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The relation between the minor chlorophyll spectral forms and fluorescence quenching in aggregated light harvesting chlorophyll *a* / *b* complex II

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Abstract

The hypothesis that fluorescence quenching in aggregated light harvesting chlorophyll *a* / *b* protein complex II is associated with the formation of minor spectral forms absorbing near 655 nm and between 680 nm–690 nm is examined. Using an homogeneous LHCII preparation, steady-state absorption changes measured at room temperature are quantitatively compared with the associated steady state fluorescence changes by means of the Stepanov relation. It is demonstrated that upon LHCII aggregation, the relative fluorescence yield is constant for chlorophyll forms absorbing between 650 nm and 690 nm. This indicates that the minor chlorophyll forms formed upon LHCII aggregation are not quenching species.

Key words: Absorption spectrum; Fluorescence emission spectrum; Fluorescence quenching; Fluorescence quantum yield; LHCII aggregation; Chlorophyll *a*/*b* complex II

1. Introduction

The principal light harvesting antenna complex of higher plants is the chlorophyll *a* / *b* protein LHCII which binds about 50% of all chlorophyll molecules and is mainly associated with Photosystem II (PS II). The complex is known to be both structurally and functionally heterogeneous [1–3]. A detailed three-dimensional crystallographic structure with 6 Å resolution of two-dimensional crystals has been presented which suggests that the basic unit is trimeric and that nearest neighbour chlorophyll distances are of the order of 9–14 Å [4]. LHCII is known to be spectroscopically complex, containing a number of Chl *a* spectral forms with Q_y absorption maxima between 660–684 nm at room temperature [5,6] and between 660 nm–678 nm at low temperatures [5,7], as well as a rather broad Chl *b* Q_y band near 650 nm. Changes in optical properties (fluorescence and circular dichroism signals), some of which are reversible, are known to be induced by light treatment in vitro [8–10].

An interesting property of isolated LHCII is its extreme sensitivity to detergent concentration, particularly in the range near the critical micellar concentration. Thus at low detergent concentrations the aggregation state increases [11,12] due to hydrophobic interactions between complexes. Pronounced quenching of Chl fluorescence also occurs together with an absorption increase near 655 nm and red shifts in the Chl *a* Q_y absorption band [11–14]. Marked increases in circular dichroism signals also occur [12–14] which may be related to the size of LHCII aggregates (Garab, G., personal communication). Ide et al. [13] have suggested that the fluorescence quenching mechanism might be associated with a strong Chl *b*-Chl *b* electronic coupling which is formed upon complex aggregation when the detergent concentration is lowered. More recently the suggestion has been made that fluorescence

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Abbreviations: Chl, chlorophyll; IEF, isoelectric focusing; LHCII, light harvesting chlorophyll *a* / *b* protein-complex II; O.G., *n*-octyl β-D-glucopyranoside; PS II, Photosystem II.

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quenching upon complex aggregation is due to the formation of small amounts of Chl species absorbing in the 680 nm–690 nm wavelength range [15]. Such a process could be associated with increased electronic coupling between Chl *a* molecules. From in vitro studies on Chl *a* this is known to bring about the formation of red shifted spectral forms with low fluorescence yields [16–18]. It has furthermore been proposed that this phenomenon, induced in vitro by modifying the detergent concentration, may constitute the mechanistic basis of the well known high energy quenching (qE) in PS II of green plants [14]. Recent picosecond time-resolved fluorescence studies show that LHCII aggregation is accompanied by decreasing lifetimes of long wavelength components at low temperature (80 K) [19]. These changes, however, are thought to be too small to explain the overall fluorescence quenching in terms of the long wavelength forms [19]. As the increase in amount of long-wavelength-absorbing Chl species is rather small, the long wavelength quenching hypothesis [14] implies that these Chl species should have a low fluorescence yield. The same comment holds true for the absorption changes near 655 nm if they are involved in fluorescence quenching. One way to examine this is to quantitatively compare the aggregation-induced changes in steady-state absorption with the fluorescence emission spectrum. In the case of isolated LHCII it is known that fluorescence is emitted from a thermally equilibrated state [20,21]. Thus one may study absorption and fluorescence changes in terms of the Stepanov relation [22]. In the present paper we present such an analysis at room temperature with an homogeneous LHCII preparation and conclude that the short and long wavelength Chl species formed upon LHCII aggregation do not have a low fluorescence yield.

2. Materials and methods

2.1. Membrane preparation

Thylakoids were prepared from maize mesophyll chloroplasts as previously described [23] and then PS II membranes were obtained according to the method of Berthold et al. [24] using the modifications described by Dunahay et al. [25].

2.2. LHCII preparation

PS II membranes (1 mg Chl ml^{-1}) were solubilised with 1% O.G. and fractionated into a 0.1 to 1 M sucrose gradient, including 1% O.G. and 5 mM Tricine (pH 8.0), which was spun at 20000 rpm in a Kontron tst 28.38 rotor for 30 h at 4°C. This yielded six green bands, the third from the top containing LHCII. As previously shown, LHCII is heterogeneous and is composed of several sub-populations which can be separated by IEF [26,27]. With the aim of using an homogeneous sample for further determinations, the LHCII preparation from sucrose gradient was further fractionated by flat bed IEF into seven bands having a p*I* ranging between 3.9 to 4.5. After a preliminary screening the fourth and the fifth IEF bands (p*I* 4.10 and 4.16), which did not form large aggregates at low detergent concentrations, were used in further work. The purity of the fractions with respect to contamination by other thylakoid components was checked by electrophoresis and Western blot.

2.3. SDS-PAGE and immunoblotting

Analytical SDS-PAGE was performed with gradient gels (12–18% acrylamide, $350 \times 350 \times 1$ mm) containing 6 M urea and run at 10 mA for 3 days using the Tris-sulphate buffer system as previously described [28]. Alternatively, a high Tris buffer system with urea (12–18% acrylamide gradient) was used [29]. For immunoblot, assay samples were separated by one of the gel systems described above and transferred to a nitrocellulose filter (Millipore, Bedford). The filters were then assayed with antibodies and antibody binding was detected by using alkaline phosphatase coupled to anti-rabbit IgG (Sigma Chemical Co.). Antibodies were raised in rabbits and characterised as previously described [30].

2.4. Spectroscopic analysis

Absorption and emission spectra were both measured using an EG & G OMAIII with an intensified diode array (model 1460) mounted on a spectrograph (Jobin-Yvon HR320) with 150 groove mm^{-1} grating. The wavelength scale was calibrated using a neon spectral calibration source (Cathodeon). The wavelength spacing between pixels is about 0.5 nm. An OG 530 filter (Schott) was placed before the collection optics to diminish stray light. Both absorption and emission spectra were made using a 3 mm pathlength cuvette, with fluorescence being collected at 90° with respect to excitation. Fluorescence was excited at 440 nm. The chlorophyll concentration was about 10 μg ml^{-1}. Under these conditions no detectable reabsorption of fluorescence occurred. When necessary the emission spectra were corrected for instrumental distortion using an intensity calibrated source (ISCO Spectroradiometer Calibration). Absorption spectra were not corrected for light scattering as this was judged not to be significant as spectra go to zero outside the absorption band. Checks were also made using the fluorescence quencher dibromothymoquinone that sample fluorescence was not detected in the absorption configuration.

3. Results

The room temperature absorption spectra of LHCII, purified by isoelectric focusing, and resuspended in *n*-octyl β-D-glucopyranoside 1% or 0.5% are presented in Fig. 1. These concentrations are respectively slightly above and slightly below the critical micellar concentration. As previously reported by others [11,12,14], lowering the detergent concentration brings about a slight red shift of the absorption spectrum. The difference spectrum has been calculated after normalising the absorption curves to their areas (Fig. 2, curve A). This shows a large positive peak near 683 nm and a smaller peak near 655 nm, indicating increased absorption near these wavelengths upon complex aggregation, as previously reported [14]. It should be mentioned that the negative values in the difference spectrum between 620–660 nm are due to the area-normalisation procedure we have used and are not thought to be associated with absorption changes. In Fig. 2, curve B the ratio of the two absorption spectra is also presented. A broad maximum is seen in the 685 nm–690 nm range, thus indicating that the difference spectrum in Fig. 2 can not be unequivocably interpreted as indicating that a specific 684 nm Chl form is produced upon aggregation. In this paper we will refer to the 'aggregation-induced increases in long wavelength forms'.

The room temperature fluorescence emission spectra of LHCII in 1% and 0.5% *n*-octyl β-D-glucopyranoside are presented in Fig. 3. As reported previously by others [11,13,14] lowering the detergent concentration leads to a marked fluorescence quenching. Further lowering of the detergent concentration caused greatly increased quenching with respect to that shown in Fig. 3. However this was accompanied by quite large decreases in absorption which were interpreted as being due to an aggregation-induced sieve effect [31]. As these absorption changes would have complicated subsequent analysis of the data, the lowest detergent concentration used in this study was 0.5%. At this concentration the emission spectrum is slightly red-shifted with respect to 1% *n*-octyl β-D-glucopyranoside. This effect is best seen in the area-normalised difference spectrum (Fig. 4, curve A) and ratio spectrum (Fig. 4, curve B), where it is evident that lowering the detergent concentration causes a relative increase in long wavelength fluorescence.

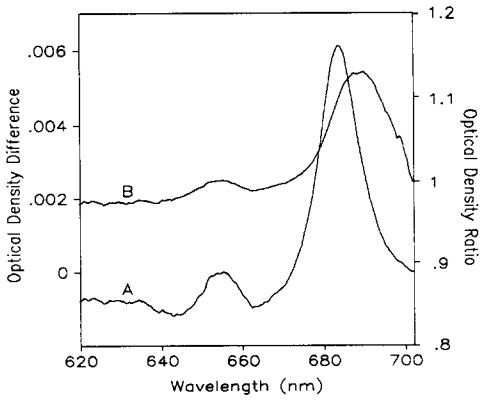

Fig. 2. Effect of *n*-octyl β-D-glucopyranoside concentration on the absorption spectrum of LHCII. Curve A is the difference spectrum (O.G. 0.5% minus OG 1%), curve B is the ratio spectrum (O.G. 0.5% divided by O.G. 1%). Absorption spectra were area-normalised prior to calculation of A and B.

If it is assumed that the excited state is thermally relaxed prior to fluorescence emission it is possible to connect the absorption and fluorescence spectra by the well known Stepanov equation [22]:

$$\frac{F(\nu)}{A(\nu)} = C(T)\nu^3 \exp\left(-\frac{h\nu}{kT}\right) \quad (1)$$

where $A(\nu)$ is the absorption spectrum, $F(\nu)$ is the emission spectrum, ν is the frequency, C is independent of frequency, k is the Boltzmann constant, T is the

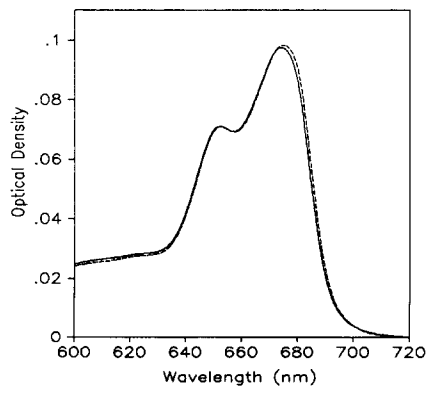

Fig. 1. Absorption spectra of LHCII suspended in *n*-octyl β-D-glucopyranoside 1% (———) or 0.5% (- - - - -). Spectra were normalised to the same area. For further details see Section 2.

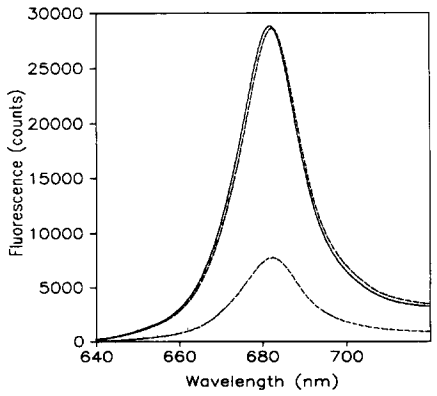

Fig. 3. Fluorescence emission spectra of LHCII suspended in *n*-octyl β-D-glucopyranoside 1% (———) or 0.5% (- - - - -; lower curve shows the original spectrum, upper curve shows the spectrum normalised to the area of 1% O.G).

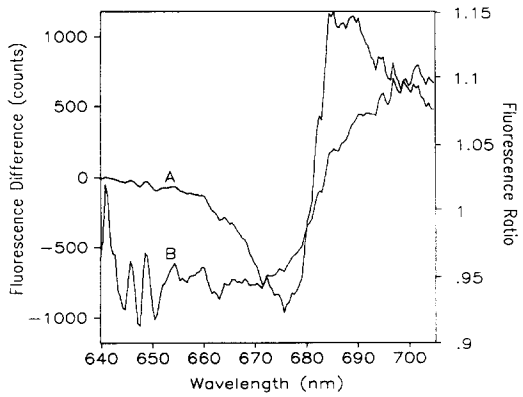

Fig. 4. Effect of *n*-octyl β-D-glucopyranoside concentration on the Fluorescence emission spectrum of LHCII. Curve A is the difference spectrum (OG 0.5% minus OG 1%), curve B is the ratio spectrum (O.G. 0.5% divided by O.G. 1%). Emission spectra were area-normalised prior to calculation of A and B.

absolute temperature, h is the Planck constant. It has been demonstrated that this expression may be reasonably applied to the absorption and emission bands of isolated LHCII [20,21]. One assumption in this expression is that the fluorescence yield of all absorbing molecules is constant. When this is not the case the following modified version of the Stepanov expression has been suggested [32]:

$$\frac{F(\nu)}{A(\nu)} = C(T)\nu^3\Phi(\nu)n^2(\nu)\exp\left(-\frac{h\nu}{kT}\right) \qquad (2)$$

where $\Phi(\nu)$ is the wavelength dependent fluorescence yield and n is the refractive index. It is therefore possible to investigate whether the Chl forms, produced upon LHCII aggregation at low detergent concentrations, have a low fluorescence yield with respect to the other chlorophylls, as would be expected if they were fluorescence quenchers. This was achieved by determining the wavelength dependent behaviour of the ratio of the F/A parameter at low with respect to high detergent concentrations. This ratio, derived from Eq. 2, is:

$$\left|\frac{F(\nu)}{A(\nu)}\right|_{0.5\%} \cdot \left|\frac{A(\nu)}{F(\nu)}\right|_{1\%} = \frac{|\Phi(\nu)n^2(\nu)|_{0.5\%}}{|\Phi(\nu)n^2(\nu)|_{1\%}} \qquad (3)$$

The relevant data are presented in Fig. 5, where it can be seen that the value of this parameter remains approximately constant between 650–690 nm. Above 690 nm it increases markedly.

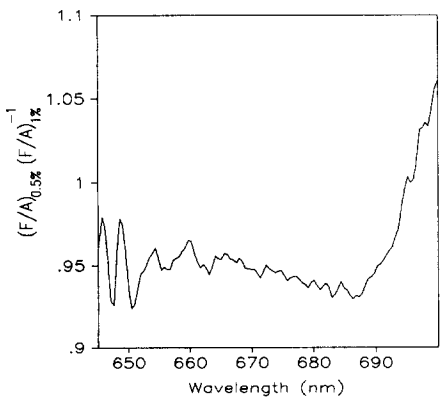

Fig. 5. The ratio of the $F(\nu)/A(\nu)$ parameter measured at 0.5% O.G. with respect to that at 1% O.G. for LHCII. This ratio is described in Eq. 3.

4. Discussion

In the present paper we have addressed the question of whether the chlorophyll absorption bands formed in isolated LHCII when the concentration of the solubilising detergent is lowered below the critical micellar concentration are responsibile for the marked fluorescence quenching which occurs. As previously reported [14], this treatment leads to a small increase in absorption near 655 nm and a larger increase at wavelengths above 680 nm. These absorption effects are considered to be associated with LHCII aggregation, possibly by the establishment of strong excitonic coupling between chlorophylls [13]. Red absorption shifts associated with Chl-Chl interactions in vitro are well known [17] and in at least one case have been demonstrated to be due to excitonic spectral splitting [18]. It is not known whether these changes might occur between Chl molecules of different LHCII complexes within the aggregated states or by modification of the mutual pigment distances and/or orientations within complexes. On the basis of the area-normalised difference absorption spectrum (Fig. 2) it is estimated that the long wavelength increase in absorption in these experiments is equivalent to about 5% of the total Q_y extinction. The changes near 655 nm are clearly a great deal less. As fluorescence is emitted from a substantially thermally equilibrated state [20,21], it is reasonable to think that if the newly formed chlorophyll forms are the fluorescence quenchers they should have a very low fluorescence yield. To investigate this we have determined the ratio of the F/A parameter for LHCII at an *n*-octyl β-D-glucopyranoside concentration of 0.5% with respect to 1.0% (Eq. 3). This ratio is equivalent to the ratio between the quantum yields for the two detergent concentrations. To a first approximation one may ignore the refractive index term as any detergent-induced changes in this parameter will be numerically very small with respect to the fluorescence yield changes necessary to explain the large fluorescence quenching. The data presented in Fig. 5 show that the F/A parameter at the two detergent concentrations used here has an approximately constant ratio over most of the Q_y absorption

band, changing slightly above 690 nm. This indicates that the minor chlorophyll species formed upon LHCII aggregation at low detergent concentrations do not have a detectably lower fluorescence yield than the other chlorophyll forms present in this complex. Thus it is concluded, contrary to previous suggestions [14,33], that these minor chlorophylls are not the fluorescence quenchers.

The relative increase in the F/A ratio above 688 nm at low detergent concentrations is in agreement with findings of Mullineaux et al. [19]. These authors, measuring fluorescence lifetimes at 77 K, demonstrated the formation of some long wavelength components with relatively long lifetime values at low detergent concentrations in LHCII.

On the basis of the present study it is not possible to identify the fluorescence quencher. While our data argue against the possibility that the long wavelength and the 655 nm absorbing forms produced upon LHCII aggregation are the quenching species, the formation of a quenching chlorophyll at very low concentrations can not be completely excluded. The approach used here would not detect the production of a very low absorbing band associated with a low fluorescence yield. These data therefore do not exclude the suggestion of Ide et al. [13] that the quencher may be a chlorophyll *b*-chlorophyll *b* exciton produced upon complex aggregation, if this is present at very low levels, not detectable in the absorption spectrum. We have, however, observed (unpublished observation) that very marked fluorescence quenching occurs upon lowering the detergent concentration in the chlorophyll–protein complexes CP47, CP43 as well as the D1/D2/cytochrome b_{559} reaction centre particle. These complexes do not contain chlorophyll *b*. Thus, if the fluorescence quenching in these complexes is mechanistically similar to that of LHCII, the quenching species cannot be a chlorophyll *b* exciton. Alternatively the possible involvement of carotenoid quenchers might be suggested. Detergent-induced circular dichroism changes in the wavelength interval of S_2 carotenoid absorption have been detected in LHCII [12,13]. Efficient energy transfer to carotenoids with 11 conjugated carbon-carbon double bonds via exchange coupling [34] is possible, as good spectral overlap between Chl emission and S_1 carotenoid absorption is expected [35,36]. This possibility is presently under investigation.

5. References

[1] Kyle, D.J., Kuang, T.-Y., Watson, J.L. and Arntzen, C.J. (1984) Biochim. Biophys. Acta 765, 89–96.

[2] Jennings, R.C., Islam, K. and Zucchelli, G. (1986) Biochim. Biophys. Acta 850, 483–489.

[3] Spangfort, M. and Andersson, B. (1989) Biochim. Biophys. Acta 977, 163–170.

[4] Kühlbrandt, W. and Wang, D.N. (1991) Nature 350, 130–134.

[5] Zucchelli, G., Jennings, R.C. and Garlaschi, F.M. (1990) J. Photochem. Photobiol. B: Biol. 6, 381–394.

[6] Jennings, R.C., Bassi, R., Garlaschi, F.M., Dainese, P. and Zucchelli, G. (1993) Biochemistry 32, 3203–3210.

[7] Hemelrijk, P.W., Kwa, S.L.S., Van Grondelle, R. and Dekker, J.P. (1992) Biochim. Biophys. Acta 1098, 159–166.

[8] Jennings, R.C., Garlaschi, F.M. and Zucchelli, G. (1991) Photosynth. Res. 27, 57–64.

[9] Garab, G. (1992) in Research in Photosynthesis, Vol. 1 (Murata, N., ed.), pp. 171–178, Kluwer, Dordrecht.

[10] Li, Chong-C. and Zhang, Zhi-Y. (1992) in Research in Photosynthesis, Vol. 2 (Murata, N., ed.), pp. 615–618, Kluwer, Dordrecht.

[11] Mullet, J.E. and Arntzen, C.J. (1980) Biochim. Biophys. Acta 589, 100–117.

[12] Bassi, R., Silvestri, M., Dainese, P., Moya, I. and Giacometti, G.M. (1991) J. Photochem. Photobiol. B: Biol. 9, 335–354.

[13] Ide, J.P., Klug, D.R., Kühlbrandt, W., Giorgi, L.B. and Porter, G. (1987) Biochim. Biophys. Acta 893, 349–364.

[14] Ruban, A.V. and Horton, P. (1992) Biochim. Biophys. Acta 1102, 30–38.

[15] Ruban, A.V., Rees, D., Noctor, G.D., Young, A. and Horton, P. (1991) Biochim. Biophys. Acta 1059, 355–360.

[16] Beddard, G.S., Carlin, S.E. and Porter, G. (1976) Chem. Phys. Lett. 43, 27–32.

[17] Butler, W.L. (1966) in The chlorophylls (Vernon, L.P. and Seely, G.R., eds.), pp. 343–379, Academic Press, New York.

[18] Shipman, L.L., Cotton, T.M., Norris, J.R. and Katz, J.J. (1976) J. Am. Chem. Soc. 98, 8222–8230.

[19] Mullineaux, C.W., Pascal, A.A., Horton, P. and Holzwarth, A.R. (1993) Biochim. Biophys. Acta 1141, 23–28.

[20] Knox, R.S. and Van Metter, R.L. (1979) in Chlorophyll Organisation and Energy Transfer in Photosynthesis, pp. 177–186, Excerpta Medica, Amsterdam.

[21] Zucchelli, G., Jennings, R.C. and Garlaschi, F.M. (1992) Biochim. Biophys. Acta 1099, 163–169.

[22] Stepanov, B.I. (1957) Sov. Phys. Dokl 2, 81–84.

[23] Bassi, R. and Simpson, D.J. (1986) Carlsberg Res. Commun. 51, 363–370.

[24] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.

[25] Dunahay, T.G., Staehelin, L.A., Seibert, M., Ogilvie, P.D. and Berg, S.P. (1984) Biochim. Biophys. Acta 764, 179–193.

[26] Bassi, R., Rigoni, F., Barbato, R. and Giacometti, G.M. (1988) Biochim. Biophys. Acta 936, 29–38.

[27] Dainese, P., Hoyer-Hansen, G. and Bassi, R. (1990) Photochem. Photobiol. 51, 693–703.

[28] Bassi, R., Hoyer-Hansen, G., Barbato, R., Giacometti, G.M. and Simpson, D.J. (1987) J. Biol. Chem. 262, 13333–13341.

[29] Fling, S.P. and Gregerson, D.S. (1986) Anal. Biochem. 155, 83–88.

[30] Di Paolo, M.L., Dal Belin Peruffo, A. and Bassi, R. (1990) Planta 181, 275–286.

[31] Duysens, L.N.M. (1956) Biochim. Biophys. Acta 19, 1–10.

[32] Ketskeméty, I., Dombi, J. and Horvai, R. (1961) Ann.Phys. 8, 342–352.

[33] Horton, P., Ruban, A.V., Rees, D., Pascal, A.A., Noctor, G.D. and Young, A.J. (1991) FEBS Lett. 292, 1–4.

[34] Dexter, D.L. (1953) J. Chem. Phys. 21, 836–850.

[35] Cosgrove, S.A., Guite, M.A., Burnell, T.B. and Christensen, R.L. (1990) J. Phys. Chem. 94, 8118–8124.

[36] Owens, T.G., Shreve, A.P. and Albrecht, A.C. (1992) in Research in Photosynthesis, Vol. 1 (Murata, N., ed.), pp. 179–186, Kluwer, Dordrecht.