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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

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The recent demonstration that the room temperature fluorescence emission spectrum of Photosystem II may be described as a linear combination of Gaussian bands associated with the well-known chlorophyll spectral forms (Zucchelli, G., Jennings, R.C., Garlaschi, F.M. (1992) Biochim. Biophys. Acta, 1099, 163–169) has permitted analysis of a number of fluorescence quenching phenomena in terms of the chlorophyll spectral forms. In this context we have analysed the fluorescence quenching due to open reaction centres and also the light-induced, non-photochemical, non-reversible quenching in isolated thylakoids, a Photosystem II membrane preparation and the major light-harvesting chlorophyll *a/b* protein complex (LHCII). Whereas open reaction centres quench the emission associated with the spectral form chl_{687}^{684} with greatest efficiency, thus indicating that it is this spectral form which is the most efficient in transferring energy to reaction centres, all the non-photochemical phenomena investigated show maximal quenching for both chl_{680}^{678} and chl_{687}^{684}. It is concluded that the light-induced, non-photochemical, non-reversible quenching involves the generation of quenching centres within the Photosystem II antenna, possibly at the level of LHCII.

Introduction

The antenna system of higher plant PS II consists of a large array of pigment molecules bound to specific polypeptides which form the so-called 'chlorophyll-protein' complexes [1,2]. Two distinct molecular species, chlorophyll *a* and chlorophyll *b*, are present at an *a/b* ratio of 2.0–2.3. Chlorophyll *a*, which is the major component, accounts for about 80% of PS II red (Q_y) absorption [3] and is present in a number of different absorption spectral forms [3–6]. While these spectral forms were initially analysed at low temperatues they may also be well-resolved in room temperature absorption spectra by gaussian band decomposition [3,5,6].

The spectral heterogeneity of PS II antenna absorption has suggested the possibility that room temperature fluorescence emission might also be heterogeneous. In a recent analysis of room temperature PS II emission spectra, using both gaussian deconvolution techniques and spectral band calculations based on the Stepanov relation [7], we have demonstrated that each of the main chlorophyll absorption spectral forms has a corresponding emission band [8]. Over 65% of the main emission band at room temperature comes from emission forms with maxima at 680 nm and 687 nm, which are Stokes-shifted by 2–3 nm with respect to their corresponding absorption.

Identification of the fluorescence bands associated with the various spectral forms is of considerable importance in understanding a number of properties of PS II. Thus, some of the energy transfer relationships between the PS II antenna and reaction centes (RCs) may be studied by determining the relative quenching of the emission bands due to energy transfer to open RCs. We have recently demonstrated that PS II fluorescence quenching is greatest near 691 nm, decreasing progressively at both longer and shorter wavelengths [9]. These data, by analogy with a previous study, in which the action spectrum of RC quenching was shown to be maximal at 683 nm [3], were interpreted to

Abbreviations: Y, Berthold, Babcock, Yocum [17]; chl_m^n, chlorophyll with absorption maximum at *n* nm and emission maximum at *m* nm; DCMU, 3-(3,4-dichlorophenyl)-dimethylurea; F_o, limiting fluorescence yield with reaction centres open; F_m, fluorescence yield with reaction centres closed; LHCII, the major light harvesting chlorophyll *a/b* protein complex; RC, reaction centre; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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indicate that it is the 684 nm antenna absorption spectral form which transfers energy to RCs with greatest efficiency, followed by the 678 nm absorption form. In the present paper we directly analyse open RC quenching in terms of the emission bands. It is demonstrated that it is, indeed, the chl_{685}^{684} which is preferentially quenched by open RCs and which is therefore, presumably, the most efficient spectral form in transferring energy to RCs. The relative quenching efficiency by RCs of the other emission bands is also established.

Another phenomenon which is amenable to analysis in terms of the emission bands is the light-induced, non-photochemical quenching of PS II fluorescence. While a number of such processes have been distinguished, most of which are reversible in the dark [10], in isolated chloroplasts a non-reversible component is also observed. This is thought to be associated with the photo-inhibition of PS II RCs [11–13] and is probably equivalent to the very slowly reversing quenching in leaves [10,14,15]. This phenomenon is generally explained in terms of increased thermal dissipation at PS II RCs associated with photo-inhibition. If this interpretation is correct one would expect this non-photochemical fluorescence quenching to be to the same emission bands as that brought about by photochemical quenching at open RCs. In the present paper we investigate this possibility. Maximal fluorescence quenching is shown to involve both the chl_{685}^{684} and chl_{680}^{678} spectral forms. As a similar fluorescence band quenching distribution is demonstrated to occur for light induced quenching in isolated LHCII, the principal PS II antenna complex, we suggest that the non-reversible, non-photochemical quenching in thylakoids may be an antenna-based phenomenon.

Materials and Methods

Thylakoids were prepared from freshly harvested spinach leaves, as previously described [16] and re-suspended in Tricine (30 mm, pH 8), NaCl (10 mM), $MgCl_2$ (5 mM) and sucrose (0.2 M).

BBY-grana were prepared from spinach leaves according to Berthold et al. [17] with omission of the last Triton X-100 treatment [18,3]. This preparation contains both LHCII and PS II core protein complexes and is substantially free of PS I chlorophyll-protein complexes (data not shown).

Light-induced, non-photochemical quenching was achieved by illuminating samples at 2°C with a broad band red light (peak transmission at 680 nm, 14.5 mW cm^{-2}) for between 2 to 10 min, depending on the sample, at a chlorophyll concentration of approx. 10 μg/ml for BBY-grana and thylakoids and 5 μg/ml for LHCII. As the fluorescence level was noticed to increase during the first few minutes after illumination had ceased, presumably due to the well characterised relaxation of the reversible non-photochemical quenching phenomena [10,14,15], samples were incubated in the dark for 15 min at 2°C and for a further 15 min at room temperature prior to commencement of the fluorescence analysis. This period of dark treatment was sufficient to ensure that all reversible quenching processes had been eliminated.

F_0 and F_m fluorescence levels were measured in the assembly previously described [19]. F_m was determined in the presence of DCMU (25 μM) and hydroxylamine (2 mM), while F_0 was measured in the same sample in the absence of these additions [19].

Fluorescence emission spectra were measured with an EG&G OMA III (model 1460) multi-channel spectrometer. The resolution of this apparatus was about 0.5 nm/pixel. Excitation light was provided by a Heath monochromator (excitation wavelength 480 nm; FWHM 1.2 nm) combined with two Corning 4-96 filters. The fluorescence emission was filtered across a Schott OG 530 filter. This arrangement ensured that stray, scattered light was not significant at wavelengths above 650 nm, as judged by using the fluorescence quencher dibromothymoquinone (280 μM). Total counts accumulated in the peak channel of each spectrum were between $8 \cdot 10^3$ and $2 \cdot 10^4$. Between 30–60 emission spectra were averaged and smoothed, according to Savitzky and Golay [20]. Fluorescence was maintained near the F_0 level by means of a weak excitation beam and with continual sample stirring, in which only a small part of the sample was illuminated at any one time. Thylakoids were illuminated in the presence of methyl viologen (0.1 mM). Emission spectra with closed RCs (F_m) were measured in the same sample after addition of 25 μM DCMU and 2 mM hydroxylamine. In all cases the chlorophyll concentration was below 2 μg/ml. The emission spectra were corrected for distortions resulting from a wavelength-dependent response of the light-collection setup, using an intensity-calibrated source (ISCO Spectroradiometer Calibrator).

Deconvolution analyses of the spectra in terms of 'asymmetric' Gaussian bands were performed as previously described [6,8,9]. Spectra were analysed to find the minimum number of bands giving the best fit, as judged using the χ^2 and the distribution of the residuals [21]. All band parameters were left free in the fitting procedure.

Results

Photochemical quenching by open RCs

Fluorescence emission spectra have been measured in both spinach thylakoids and the BBY-grana (PS II)

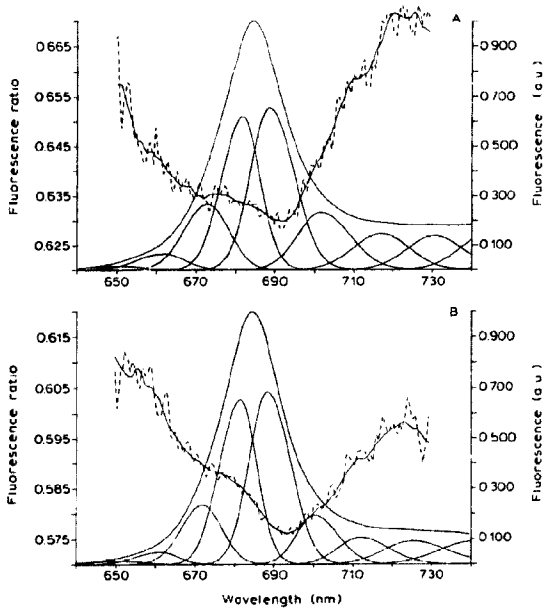

Fig. 1. The ratio of the fluorescence emission spectra measured with PSII RCS open to that measured with closed RCs (- - - - - -) for: (A) thylakoids and; (B) BBY-grana. Also presented are the fluorescence emission spectra of both membrane preparations (———, closed RC's) with the relative gaussian bands obtained by numerical analysis. The emission spectra have been corrected for the sensitivity of the measuring apparatus (see Materials and Methods). Both smoothed (continuous line) and non-smoothed (broken line) ratio spectra are presented. For further details see Materials and Methods.

preparation with RCs either partially open or fully closed. The ratios of these spectra are presented in Fig. 1. As previously reported, for the BBY-grana [9] there is a distinct minimum near 690–691 nm, indicating maximal RC quenching at this wavelength.

The fluorescence emission ratio spectrum previously reported for thylakoids [9] was somewhat blue-shifted with respect to the BBY-grana and to that for thylakoids presented in Fig. 1. This was interpreted as being due to a minor, long-wavelength fluorescence contribution by PS I, proportionally greater at open RCs than at closed RCs. In the present study we determined the 'open RC' emission spectrum at lower steady-state levels of open RCs than previously (higher excitation beam intensity). This has the effect of reducing the open-to-closed-RC fluorescence ratio and thus minimises the effect of a small, but fixed, PS I emission. Under these conditions both BBY-grana and thylakoids display maximal open RC quenching at 690–691 nm.

In Fig. 1 the fluorescence ratio spectra are superimposed on the emission spectra (closed RCs) for both thylakoids and BBY-grana, in which the different gaussian emission bands are also indicated. As previously demonstrated, these components, up to and including that emitting near 700 nm, represent the emission of the various chlorophyll *a* spectral forms and chlorophyll *b* [8]. The dominant emission bands, peaking near 680 nm and 687 nm, are associated with the 678 nm and 684 nm absorption forms, respectively. It is evident that maximal RC quenching in both thylakoids and BBY-grana is associated with the 687 nm emission form, with decreased quenching of the components emitting at longer and shorter wavelengths. From the data presented in Fig. 1 we have calculated the relative quenching of each of the spectral bands for both BBY-grana and thylakoids. The relative quenching order for thylakoids is:

687 > 680, 672 > 662, 700 > 652

and for BBY-grana is:

687 > 680, 700 > 672 > 662 > 652

Thus the two quenching order sequences are in good agreement, except for the emission band around 700 nm.

Light-induced, non-photochemical, non-reversible quenching in PS II

Fluorescence emission spectra have been measured at the F_m fluorescence level in thylakoids and BBY-grana both before, and after, illumination with high-intensity light. This treatment was sufficient to induce non-reversible, non-photochemical quenching in the samples, while having no measurable chlorophyll photobleaching activity. As can be seen in Table I, the high light intensity treatment brought about a marked decrease in the F_m level fluorescence in both samples with only a small decline in the F_o level in chloroplasts, as is usually observed [11–13]. Quenching of F_o fluorescence was, however, more pronounced in BBY-grana. The emission spectra ratios are presented in Fig. 2, together with the band deconvolution of the

TABLE I

Effect of illumination with high light intensity on the fluorescence induction parameters of BBY-grana and thylakoids

BBY-grana were illuminated for 2 min and thylakoids for 10 min. For other details see Materials and Methods.

	Non-illuminated	Illuminated
BBY-grana		
F_o	34	28
F_m	100	48
Thylakoids		
F_o	35	33
F_m	100	64

relevant emission spectra. In both membrane samples the fluorescence ratio spectra have a distinct minimum near 685 nm, which indicates maximal fluorescence quenching at this wavelength. Comparison with the emission bands (Fig. 2) indicates that maximal quenching is associated approximately equally with the two principal components, peaking at 680 nm and 687 nm, with decreased quenching of both the longer and shorter wavelength components.

Light-induced non-photochemical quenching in isolated LHCII

We have recently demonstrated that illumination of isolated LHCII with relatively high light intensities provokes pronounced fluorescence quenching, which is, in part, reversible [22]. We have now analysed the emission spectrum of the non-reversible component by comparing the spectra of quenched and non-quenched samples (Fig. 3). From Fig. 3 it can be seen that maximal quenching occurs in the 680 nm–690 nm region and is seen to be greatest for the emission components peaking at 680 nm and 687 nm, with decreased quenching of both the longer and shorter wavelength components.

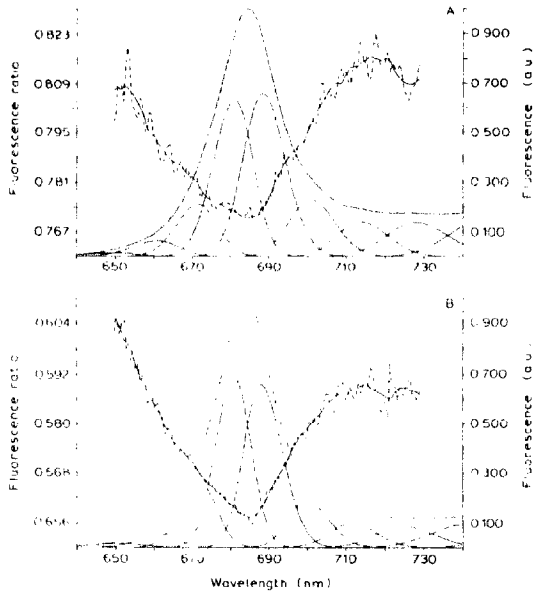

Fig. 2. The ratio of the fluorescence emission spectra measured with samples in which the non-photochemical, non-reversible quenching was induced by pre-illumination to that of non-preilluminated samples (- - - - - -) for: (A) thylakoids and; (B) BBY-grana. Also presented are the fluorescence emission spectra of both membrane preparations (———, non-pre-illuminated samples) with the relative gaussian bands obtained by numerical analysis. All spectra were measured with RCs closed. The emission spectra have been corrected for instrument sensitivity (see Materials and Methods). Both smoothed (———) and non-smoothed (- - - - - -) ratio spectra are presented. Pre-illumination of thylakoids was for 10 min and 2 min for BBY-grana. For further details see Materials and Methods.

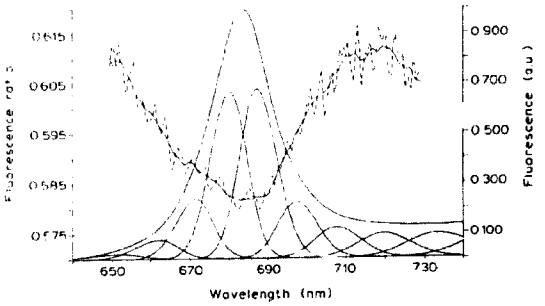

Fig. 3. The ratio of the fluorescence emission spectra for isolated LHCII measured with samples in which fluorescence quenching was induced by pre-illumination to that of non-pre-illuminated samples (- - - - - -). Also presented is the emission spectrum with relative Gaussian bands obtained by numerical analysis (non-preilluminated sample, ———). The emission spectrum was corrected for instrument sensitivity (see Materials and Methods). Both smoothed (———) and non-smoothed (- - - - - -) ratio spectra are presented. Pre-illumination was for 2 min. For further details see Materials and Methods.

Discussion

We have previously reported that small, emission wavelength-dependent differences in fluorescence quenching due to open PS II RCs can be demonstrated by analysing the steady-state emission spectra at open and closed RCs. Thus, in a spinach PS II grana preparation, maximal RC quenching was observed near 691 nm. This was interpreted as indicating that excitation energy is transferred from those molecules emitting at this wavelength to RCs with greatest efficiency [9]. In the present paper this observation has been confirmed for the BBY-grana preparation and spinach thylakoids and it has been possible to interpret the different RC quenching efficiencies, indicative of relative transfer efficiencies to RCs, in terms of the fluorescence emission bands [8]. As these emission bands are associated with the well-known chlorophyll absorption spectral forms, one may write the following transfer efficiency sequence in terms of the chlorophyll spectral forms:

$$chl_{685}^{684} > chl_{680}^{678} > chl_{672}^{670} > chl_{664}^{660} > chl_{652}^{648}$$

This sequence, based on the emission bands, is very similar to that previously suggested for the absorption spectral forms from an analysis of the dependence of RC quenching efficiency on the excitation wavelength [3]. We have not included the long wavelength band chl_{700}^{695} in this sequence, owing to the differences encountered between the BBY-grana and thylakoids membrane preparations. However, in both cases, this long wavelength form is more efficient than chlorophyll

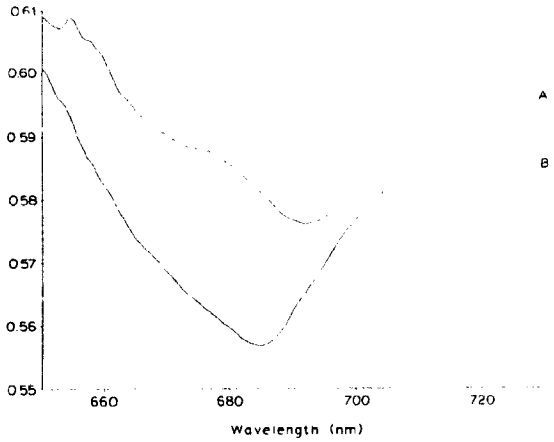

Fig. 4. The ratio of fluorescence emission spectra for: (A) open RC quenching and; (B) light-induced, non-reversible, non-photochemical quenching for BBY-grana. The spectra are the same as those presented in Fig. 1 and Fig. 2, respectively and are shown for direct comparison. For details see legends to Figs. 1 and 2.

b (chl_{652}^{648}) in transferring energy to RCs, even though it is red-shifted by about 15 nm with respect to the RC.

We have determined the fluorescence quenching spectrum in both BBY-grana and thylakoids of the light-induced, non-reversible quenching. In both cases maximal quenching was observed near 685 nm. This is significantly blue-shifted, with respect to RC quenching. This blue shift is due to the high sensitivity of chl_{680}^{678}, which is quenched equally to chl_{687}^{684}. In Fig. 4 the emission ratio spectra for open RCs (a) and light-induced quenching (b), determined for similar overall quenching levels in BBY-grana, are directly compared. It is evident that not only is the light-induced phenomenon blue-shifted with respect to RC quenching, but that the amplitude of the ratio spectrum is much greater in the case of the light-induced phenomenon. Thus it is clear that the light-induced, non-reversible quenching in PS II is spectrally different to that due to open RCs, which suggests that it is incorrect to interpret this light-induced quenching exclusively in terms of RCs (see Introduction). We thus propose that this quenching phenomenon involves the generation of quenching centres in the PSII antenna. This suggestion is supported by the observation (Fig. 3) that light-induced quenching in isolated LHCII, the principal PSII antenna complex, is greatest for the same spectral forms as the light-induced quenching in PS II, i.e., chl_{680}^{678} and chl_{687}^{684}. The generation of light-induced quenching processes within the PS II antenna has been previously suggested by Bradbury and Baker [23], Demmig et al. [24] and Ruban et al. [25].

As mentioned above, the light-induced quenching is greatest for chl_{680}^{678} and chl_{687}^{684} in all three systems investigated here. This suggests either: (a) that these spectral forms interact preferentially with the fluorescence quenchers generated by light or; (b) that at least some molecules of these spectral forms become, themselves, fluorescence quenchers, i.e., are converted to forms with increased thermal dissipation. It is not possible at present to distinguish between these two possibilities.

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