Chlorophyll fluorescence quenching in isolated light harvesting complexes induced by zeaxanthin

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Abstract Non-photochemical quenching of chlorophyll fluorescence in plants occurs in the light harvesting antenna of photosystem II and is regulated by the xanthophyll cycle. A new in vitro model for this process has been developed. Purified light harvesting complexes above the detergent critical micelle concentration have a stable high fluorescence yield but a rapidly inducible fluorescence quenching occurs upon addition of zeaxanthin. Violaxanthin was without effect, lutein and antheraxanthin induced a marginal response, whereas the violaxanthin analogue, auroxanthin, induced strong quenching. Quenching was not caused by aggregation of the complexes but was accompanied by a spectral broadening and red shift, indicating a zeaxanthin-dependent alteration in the chlorophyll environment.

Key words: Non-photochemical quenching; Light harvesting complex; Xanthophyll cycle; Chlorophyll fluorescence; Thylakoid membrane

1. Introduction

The importance of the functional flexibility of the light harvesting antenna of photosystem II (PS II) in accommodating the fluctuation in the balance between light input and metabolic capacity in plants is well documented – in limiting irradiance, light energy is efficiently harvested, in excess irradiance, energy is effectively dissipated [1]. The peripheral PS II antenna is a relatively complex assembly of at least six Lhcb (proteins encoded by the *Lhcb* genes) protein subunits (together called light harvesting complexes of photosystem II (LHC II)), together with a number of Lhc-related proteins including ELIPs and PsbS [2]. In excess light, the transthylakoid pH gradient (ΔpH) plays a key role in the induction of energy dissipation, which is measured as a component of the non-photochemical quenching of chlorophyll fluorescence (NPQ) referred to as non-photochemical quenching dependent upon the ΔpH (*q*E) [3]. The acidification of the thylakoid lumen appears to result in a conformational change that is correlated to the formation of the quencher [4,5], and which is proposed to result from protonation of key carboxyl residues on one or more of the Lhc proteins [6–8]. In addition, the ΔpH activates the xanthophyll cycle [9], bringing about de-epoxidation of LHC II-bound violaxanthin to form zeaxanthin [10]. The formation of zeaxanthin also correlates with the induction of *q*E [11].

Although it is recognised that the xanthophyll cycle plays a key role in *q*E, the mechanism involved has not been elucidated. It has been proposed that zeaxanthin may act as a direct quencher of Chl excited states [11]: the principle argument in support of this was the predicted difference in S1 energy level between violaxanthin and zeaxanthin that would allow only the latter carotenoid to be a quencher [12,13]. However, recent data suggest the difference in energy levels would not provide differential capacity for quenching [14]. Alternatively, it has been proposed that violaxanthin and zeaxanthin control quenching indirectly, by modulating the structural changes in the antenna proteins that give rise to quenching. Evidence to support this idea has been reviewed [1,15].

In order to understand fully the mechanism of *q*E and in particular the role played by the xanthophyll cycle it is necessary to develop an in vitro system using purified components of the PS II antenna. It has been observed in fact that all of the isolated Lhcb pigment protein complexes so far examined can display the level of photophysical flexibility consistent with the extent of *q*E in vivo [16–18]. Quenching occurs in these proteins if they are induced to aggregate at detergent concentrations below the critical micelle concentration (CMC). It was found that in vitro quenching was controlled by pH, by reagents such as DCCD, dibucaine and antimycin A, and by the presence of carotenoids, therefore having the main properties of in vivo *q*E [16–19]. In particular, zeaxanthin was found to stimulate quenching and violaxanthin was found to be a quenching inhibitor.

Although this system has proved useful in establishing many facets of the properties of the PS II antenna with respect to quenching, it has the disadvantage of lack of stability. Well below the CMC, the complexes tend to form aggregates the size of which can be difficult to control. Also the process of aggregation is rather non-specific and can be displayed by core antenna proteins such as CP47 not involved in *q*E in vivo [20]. Finally, a most important question is whether aggregation is actually required for quenching, or whether the conditions that induce quenching in this system also promote aggregation [1,15].

In this paper we describe a new experimental system with purified LHC II components in higher detergent concentration. In this case the fluorescence yield is stable and at a

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-ρ-benzoquinone; DM, *n*-dodecyl β-maltoside; Lhcb, proteins encoded by the *Lhcb* genes; LHC II, the light harvesting complexes of photosystem II; NPQ, non-photochemical quenching; PS II, photosystem II; *q*E, non-photochemical quenching dependent upon the ΔpH; ΔpH, transthylakoid pH gradient

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maximum value, and most significantly, addition of zeaxanthin can bring about a rapid and large quenching, in the absence of measurable complex aggregation.

2. Materials and methods

The LHC II components LHC IIb, CP29 and CP26 were prepared from spinach BBY particles are described previously [10,17]. Monomers of LHC IIb were prepared by phospholipase treatment, and purification on a sucrose gradient, as described before [10]. Sucrose gradient centrifugation used a 0.15–1.0 M seven step exponential gradient of sucrose, with a centrifugation at $100\,000\times g$ for 20 h in a Beckman SW41 rotor [10]. Before centrifugation, samples of CP29 at a Chl concentration of 0.5 mg/ml were incubated for 10 min at room temperature in the presence of 200 μM *n*-dodecyl β-maltoside (DM) with either zero or an excess of zeaxanthin (Carotenoid/Chl of 4). Chl fluorescence assays at room temperature were carried out using a Walz PAM 101 Chlorophyll fluorimeter at a [Chl] of 2 μg/ml in a medium containing 200 μM DM, 20 mM HEPES, 10 mM MES, pH 8.0 as previously described [16,17]. Quenching was calculated as $(F_{max}-F_t)/F_t$, where F_{max} is the maximum yield at 200 μM DM, and F_t is the yield at any time after initiation of quenching. The fluorescence level after maximum quenching (the theoretical unquenched fluorescence when $t=\infty$) is referred to as F_U. Fluorescence emission spectra were recorded at 77 K as before [17]. Absorption difference spectra were recorded at 20°C using an Aminco DW2000 spectrophotometer [17]. Carotenoid analyses were carried out using HPLC with at least three different extracts [10].

3. Results and discussion

The important assumption underlying these experiments is that the xanthophyll cycle carotenoids bind to sites on LHC II that are easily accessible from the external medium. Evidence to support this view has come from determination of the binding of these carotenoids to isolated LHC II [10]. Also, it is implicit in the fact that violaxanthin can be reversibly de-epoxidised by enzymes bound extrinsically to the thylakoid membrane, that these carotenoid must be readily exchangeable with the lipid phase of the membrane [9]. Although the simplest idea is that these pigments would be bound loosely to peripheral sites, it does not exclude the possibility that the sites are internal so long as they are accessible.

When LHC IIb, CP29 or CP26 are dissolved in 200 μM DM, the fluorescence yield of the complex is at a maximum. The CMC of DM is approx. 150 μM. A further increase in detergent concentration does not give any increase in fluorescence yield. This level of fluorescence is stable for over 30 min with stirring at room temperature. Addition of zeaxanthin to the complexes results in rapid quenching of fluorescence (Fig. 1A). For LHC IIb, this is only about 15–20% F_{max}, but for CP29 a quenching of over 50% was observed. A similarly strong response was found with CP26. Monomers of LHC IIb showed a response similar to CP29 and CP26, showing that the trimeric state of LHC IIb stabilises the unquenched state. The kinetics of quenching in every case fitted a second order rate equation (Fig. 1B), as observed previously for LHC II in low detergent conditions, and for NPQ in chloroplasts and leaves [18].

The extent of quenching was dependent upon the concentration of zeaxanthin, but showed saturation at approx. 50 μM with half maximal quenching at approx. 18 μM. The effect of zeaxanthin was dissimilar from the quenching caused by the addition of a non-specific homogeneous quencher such as 2,5-dibromo-3-methyl-6-isopropyl-ρ-benzoquinone (DBMIB),

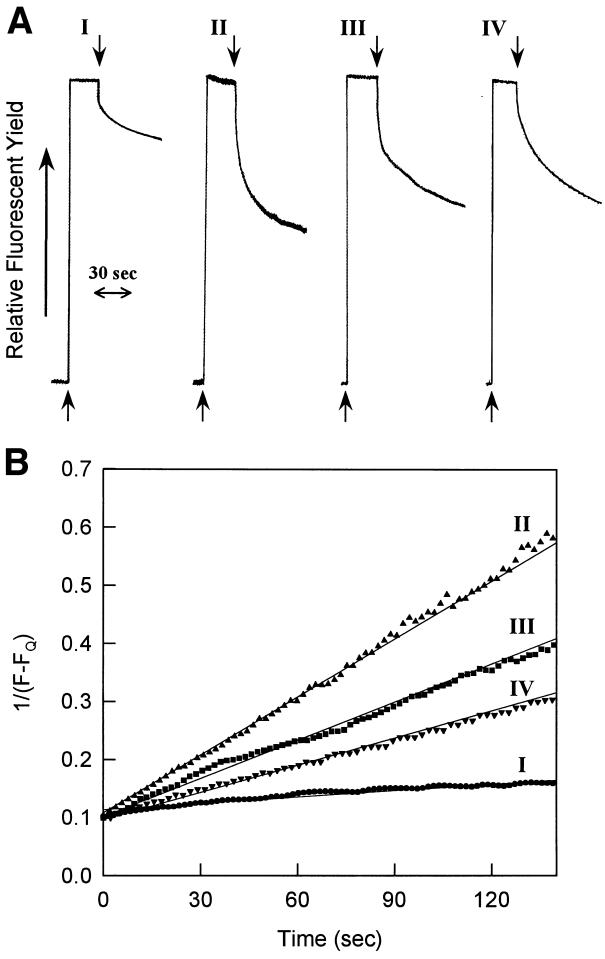

Fig. 1. Zeaxanthin-dependent chlorophyll fluorescence quenching in isolated PS II antenna complexes at 20°C. A: Fluorescence yield vs. time for LHC IIIb trimers (I), CP29 (II), CP26 (III) and LHC IIb monomers (IV). ↑, Measuring beam on; ↓, addition of 20 μM zeaxanthin. B: Reciprocal plot of fluorescence to show fitting to second order kinetics – data points obtained from digitisation of the curves in A. *F* is fluorescence at any time, and *F*u is the final level of unquenched fluorescence. r^2 Values are 0.938, 0.995, 0.995 and 0.995 for I (LHC IIb), II (CP29), III (CP26) and LHC IIb monomers respectively.

Table 1
The effects of different carotenoids on Chl fluorescence quenching in isolated light harvesting complexes

Complex	Carotenoid	Quenching $(F_{max}-F_t)/F_t$
CP26	None	0.016 ± 0.004
	Zeaxanthin	0.712 ± 0.014
	Violaxanthin	0.020 ± 0.008
	Antheraxanthin	0.083 ± 0.009
	Lutein	0.054 ± 0.005
	Auroxanthin[a]	1.673 ± 0.035
LHC IIb	None	0.012 ± 0.004
	Zeaxanthin	0.240 ± 0.019
	Violaxanthin	0.012 ± 0.007
	Antheraxanthin	0.070 ± 0.006
	Lutein	0.033 ± 0.008
	Auroxanthin[a]	0.570 ± 0.028

F_t was recorded 2 min after addition of the carotenoid (see Fig. 1). Data are the means ± S.E.M. for at least three separate experiments. The control (none) was the addition of ethanol. Carotenoid concentrations were 20 μM.
[a]Auroxanthin was used at a concentration of 10 μM.

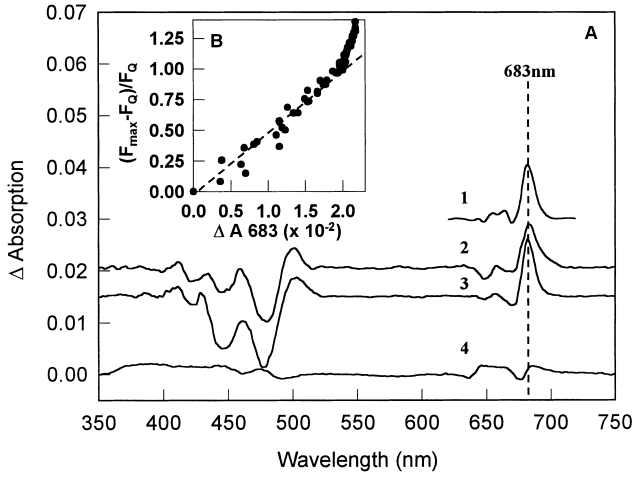

Fig. 2. Absorption difference spectra (quenched minus unquenched); (1) zeaxanthin dependent quenching for CP26 as in Fig. 1; (2) quenching induced by dilution of the complex into zero DM as in [17]; (3) quenching induced by decrease in pH as in [20]; (4) quenching under the conditions of Fig. 1 but induced by addition of 40 μM DBMIB to give the same quench as for (1). Insert: relationship between the amplitude of the absorption band at 683 nm (ΔA_{683}) and the extent fluorescence quenching recorded at different times after zeaxanthin addition.

which showed a continuous increase in quenching with increase in concentration.

The specificity of the effect of zeaxanthin was explored (Table 1). Addition of violaxanthin had no effect, whereas the mono-epoxy carotenoid antheraxanthin exerted less than 20% of the response found for zeaxanthin. Antheraxanthin is an intermediate in the conversion of violaxanthin to zeaxanthin – it has been proposed that it exerts an equal effect on *q*E as zeaxanthin in vivo [21], but these data make this proposition less likely. It has also been suggested that in the absence of zeaxanthin, lutein could be a non-photochemical quencher [22]. Addition of lutein to CP26 had no effect on fluorescence yield, again making this suggestion less likely to be correct. However, it can not be excluded that for lutein to act as a quencher it must be in the central axial position in the complex [23].

Auroxanthin is a C5,8 epoxy carotenoid that has previously been shown to enhance aggregation-associated quenching in isolated LHC IIb [24]. Table 1 shows the potency of this carotenoid in the new assay conditions for CP26. Auroxanthin was even able to induce a strong quenching in LHC IIb. The effectiveness of auroxanthin suggests that the quenching induced by zeaxanthin addition is not related to S1 energy level – this is estimated for auroxanthin to be higher than violaxanthin (H. Frank, personal communication). Instead, the effect is explained by its structure – it is predicted that the end groups of auroxanthin lie in the plane of the conjugated carbon double bond chain, as in zeaxanthin, whereas they are expected to be out-of-plane in violaxanthin. In fact, the C5,8 epoxide would hold the end group rigidly in-plane, explaining why it is even more effective than zeaxanthin [15,24].

In previous work using isolated LHC II it has been shown that quenching is associated with an absorption change in the Chl red band [15,17,20]. Because in those conditions quenching was associated with protein aggregation it could not be ascertained whether the absorption change was directly linked to quenching or only to aggregate formation. In the conditions used in Fig. 1 it is unlikely that aggregates would be formed. At this concentration of DM, even pre-formed aggregates are dispersed (Wentworth, M, unpublished data). Fig. 2 shows a difference absorption spectrum (plus zeaxanthin minus control), which has a positive band at 682–683 nm, a negative band at 670 nm from Chl *a* and a broader band in the Chl *b* region – positive at 657 nm and negative around 648 nm. Also shown in Fig. 2 are the difference spectra associated with quenching in LHC II induced under aggregation conditions as in [17,20]. A spectrum for spontaneous quenching induced by dilution of detergent well below the CMC and a spectrum for the low pH stimulation of that process show the same features as for zeaxanthin-dependent quenching. In contrast, the quenching spectrum for DBMIB induced quenching is relatively featureless.

Thus quenching is associated in all cases with the appearance of the 683 nm band. In fact these two features are also kinetically correlated. The absorption change and the extent of quenching are linearly correlated for the major part of the quenching following zeaxanthin addition (inset, Fig. 2). Thus unlike the relationship between [zeaxanthin] and quenching, the ΔA at 683 nm behaves as if it emanates from a species that is either closely related to the quencher, or is the quencher itself. A red shifted Chl absorption may arise from alterations in pigment–pigment and/or pigment–protein interactions in the complex; for example, it is consistent with formation of a Chl dimer of the kind that has been shown in model systems to be a quencher [15].

LHC II aggregation has been associated with enhancement of strongly red-shifted emission bands at low temperature [17,25,26]. Fluorescence spectra for samples before and after zeaxanthin treatment are different, the carotenoid addition inducing a red shift in the peak maximum and a broadening of the spectrum (Fig. 3). However, this change is different from that associated with LHC II aggregation, when a pronounced band at 700–710 is observed. Quenching of fluorescence by DBMIB does not give rise to any alteration in the 77 K emission spectrum (not shown).

In an attempt to demonstrate directly whether zeaxanthin was causing association between LHC II monomers, CP29 samples pre-incubated with zeaxanthin were analysed by su-

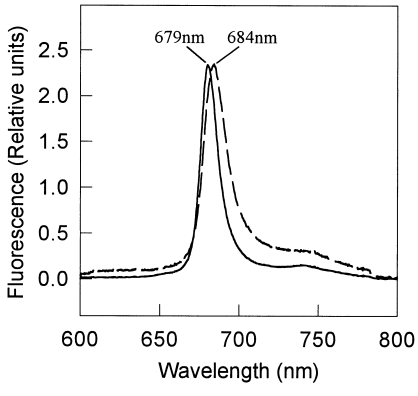

Fig. 3. 77 K Fluorescence emission spectra of CP26 before (solid) and after addition (dashed) of zeaxanthin as in Fig. 1. Samples were incubated at 20°C for 2 min with and without zeaxanthin and then frozen in liquid nitrogen before measurement of the emission spectra.

crose gradient centrifugation. There was no evidence of oligomer formation in the samples incubated with zeaxanthin, all of the CP29 being found as a single monomeric band. The sample showed a nearly 20% reduction in fluorescence yield compared to the sample prepared in the absence of zeaxanthin, indicating retention of a part of the zeaxanthin-dependent quenching. During the sucrose gradient centrifugation excess zeaxanthin was removed, and thus it is to be expected that a saturated level of quenching would not be found. HPLC analysis of the CP29 samples showed that the zeaxanthin content increased from zero to 49.0 ± 0.89% of the bound carotenoid, with the total carotenoid bound increasing from 0.22 ± 0.01 car/Chl to 0.37 ± 0.02 car/Chl. We estimate from these data that each control CP29 molecule binds 1 lutein, 0.54 neoxanthin, and 1.3 violaxanthin (in agreement with the data in [10]), changing to 1 lutein, 0.45 neoxanthin 0.96 violaxanthin and 2.5 zeaxanthin in the zeaxanthin-treated complex. The data suggest that a part of the violaxanthin is replaced by zeaxanthin, and that additional zeaxanthin can be bound to the complex, above that normally shown to be associated with the complexes in vivo.

In previous work, we showed that when complexes were enriched in zeaxanthin, they showed an increased tendency for quenching in low detergent [16–18]. It was found that CP29 removed from the sucrose gradient showed an increased rate of quenching in the zeaxanthin-treated samples compared to the control. Half-lives of 1.01 ± 0.06 and 0.43 ± 0.02 s were determined from triplicate assays of the control and zeaxanthin-treated sample respectively. The increased tendency for quenching was also revealed by an alkaline shift of approx. 0.5 pH units (from approx. 5.0 to 5.5) in the apparent p*K* for quenching, determined as in [18].

The present data represent the first demonstration of quenching of Chl fluorescence induced only by addition of zeaxanthin to isolated PS II antenna complexes. This quenching is saturatable, results in binding to the complex, and shows specificity for zeaxanthin compared to other chloroplast xanthophylls. The quenching interaction results in an alteration in absorption and fluorescence spectra of Chl bound to the complex that occurs within the Lhcb protein monomer, and does not depend upon oligomer formation. The quenched complex also shows an increased tendency for further quenching when put under conditions of low pH or low detergent induced quenching when oligomerisation is promoted.

This behaviour seems to be shared by all of the Lhcb proteins; the relative insensitivity of LHC IIb is explained by its trimeric state, which stabilises the unquenched state since monomers behave similarly to CP29 and CP26. The similarity of this behaviour across all Lhcb pigment–protein complexes raises the question of whether a single protein is the site of action of *q*E in vivo. Previous work has suggested that CP29 and CP26 are the key sites [6,27–29], whereas an alternative view is that quenching is distributed throughout the PS II antenna [18]. Since in vivo NPQ is heterogeneous [30,31], it may be associated with particular complexes under different quenching conditions. Most recently, it has been found that an *Arabidopsis* mutant deficient in the Lhc-related protein PsbS does not exhibit the rapidly relaxing *q*E-type of NPQ [32]. One explanation of this observation is that PsbS is the unique or primary site of *q*E, and in this case the data described here suggest that this protein would also show zeaxanthin-dependent quenching. Alternatively, PsbS may act as an organiser in the antenna, promoting and controlling *q*E in neighbouring Lhc proteins [32].

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