennings, Excited state equilibration in the photosystem I–light harvesting complex I: P700 is almost isoenergetic with its antenna, Biochemistry 35 (1996) 8572–8579.
[39] G. Zucchelli, R.C. Jennings, F.M. Garlaschi, The presence of long-wavelength chlorophyll a spectral forms in the light-harvesting chlorophyll a/b binding complex, J. Photochem. Photobiol., B Biol. 6 (1990) 381–394.
[40] H.K. Lichtenthaler, Chlorophyll and carotenoids: the pigments of photosynthetic membranes, Methods Enzymol. 148 (1987) 350–382.
[41] W.L. Butler, M. Kitajima, Fluorescence quenching in Photosystem II of chloroplasts, Biochim. Biophys. Acta 376 (1975) 116–125.
[42] A. Sonneveld, H. Rademaker, L.M.N. Duysens, Transfer and trapping of excitation energy transfer in Photosystem II as studied by chlorophyll a fluorescence quenching by dinitrobenzene and carotenoid triplets. The matrix model, Biochim. Biophys. Acta 593 (1980) 272–289.
[43] K.K. Karukstis, S.M. Gruber, J.A. Fruetel, C.S. Boegerman, Quenching of chlorophyll fluorescence by substituted anthraquinones, Biochim. Biophys. Acta, Bioenerg. 932 (1988) 84–90.
[44] R.C. Jennings, G. Zucchelli, F.M. Garlaschi, A. Vianelli, A comparison of the light-induced, non-reversible, fluorescence quenching in Photosystem II with quenching due to open reaction centres in terms of the chlorophyll emission spectral forms, Biochim. Biophys. Acta, Bioenerg. 1101 (1992) 79–83.
[45] M. Du, X.L. Xie, Y. Jia, L. Mets, G.R. Fleming, Direct observation of ultrafast energy transfer in PSI core antenna, Chem. Phys. Lett. 201 (1993) 535–542.
[46] R. vanGrondelle, J.P. Dekker, T. Gillbro, V. Sundstrom, Energy trapping and transfer in photosynthesis, Biochim. Biophys. Acta, Bioenerg. 1187 (1994) 1–65.
[47] T. Bittner, G.P. Wiederrecht, K.D. Irrgang, G. Renger, M.R. Wasielewski, Femtosecond transient absorption spectroscopy on the light harvesting chlorophyll *a/b* protein complex of Photosystem II at room temperature and 12 K, Chem. Phys. 194 (1995) 311–322.
[48] J.P. Connelly, M.G. Muller, M. Hucke, G. Gatzen, C.W. Mullineaux, A.V. Ruban, P. Horton, A.R. Holzwarth, Ultrafast spectroscopy of trimeric light-harvesting complex II from higher plants, J. Phys. Chem., B 101 (1997) 1902–1909.
[49] W. Haehnel, J.A. Nairn, P. Reisberg, K. Sauer, Picosecond fluorescence kinetics and energy transfer in chloroplasts and algae, Biochim. Biophys. Acta 680 (1982) 161–173.
[50] M. Hodges, I. Moya, I. Time-resolved chlorophyll fluorescence studies of photosynthetic membranes: resolution and characterization of four kinetic components, Biochim. Biophys. Acta, Bioenerg. 849 (1986) 193–202.
[51] G.H. Schatz, H. Brock, A.R. Holzwarth, Kinetic and energetic model for the primary processes in Photosystem II, Biophys. J. 54 (1988) 397–405.
[52] R.J. Gulotty, L. Mets, R. Alberte, G.R. Fleming, Picosecond fluorescence study of photosynthetic mutants of *Chlamydomonas reinhardtii*: origin of the fluorescence decay kinetics of chloroplasts, Photochem. Photobiol. 41 (1985) 487–496.
[53] A.R. Holzwarth, J. Wendler, W. Haehnel, Time-resolved picosecond fluorescence spectra of the antenna chlorophylls in *Chlorella vulgaris*. Resolution of photosystem I fluorescence, Biochim. Biophys. Acta, Bioenerg. 807 (1985) 155–167.
[54] R.C. Jennings, R. Croce, D. Dorra, F.M. Garlaschi, A.R. Holzwarth, A. Rivadossi, G. Zucchelli, Photosystem I red spectral forms: diffusion limited energy transfer, optical reorganisation energy and absorption cross section, in: G. Garab (Ed.), Photosynthesis: Mechanisms and Effects, vol. 1, Kluwer Academic Publishers, Dordrecht, 1996, pp. 271–276.
[55] N.E. Holt, D. Zigmantas, L. Valkunas, X.-P. Li, K.K. Niyogi, G.R. Fleming, Carotenoid cation formation and the regulation of photosynthetic light harvesting, Science 307 (2005) 433–436.
[56] R.C. Jennings, G. Elli, F.M. Garlaschi, S. Santabarbara, G. Zucchelli, Selective quenching of the fluorescence of core chlorophyll–protein complexes by photochemistry indicates that Photosystem II is partly diffusion limited, Photosynth. Res. 66 (2000) 225–233.
[57] S. Vassiliev, C.I. Lee, G.W. Brudvig, D. Bruce, Structure-based kinetic modeling of excited-state transfer and trapping in histidine-tagged Photosystem II core complexes from synechocystis, Biochemistry 41 (2002) 12236–12243.
[58] J.P. Dekker, R. van Grondelle, Primary charge separation in Photosystem II, Photosynth. Res. 63 (2000) 195–208.
[59] G. Zucchelli, P. Dainese, R.C. Jennings, J. Breton, F.M. Garlaschi, R. Bassi, Gaussian decomposition of absorption and linear dichroism spectra of outer antenna complexes of Photosystem II, Biochemistry 33 (1994) 8982–8990.
[60] R.C. Jennings, G. Zucchelli, F.M. Garlaschi, The influence of quenching by open reaction centres on the Photosystem II emission spectrum, Biochim. Biophys. Acta, Bioenerg. 1060 (1991) 245–250.
[61] H. Dau, K. Sauer, Exciton equilibration and Photosystem II exciton dynamics. A fluorescence study on Photosystem II particles of spinach, Biochim. Biophys. Acta, Bioenerg. 1273 (1996) 175–190.
[62] D.L. Dexter, A theory of sensitized luminescence in solids, J. Chem. Phys. 21 (1953) 836–850.
[63] L.L. Shipman, D.L. Housman, Förster transfer rate for chlorophyll *a*, Photochem. Photobiol. 29 (1979) 1163–1167.
[64] R.M. Pearlstein, Chlorophyll singlet excitons, in: Govindjee (Ed.), Photosynthesis. Energy Conversion by Plants and Bacteria, vol. 1, Academic Press, New York (N.Y.), 1982, pp. 293–330.
[65] A.Y. Borisov, Specific Features of Excitation Migration in Photosynthesis in: R.C. Jennings, et al. (Eds.), Light as an Energy Source and Information Carrier in Plant Physiology, Plenum, New York (N.Y.), 1996, pp. 31–40.
[66] S. Santabarbara, R.C. Jennings, D. Carbonera, Analysis of Photosystem II triplet states in thylakoids by Fluorescence Detected Magnetic Resonance (FDMR) in relation to the redox state of the primary quinone acceptor Q_A, Chem. Phys. 294 (2003) 257–266.