Involvement of uncoupled antenna chlorophylls in photoinhibition in thylakoids

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Abstract Evidence is presented, by means of both fluorescence and action spectroscopy, that a small, spectroscopically heterogeneous population of both Chl *a* and Chl *b* molecules is present in isolated spinach thylakoids and is active in photoinhibition. The broadness of the action spectrum suggests that degraded or incompletely assembled pigment–protein complexes may be involved.

Key words: Photoinhibition; Action spectrum; Excited state quenching; Uncoupled pigment; Thylakoid

1. Introduction

When exposed to high photon fluences, plants are subject to oxidative damage, mainly at the level of photosystem II (PS II), which seems to be primarily caused by singlet oxygen [1,2]. This reactive oxygen species is generated by an interaction between the ground state electrons of triplet oxygen with the triplet state of chlorophyll (Chl), formed by either intersystem crossing from the first excited singlet state of Chl [3] or by the primary charge separated state involving P_{680} and pheophytin (the recombination triplet). This latter process is often suggested as being the primary cause of photoinhibition (e.g. [4,5]). Another possibility for the formation of potentially damaging Chl triplets involves Chl molecules which are either weakly coupled to or completely uncoupled from the antenna pigment matrix and hence from the carotenoids (Car) which efficiently quench them. Thus Sinclair et al. [6] observed that the degree of photoinhibition in thylakoids was largely insensitive to both the PS II antenna size and the so-called high energy quenching (q_{NP}) of PS II fluorescence. Santabarbara et al. [7] showed that while PS II photoinhibition in isolated thylakoids displayed a linear light dosage/response plot, it was only slightly decreased by singlet excited state quenchers. Similar data with singlet quenchers were also published by Tyystjarvy et al. [8]. During the greening of etiolated leaves high levels of Chl triplets have been detected [9,10] and Carspi et al. [11] have shown that singlet oxygen is also formed. This suggests the involvement of pigment–protein complexes in which Chl–Car coupling for triplet transfer is not correctly established during synthesis. Interestingly, Vassil'ev et al. [12] detected a minor, long-lived ns fluorescence decay component in thylakoids which was insensitive to singlet quenchers. The decay-associated spectrum of this component seemed to be somewhat blue-shifted with respect to the main emission maximum though precise details on this point are lacking. The data were interpreted in terms of a small population of uncoupled Chls. If this is correct it should be possible to directly observe this population of uncoupled Chls by its reduced sensitivity to singlet quenchers also in steady state fluorescence spectra and gain additional information on some of its spectroscopic characteristics. In the first part of this paper we present evidence of this kind, using singlet quenchers, with freshly prepared spinach thylakoids, and demonstrate that both Chl *a* and *b* forms are involved. In the second part we attempt to demonstrate a 'cause–effect' relationship between the uncoupled pigments and photoinhibition by determining the photoinhibition action spectrum in isolated thylakoids. This action spectrum points to uncoupled pigments associated with incompletely assembled or damaged Chl *a*/*b* protein complexes being involved in thylakoid photoinhibition.

2. Materials and methods

Thylakoids were prepared from freshly harvested spinach leaves as previously described [13]. PS II-enriched membranes were prepared according to Berthold et al. [14] as modified by Dunahay et al. [15]. Pigments were extracted in 80% buffered acetone and the concentrations estimated according to Lichtenthaler [16]. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), *m*-dinitrobenzene (DNB) and 2-5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB) were added in alcohol and the final v/v ratio was less than 1%.

Steady state absorption and instrument-corrected fluorescence emission spectra were measured with an EG&G OMAIII (model 1460) as previously described [17]. Excitation wavelengths were selected by a Heath monochromator and two Corning CS 4-96 filters. In order to have an adequate signal to noise ratio, fluorescence emission spectra were accumulated to around 10^7 counts at the emission maximum. Absorption spectra were determined using opal glass diffuser plates to minimise light scattering distortion. Fluorescence excitation spectra were recorded with an SLM4800 fluorometer, corrected instrumentally for the incident light and also for the small amount of residual scattered light by subtracting the spectra of the same sample incubated with a saturating concentration of the quencher DBMIB (120 μM).

For the action spectrum light from a 900 W xenon lamp was filtered through an F3.4 monochromator (Applied Photophysics), and a cut-on filter RG 630 (Schott). The band-shape and relative intensity of monochromatic light were determined using the above described OMAIII detector. The band-shape is approximately Lorentzian with an FWHM of about 3.5 nm. The Chl concentration of the sample was

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Abbreviations: PS II, photosystem II; PS I, photosystem I; Chl, chlorophyll; Car, carotenoids; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2-5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone; DNB, *m*-dinitrobenzene

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4 μg ml^{-1}, and the temperature 4°C. Thylakoids were illuminated in the presence of 10 μM DCMU unless otherwise stated. Photoinhibition was estimated by the initial slope of the relative decrease of the F_V/F_M ratio by a least-square regression or by the light-induced F_M quenching rate parameter (K_I) as previously described [7] using the standard Stern–Völmer equation:

$$\Phi_M = \frac{k_f}{(\sum K_t + k_f + Q\,k_q + K_I)}$$

where k_f is the fluorescence rate constant, Σk_t is the sum of the other intrinsic decay processes, k_q is the quencher rate constant as modulated by the quencher concentration Q and K_I is the macroscopic, light-induced quenching rate constant. Fluorescence induction was measured as previously described [13] after 15 min of dark incubation at 4°C to allow complete Q_A oxidation.

PS II electron transport from H_2O to *p*-phenylenediamine (500 μM) was measured with a Clark-type oxygen electrode in a reaction mixture containing 0.1 M sucrose, 30 mM tricine–NaOH pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$ and 1 mM $Fe_3(CN)_6$ [18] under saturating light. The Chl concentration was 10 μg ml^{-1}.

3. Results and discussion

3.1. Effect of singlet quenchers on steady state fluorescence emission spectra

If a singlet quencher is added to a pigment system such as PS II with a large, energetically well coupled antenna matrix and which may also contain a small domain or population of uncoupled Chls it is expected that the uncoupled pigments will be less quenched than the coupled pigment matrix. This situation is approximated by Eq. 1 where the first term represents the well coupled matrix and the second term the hypothetical uncoupled pigments:

$$\Phi_{F_M} = \frac{N}{N+n}\frac{k_f}{(\sum k_t + k_f + p_N\,Q\,k_q)} + \frac{n}{N+n}\frac{k_f'}{(\sum k'_t + k_f' + p_n\,Q\,k_q)} \quad (1)$$

where Φ_{F_M} is the fluorescence yield at closed reaction centres; k_f and k_f' are the fluorescence rate constants for the coupled and uncoupled domains respectively and are probably numerically similar. Σk_t and $\Sigma k_t'$ represent the sum of all other trivial decay processes in the coupled and uncoupled domains and also in this case they are probably numerically similar. N and n are the respective sizes of the coupled and uncoupled pigment domains with $N \gg n$. Q is the quencher concentration weighted for the probability terms p_N and p_n that the N and n populations respectively bind a quencher and are expected to scale with the population size, i.e. $p_N \propto N/(N+n)$ and $p_n \propto n/(N+n)$. Due to ultrafast energy transfer between pigment sites within the well coupled matrix (ps and sub-ps) the N matrix is expected to be uniformly quenched. Thus, even though $N \gg n$, it is clear that in a quenching experiment, with sufficient instrumental resolution, the fluorescence from the uncoupled pigments should be detectable.

In Fig. 1 the steady state emission spectra of thylakoids at F_M are shown in the presence and absence of two different singlet quenchers, the substituted quinone DBMIB and the

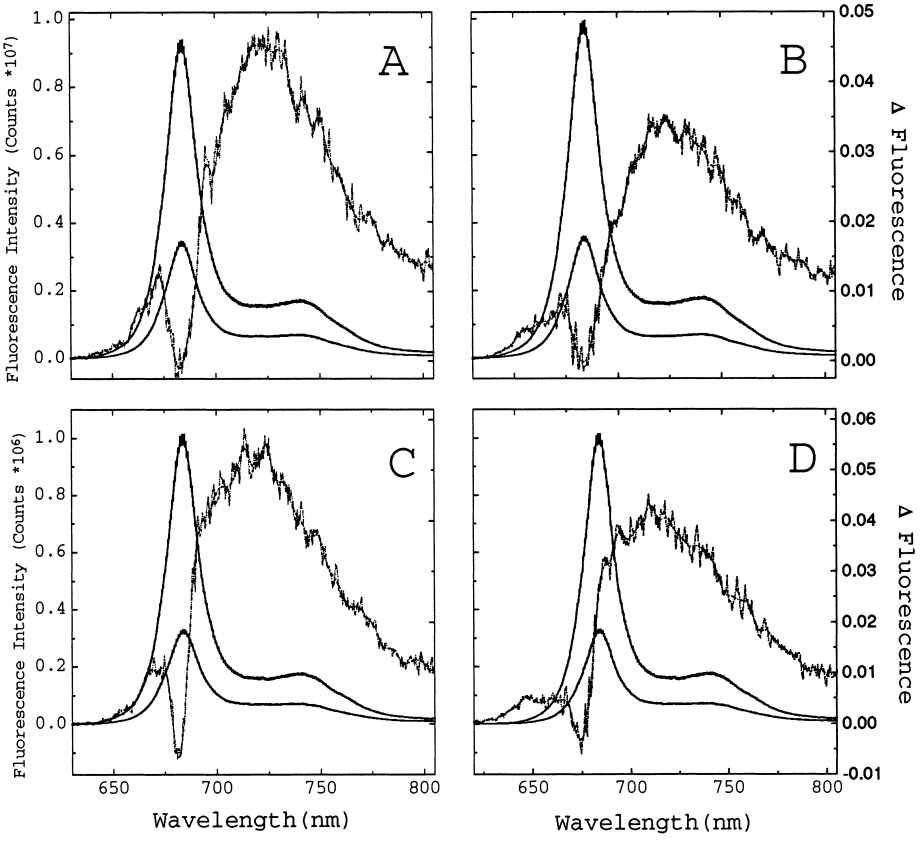

Fig. 1. Effects of DBMIB and DNB on fluorescence emission spectra of thylakoids at F_M. A, C: excitation at 435 nm. B, D: excitation at 475 nm. A, B: quencher DBMIB (1.5 μM) C, D: quencher DNB (500 μM). Solid lines, measured emission spectra. Dashed lines, difference quenched minus unquenched spectrum, after normalisation of each spectrum at its maximum (683 nm).

quinone analogue DNB, for excitation wavelengths dominated by either Chl *a* absorption (435 nm) or Chl *b* absorption (475 nm). For both DNB and DBMIB, quenching was approximately 70% at the PS II emission maximum near 683 nm. Also presented in Fig. 1 are the difference spectra, calculated after normalisation to the maximum emission. These difference spectra are dominated by a long wavelength structure, with a maximum near 720–725 nm, which we associate with photosystem I (PS I) on the basis of comparison with published RT spectra of isolated PS I particles [19]. The reduced sensitivity of PS I to singlet quenchers is expected on the basis of its very short excited state lifetime (120 ps; [20]) with respect to that of the F_M fluorescence of PS II (2–3 ns; [21]). In addition a smaller, rather broad, difference spectrum feature is evident on the short wavelength side of the PS II emission maximum, the exact structure of which depends on the excitation wavelength. The peak position, under 435 nm excitation, is in the 670–675 nm region and is clearly associated with Chl *a* emission. The broadness of this band (FWHM $\approx$30 nm) suggests that more than one emission form is present as protein-bound Chl forms have a half bandwidth of 10–12 nm in the Q_y region at room temperature [17]. Under 475 nm excitation a clear emission structure near 650 nm emerges which is presumably due to Chl *b* molecules. It should be underlined that these short wavelength difference spectra structures can not be interpreted in terms of a broad, blue tailing PS I emission as we have carefully compared both the absorption and emission spectra of an intact PS I-200 preparation [19] with that of PS II membranes (BBY particles). Our observations indicate that PS I is red-shifted with respect to PS II at all wavelengths and this increases with decreasing wavelength (data not presented). Similar structures have been observed in thylakoids subject to high intensity, light-induced non-photochemical quenching of F_M (unpublished observation) thus excluding the possibility that they are artefactually induced by addition of the singlet quenchers. The data would therefore seem to be most readily interpreted in terms of a mixed population of uncoupled Chl *a* and Chl *b* forms according to Eq. 1. The Chl *a* emitting forms are somewhat blue-shifted with respect to the overall PS II emission, in general agreement with Vassil'ev et al. [12].

3.2. Photoinhibition action spectrum

In order to ascertain whether uncoupled Chl *a* and *b* molecules might be involved in inducing photoinhibitory damage in isolated thylakoids we have determined the action spectrum of this process. This was directly achieved by determining both the photoinhibition-induced decrease in the F_V/F_M ratio and by evaluating the macroscopic fluorescence quenching rate constant, K_I (see Section 2 and [7]), of F_M lowering, which is the dominant process involved in the F_V/F_M decrease (e.g. [7]). We initially checked whether under our experimental conditions the F_V/F_M ratio is a correct indicator of PS II photoinhibition, as has often been observed for plant systems (e.g. [22]), though not always with algae [23]. To this end thylakoids were subjected to photoinhibitory conditions and the two parameters measured after different times of treatment. The results (Fig. 2) show a linear correlation between PS II-catalysed oxygen evolution and the F_V/F_M ratio thus confirming that the fluorescence ratio is an accurate measure of PS II photoinhibition. Furthermore, for the small levels of photoinhibitory damage measured in the present study, the response was shown to be approximately linear with both fluence rate and time (data not presented), as previously described [7]. In addition, immunoblotting showed that D1 protein degradation did not occur on the time scale and fluence rate used here (data not presented).

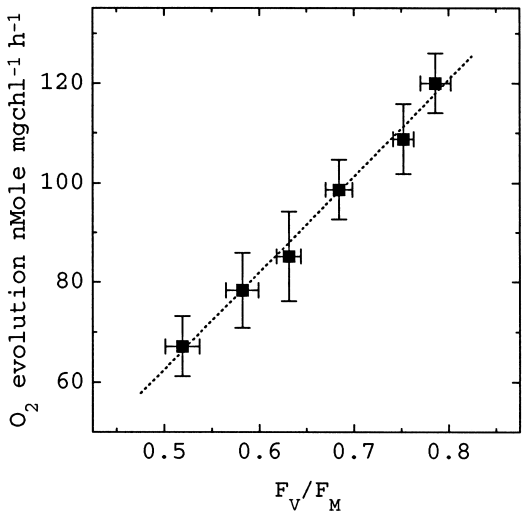

Fig. 2. Correlation between oxygen evolution and the decrease of F_V/F_M in isolated thylakoids during photoinhibition. Errors are standard deviations of five different preparations.

We also checked whether our previous observation of a substantial insensitivity of photoinhibition to the excited state levels [7], obtained with white light of high photon fluence rates (2000–20 000 μE m^{-2} s^{-1}), also occurred at the much lower monochromatic fluence rates (about 250 μE m^{-2} s^{-1}) used to determine the action spectrum. In Fig. 3 data for the K_I parameter are presented for several experiments with different monochromatic wavelengths in the intensity range used to determine the action spectrum. It is clear that, also in this

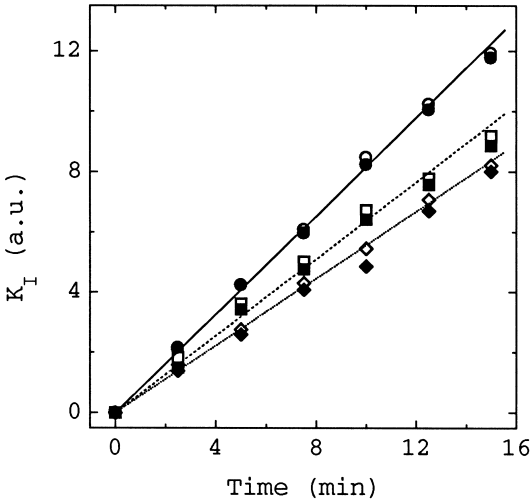

Fig. 3. The effect of reducing the excited state population on the photoinhibition parameter, K_I, under low intensity, monochromatic light, as a function of treatment time. 651 nm: Squares and dashed line. 672 nm: Circles, solid line. 681.5 nm: Diamonds, dash-dotted lines. Open symbols are controls and closed symbols thylakoids incubated with 1.5 μM DBMIB. Quenching in both cases was approximately 70%. The standard deviations are between 4 and 7%.

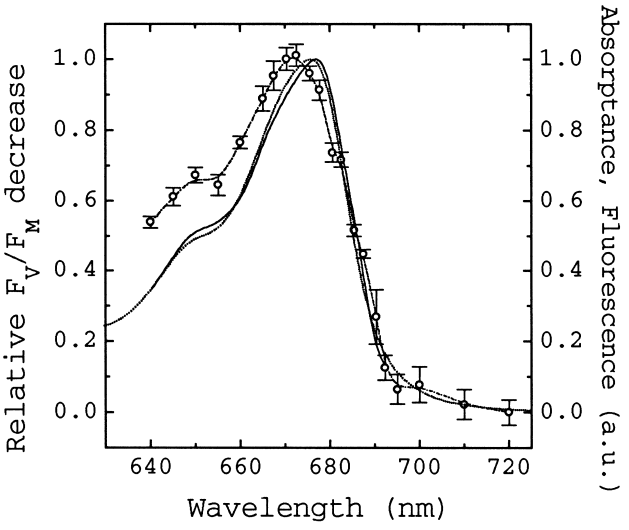

Fig. 4. Action spectrum of light-induced F_V/F_M lowering (open circles and dotted-line interpolation). Error bars are the standard deviation of the weighted mean. The dotted line is the normalised 1-T spectrum of BBY membrane and the solid line is the excitation spectrum of thylakoid fluorescence at F_M, monitored at 700 nm.

case, lowering the excited state population does not significantly reduce K_I.

The action spectrum for PS II photoinhibition in spinach thylakoids, determined as the F_V/F_M ratio lowering, for the spectral interval between 640 and 720 nm and corrected for the small differences in incident photon fluence rates (see Section 2), is shown in Fig. 4. Almost identical results were obtained when photoinhibition was estimated by the F_M quenching rate constant K_I (data not presented). Also shown for comparison are:

1. the photon absorption spectrum (1-T) of an isolated PS II preparation (BBY particles). This was calculated for a peak OD of 0.065 which was half that of the thylakoid sample used for experiments and hence is expected to approximate that of the thylakoid bound PS II.
2. The F_M fluorescence excitation spectrum, corrected for minor variations in the incident photon fluence. As significant variations in the fluorescence yield across the PS II absorption band are not expected, this spectrum should accurately mirror the PS II photon absorption spectrum, as is seen. It should be mentioned that both the 1-T and the fluorescence excitation spectra are similar to the published action spectra for PS II-mediated electron transport [24,25].

It is clear from the results presented in Fig. 4 that the action spectrum does not coincide with PS II absorption as would be expected if photoinhibition was determined by absorption into the coupled antenna of PS II. Over most of the spectral interval investigated the action spectrum is significantly blue-shifted with respect to PS II, with a peak value near 672–673 nm whereas PS II is close to 677 nm. It can also be seen to be very broad and is clearly not due to a spectroscopically homogeneous population of Chls. The pronounced shoulder near 650 nm is characteristic of Chl *b* and the broadness in the Chl *a* absorption region suggests the involvement of a number of Chl *a* spectral forms. Though precise comparison is impossible, these characteristics are similar, in general terms, to those of the putative uncoupled Chls observed in the singlet quenching experiments (Fig. 1). Moreover, the photoinhibition action spectrum shares common features with the excitation spectrum of Chl *a* phosphorescence in greening and mature leaves [10], as well as in chloroplast and thylakoids [9]. We therefore believe that these experiments provide strong evidence for the involvement of uncoupled, or weakly coupled Chls, in photoinhibition, at least in isolated thylakoids. The broadness of the photoinhibition action spectrum may indicate that we are dealing with damaged or incompletely assembled Chl–protein complexes rather than with stochastically uncoupled pigments [7,8] though the difference may be largely one of semantics as uncoupled pigments are most likely bound to complexes and not free in the membrane. In this suggestion it is not unreasonable that the transfer of the Chl triplet to Car should be interrupted as this is very sensitive to both Chl–Car distance (van der Waals contact is required) and dipole orientation [26]. At this point it is not possible to identify the denatured complex(s) involved thought the involvement of Chl *b* implicates external antenna components. The direct biochemical demonstration of such complexes is hampered by the universal use of detergents in their preparation, which in itself leads to pigment uncoupling, e.g. [19], though attempts are underway.

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