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Absolute absorption and relative fluorescence excitation spectra of the five major chlorophyll-protein complexes from spinach thylakoid membranes

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The absolute absorption and relative fluorescence excitation spectra have been obtained for the five major chlorophyll-protein complexes from spinach chloroplasts, resolved by mild SDS-polyacrylamide gel electrophoresis. Electro-elution of the chlorophyll-protein complexes from the gels enabled their absolute absorption spectra to be determined. The mean molar extinction coefficient from 400 to 720 nm was independent of the type of protein complex and the chlorophyll *a*/*b* ratio, averaging 2230 ± 40 m^2 per mol chlorophyll. The mean molar extinction coefficient was used to normalize the absorption spectra of the protein complexes while in the gel slices. The distribution of chlorophyll between the pigment complexes can be determined at 662 nm rather than from the mean of two scans at 650 nm and 675 nm. Recombining the absorption spectra from the pigment-protein complexes gave reasonable absolute agreement with the spectrum of spinach thylakoids. However, the solubilization led to (i) a shift in the red peak to shorter wavelengths by 4.5 nm, and (ii) a loss of carotenoids which also shifted the blue shoulder to shorter wavelengths. The absorption spectra for the two photosystems were predicted with phosphorylated (State 2) and nonphosphorylated (State 1) light-harvesting complexes. The 77 K fluorescence excitation spectra of the chlorophyll-protein complexes were measured in an attempt to assess the excitation spectra of PS I and PS II in terms of their chlorophyll-protein complexes. Due to the relative nature of fluorescence detection, the ratio of fluorescence excitation at 470 relative to that at 400 nm was used to compare the spectra. The discrepancies between the absorption and fluorescence spectra for the Photosystem I complexes meant that fluorescence emission was an insensitive method for assessing the degree of association of the light-harvesting chlorophyll *a*/*b*-complex with each photosystem.

Introduction

The chlorophyll-protein complexes of chloroplast thylakoid membranes can be resolved by SDS-polyacrylamide gel electrophoresis with very little free chlorophyll being liberated [1]. The proportion of chlorophyll associated with each complex can be estimated by scanning the gels. Modulations in the distribution of chlorophyll between the chlorophyll-protein complexes result from altering the irradiance during growth [2,3] and more dramatic differences have been observed for sun and shade plants [4–7]. To understand the consequences of these modulations of chlorophyll-pro-

Abbreviations: LHC II, light-harvesting chlorophyll *a*/*b*-protein; PS II, Photosystem II; PS I, Photosystem I, Chl, chlorophyll.

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tein complexes in terms of light absorption and distribution between the two photosystems, the absolute absorption spectra must be known. An initial attempt was made using published relative absorption spectra assuming that all of the chlorophyll protein complexes had the same mean molar extinction coefficient from 400–720 nm [8]. The assumption was then necessary because the absolute spectra have never been obtained due to the difficulty in extracting the chlorophyll proteins from the gels following electrophoresis. It was concluded that shade light, which is enriched in far-red wavelengths, was not utilised with better efficiency by shade-adapted leaves and that a substantial proportion of light absorbed by the light-harvesting chlorophyll *a/b*-protein complex must be transferred to PS I. This paper confirms the fundamental assumption that the mean molar extinction coefficients from 400–720 nm are the same for the different chlorophyll-protein complexes.

This study has two main aims. The first was to obtain absolute absorption spectra for the major chlorophyll-protein complexes that had been electro-eluted from gel pieces which enabled the chlorophyll content to be determined so that absolute spectra could be obtained. As electro-elution in the continued presence of SDS caused further degradation of the complexes, the results were used to normalize the truer absorption spectra obtained from gel slices. Secondly, to determine the functional association of the chlorophyll-protein complexes in thylakoid membranes, the 77 K fluorescence excitation spectra of the complexes were examined.

Materials and Methods

Spinacia oleracea L. (Henderson's hybrid 102) plants were grown hydroponically in a glasshouse. Thylakoid membranes were isolated as previously described [9]. PS I complex was isolated from a sucrose density gradient following fragmentation of thylakoids with Triton X-100 according to the procedure of Ryrie [10]. Thylakoid membranes (50 μg Chl) were solubilised at 0 °C in 50 mM Tris (pH 8.0) containing 10% glycerol and 0.75% SDS (100 μl) to give an SDS/Chl ratio of 7.5 (w/w) [9]. Samples were immediately applied to SDS-polyacrylamide electrophoresis gels (4% stacking, 8% resolving gels) prepared as in Ref. 9. The gels were run at 3 mA per tube (6 mm diameter) for 15 min at 4 °C prior to loading the sample.

Following electrophoresis at 3 mA per gel for 45 min at 4 °C [9], the bands were cut from the gels and absorption spectra of the gel slices were recorded at room temperature with a double-beam spectrophotometer (Perkin Elmer 557) attached to a computer. To correct for light scattering, the baseline for each spectrum was taken as the average of the absorbances between 740 and 750 nm. The bands from other gels were finely diced and the chlorophyll-protein complexes were eluted electrophoretically in 0.43 M Tris (pH 9.35) with 0.05% SDS at 4 °C. After recording the absorption spectra of the electro-eluted complexes their chlorophyll concentration was determined in 80% acetone by the method of Arnon [11]. The mean molar extinction coefficient for each spectrum was then calculated by the computer from the mean of the absorbances at 1 nm intervals between 400 and 720 nm, divided by the chlorophyll concentration. The distribution of chlorophyll between the bands was determined by scanning the gels immediately following electrophoresis in a Varian 635 spectrophotometer [12] and then measuring the areas under each peak. Scans were made at 650, 662 and 675 nm. The areas were divided by the relevant extinction coefficient and then expressed as a percentage of the total corrected area for each wavelength scan.

The fluorescence excitation spectrum of each chlorophyll-protein complex was recorded at 77 K in a fluorescence spectrophotometer (Perkin Elmer MPF-44B and DCSU-2) with excitation and emission half-bandwidths of 3 and 10 nm, respectively. Gel pieces were immersed in glycerol and immediately frozen, while spinach thylakoids (5 μM Chl) were frozen in 50 mM Tricine (pH 8.0). The spectra were corrected for the spectral sensitivity of the photomultiplier and monochromator as well as for variation in the output of the light source. For each complex, the excitation spectrum was obtained by recording fluorescence emission intensity at the wavelength of maximum emission.

Results and Discussion

The six chlorophyll-protein complexes of spinach thylakoids, resolved by discontinuous SDS-polyacrylamide gel electrophoresis, have been previously characterized [1,9,13]. Two complexes have been ascribed to PS I: CP1, the core reaction centre complex of PS I, and CP1a, which also contains the light-harvesting chlorophyll *a/b*-protein complex associated with PS I. The remaining four complexes include CPa, which is the PS II reaction centre, and three bands LHC_1, LHC_2 and LHC_3, which derive from the associated light-harvesting chlorophyll *a/b*-protein complex (LHC II). The minor band LHC_2, which contains only 14% of the chlorophyll associated with LHC II [3], was not characterized further but is similar to LHC_1 (data not shown).

Mean molar extinction coefficient

The mean molar extinction coefficients (400–720 nm) for the chlorophyll-protein complexes are shown in relation to their chlorophyll *a/b* ratio (Fig. 1). It is clear that the mean molar extinction coefficients are independent of the chlorophyll *a/b* ratios greater than 5 being only approximate. No significant variation exists in the mean molar extinction coefficients of the various chlorophyll-protein complexes and the mean is 2230 ± 40 m^2 per mol Chl (equivalent to 22 300 $l \cdot mol^{-1} \cdot cm^{-1}$). This differs from values of 1907 and 2063 for pure chlorophylls *a* and *b*, respectively, in ether [14]. Anderson [15] suggested that, since the chlorophylls in the various chlorophyll-protein complexes are complexed with different polypeptides, there was no reason why they would be equally effective at light absorption. Previously, in order to quantify the absorption spectra of the two photosystems, Evans assumed that the chlorophyll-protein complexes all had mean molar extinction coefficients (400–720 nm) of 2180 $m^2 \cdot mol^{-1}$ [8] despite having different chlorophyll *a/b* ratios and carotenoid contents [16]. It is clear from Fig. 1, that this fundamental assumption was justified.

Electro-elution of the chlorophyll-protein complexes was accompanied by a shift of the absorbance peak at 678 nm to shorter wavelengths. Consequently, it was thought that absorption spectra obtained with gel slices would be closer to the in vivo spectra. Since the mean molar extinction coefficient was similar for all of the electro-eluted chlorophyll-protein complexes (Fig. 1), it

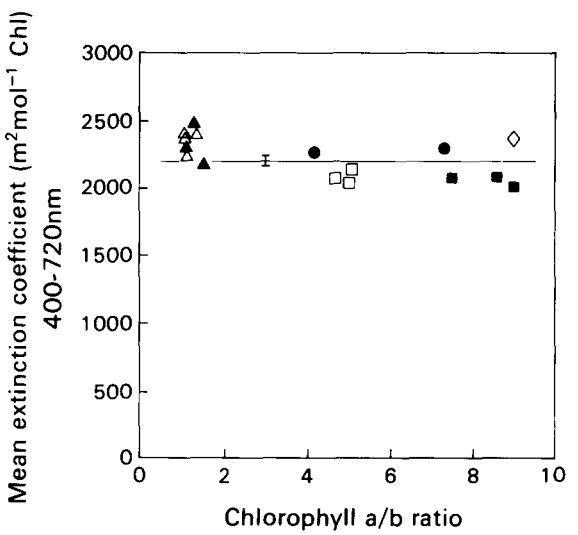

Fig. 1. Mean molar extinction coefficients over the wavelength range 400–720 nm for electro-eluted chlorophyll-protein complexes as a function of their chlorophyll *a/b* ratios. The mean molar extinction coefficient for all the complexes was 2230 ± 40 m^2/mol. LHC_1 (△), LHC_3 (▲), CPa (●), CP1a (□), CP1 (■), PS I (◇).

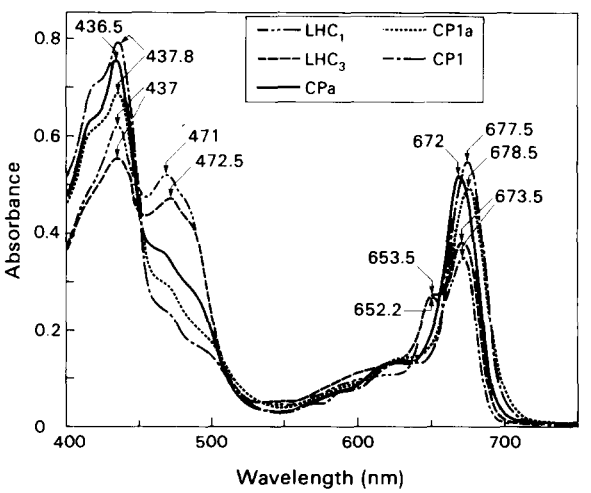

Fig. 2. In situ absorption spectra for the five chlorophyll-protein complexes in gel slices. The spectra are normalized to 10 μM solutions for a 1 cm pathlength.

was possible to normalize the absorption spectra of gel slices. These normalized spectra are shown for the equivalent of 10 μM Chl in a 1 cm pathlength (Fig. 2). The absorbances at 472 and 652 nm reflect the relative amount of Chl *b*.

The extinction coefficients for the chlorophyll-protein complexes at wavelengths useful for quantifying the chlorophyll distribution between the chlorophyll-protein complexes, are shown in Table I. The percentage of chlorophyll in each chlorophyll-protein complex was then calculated from the mean of scans at 650 and 675 nm or the scan at 662 nm (see Materials and Methods). 650 and 675 nm are the wavelengths of peak absorption by Chl *b* and *a*, respectively, in situ. In both cases, allowance needs to be made for the differences in the extinction coefficients between the various chlorophyll-protein complexes. Results obtained by the two methods agree within experimental error; however, use of the scan at 662 nm is more convenient.

Comparison with the original thylakoid absorption spectrum

Some disruption to the chlorophyll-protein complexes during solubilisation of the thylakoids and subsequent SDS-polyacrylamide gel electrophoresis is unavoidable as indicated by the ever-present bands of free chlorophyll and carotenoids at the electrophoretic front. The importance of this can be assessed by comparing the absorption spectrum of spinach thylakoids with that obtained by summing the absorption spectra of the complexes in proportion to their chlorophyll contents from Table I (Fig. 3). Overall, there is good absolute agreement between the actual and the calculated spectra. There are three regions where minor discrepancies occur. The red peak is shifted to shorter wavelengths by 4.5 nm, presumably due to the SDS solubilisation [17]. The shoulder at 500 nm is also shifted to shorter wavelengths, possibly as a result of the partial loss of carotenoids from the complexes which can be seen along with the

TABLE IA

EXTINCTION COEFFICIENTS FOR THE FIVE CHLOROPHYLL-PROTEIN COMPLEXES IN GEL SLICES AT WAVELENGTHS USEFUL FOR GREEN GELS

Wavelength (nm)	Extinction coefficient for Chl-protein complexes: (m^2 per mol Chl)				
	LHC_1	LHC_3	CPIa	CP1	CPa
451	4850 (0.918) [a]	4450	4450	4450	4450
650	2630	2700	1650	1450	1880
662	2530 (1.126) [a]	2850	2850	2850	3350 (0.851) [a]
675	3380	3730	4750	5280	4900

[a] Multiplication factor to give true proportion of Chl in the complex.

TABLE IB

THE CALCULATED DISTRIBUTION OF CHLOROPHYLL BETWEEN THE PROTEIN-COMPLEXES

Wavelength (nm)	Percentage of chlorophyll in chlorophyll-protein complexes:				
	LHC_1	LHC_3	CPIa	CP1	CPa
650 + 675 [b]	41.3	15.1	26.3	6.3	11.0
662	40.8	16.0	26.0	5.8	11.3
(650 + 675)/662	1.01	0.94	1.01	1.09	0.97

[b] Percentage of chlorophyll estimated from the sum of the scan areas at 650 and 675 nm, accounting for the unequal extinction coefficients.

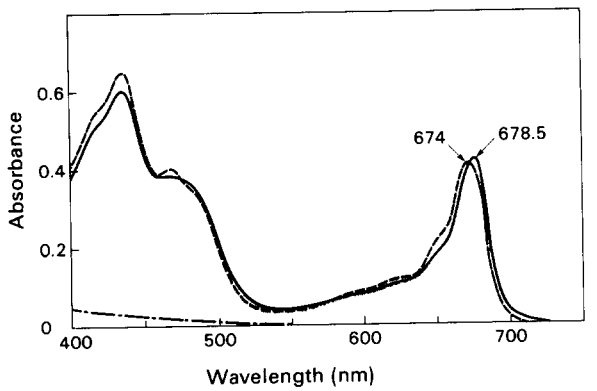

Fig. 3. Absorption spectrum for spinach thylakoids and the spectrum obtained from a combination of the chlorophyll-protein complex spectra in gel slices. The proportion of chlorophyll associated with each complex is shown in Table I. Each spectrum is calculated for a 10 μM solution. Thylakoid preparation (25 mM Tricine (pH 8.0)/50 mM NaCl/5 mM $MgCl_2$) (————), combination (— — —), scattering by gel (— · —).

free chlorophyll at the electrophoretic front. There is also an enhancement of the absorption between 400 and 450 nm which cannot be attributed to light scattering by the gel which increases in this region (Fig. 3), because in this region the spectra of eluted complexes were comparable to those in the gel slices (data not shown). Rather, nonhomogeneous dispersion of the pigments in the thylakoids may give rise to flattening of the absorption spectrum.

Calculated absorption spectra for PS I and PS II

Given the reasonable agreement between the absorption spectrum of the spinach thylakoids and the summation of the chlorophyll-protein complexes in gel slices, the absorption spectra for the two photosystems were calculated. The distribution of chlorophyll between the complexes was based on that of pea plants grown at 840 μmol quanta/m^2 per s [3] because this was the basis for the previous calculations [8]. The free chlorophyll was redistributed to CP1, CP1a and CPa in proportion to their chlorophyll content as these are the most labile complexes and the free chlorophyll is predominantly Chl *a* [1]. Since 15% of the chlorophyll in LHC II is located in the stromal thylakoids [18] it was assumed that this contributed to PS I, as PS I is predominantly located in stroma-exposed thylakoids. Energy transfer from LHC II to PS I has been demonstrated for these complexes reconstituted into proteoliposomes [19]. Some LHC II has also been localized in stromal thylakoids with immunogold labelling [20,21]. With this distribution, the ratio of chlorophyll associated with PS II to that with PS I was 1.26, which means that PS II has a greater absorption than PS I between 400 and 672 nm (Fig. 4A). This distribution of chlorophyll between PS I and PS II may be equivalent to State 1 [22,23]. To simulate the transition to State 2, a further 15% of the LHC II was assumed to transfer energy to PS I, resulting in equal chlorophyll distribution between the two photosystems (Fig. 4B). In State 2, some LHC II becomes phosphorylated and the change in the surface charge density results in the migration of this LHC II away from regions of membrane appression, thereby preventing energy transfer to

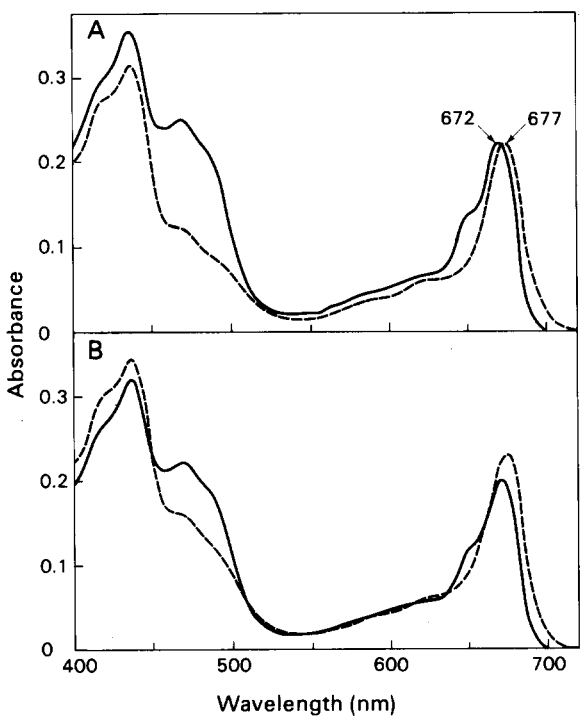

Fig. 4. Calculated absorption spectra for PS I (- - - - -) and PS II (————). (A) Distribution of chlorophyll between the chlorophyll protein complexes: 28.3% LHC_1, 17.3% LHC_3, 22.6% CPIa, 14.8% CP1, 17% CPa, with 15% of $LHC_1 + LHC_3$ assumed to contribute to PS I (State 1). (B) A further 15% of the $LHC_1 + LHC_3$ associated with PS II is 'phosphorylated' and assumed to contribute to PS I (State 2).

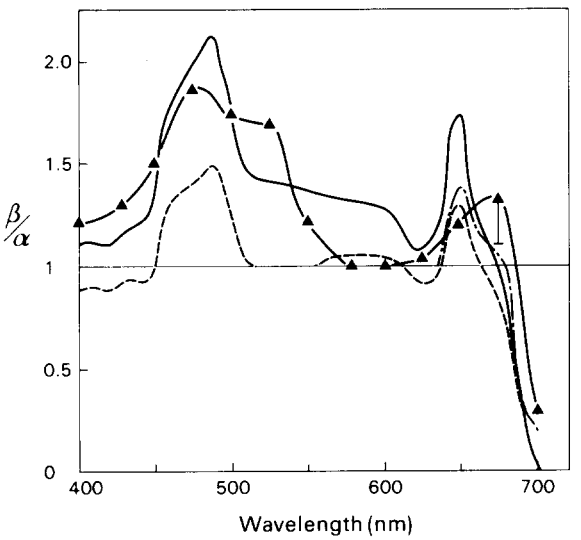

Fig. 5. Ratios of the light absorbed by PS II (β) to PS I (α) vs. wavelength. Two ratios were calculated from the absorption spectra in Fig. 4. State 1 (————); State 2 (— — —). Another ratio (▲———▲) was calculated from quantum yield data for clover (Ref. 24, see also Ref. 25). A further ratio (— · —) was also calculated from the action spectra of PS II and PS I [23] assuming that the two photosystems absorbed equally at 680 nm.

PS II while enabling energy transfer to PS I.

The ratio of light absorbed by PS II to that absorbed by PS I (β/α), calculated from Fig. 4 for thylakoids in both State 1 and State 2, is shown in Fig. 5. Two peaks occur at 487 and 650 nm. Due to very low absorption between 500 and 620 nm, the estimate is considerably less certain in this region. The features of the spectrum for the calculated ratio agree with experimental data obtained from relative action spectra of PS II and PS I [24] or derived from quantum yield measured as a function of wavelength [25]. The ratio calculated from the data of Joliot et al. [24] assumes that the peak absorptions for the two photosystems are equivalent, which was predicted in Fig. 4A. The ratio calculated from quantum yield as a function of wavelength for clover [25] is explained in detail by Evans [26]. The ratios calculated from the absorption spectra deviate from that calculated from the quantum yield in two places. Firstly, between 500 and 540 nm, the ratio is probably underestimated due to the loss of carotenoids from LHC II. This error is consistent with the difference between the spectra from spinach thylakoids and the recombined complexes (Fig. 3). If the difference between the two spectra is ascribed to carotenoids associated with PS II then the ratio β/α becomes 1.74 at 525 nm, which is close to that calculated from the quantum yield. The second deviation is the shift to shorter wavelengths of the absorption peak and crossover in the red. As noted above, the peak in the red wavelengths for the chlorophyll-protein complexes was shifted to the blue by about 5 nm. The absorbance peaks for PS I and PS II, 677 + 5 nm and 672 + 5 nm, respectively, then correspond well with the 681 and 677 nm peaks observed by Joliot et al. [24]. It would be expected that if the spectra had their absorption maxima at these longer wavelengths, that the spectrum of β/α would have a broader peak around 650 nm, equalling unity near 680 nm. The high ratio calculated from the quantum yield for clover at 675 nm was atypical, as the ratio ranged from 1.1 to 1.3 for the different species. When the effects of carotenoid loss and absorbance peak shift by SDS are included, the ratio of β/α predicted from the chlorophyll-pro-

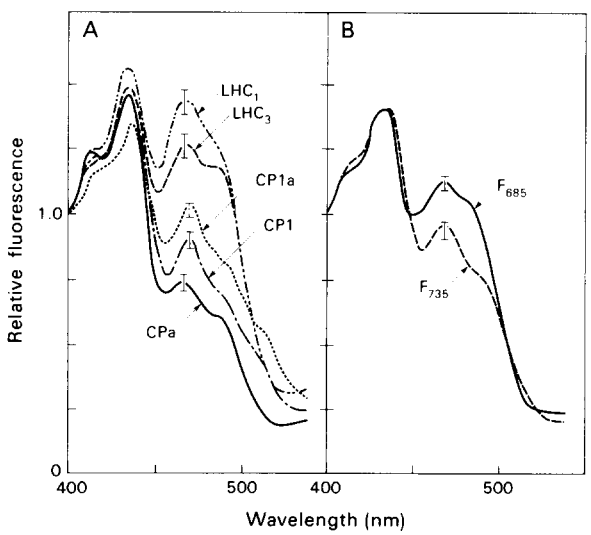

Fig. 6. Corrected 77 K fluorescence excitation spectra for the chlorophyll-protein complexes in situ (A) and spinach thylakoids (B). Spectra for the complexes were obtained with gel pieces suspended in glycerol, while spinach thylakoids (5 μM Chl) were frozen in 50 mM Tricine (pH 8). The spectra are normalized to 1.0 at 400 nm. (A) LHC_1, 681 nm fluorescence emission peak wavelength; LHC_3, 681; CP1a, 725 nm; CP1, 722 nm; CPa, 681 nm. (B) PS II, 685 nm; PS I, 735 nm.

TABLE II

COMPARISON OF THE RATIOS OF FLUORESCENCE EMISSION IN RESPONSE TO EXCITATION AT 470 AND 400 nm (E_{470}/E_{400}) WITH THE RATIO OF ABSORBANCE (A) AT 470 AND 400 nm FOR THE FIVE CHLOROPHYLL-PROTEIN COMPLEXES IN GEL SLICES AND FOR THE TWO PHOTOSYSTEMS FROM A THYLAKOID PREPARATION

E_{470}/E_{400} is the ratio of fluorescence emission due to excitation at 470 nm/400 nm.

	Fluorescence emission wavelength (nm)	E_{470}/E_{400} [a]	A_{470}/A_{400} [b]
Chl-protein complexes			
LHC_1	681	1.43	1.35
LHC_3	681	1.26	1.26
CP1a	725	1.02	0.66
CP1	722	0.90	0.45
CPa	681	0.74	0.76
Photosystems			
PS I	735	0.95 [c]	0.61 for State 1 [d]
			0.73 for State 2 [e]
PS II	685	1.12 [c]	1.14 for State 1 [d]
			1.11 for State 2 [e]

[a] Fig. 6A.
[b] Fig. 2.
[c] Fig. 6B.
[d] Fig. 4A.
[e] Fig. 4B.

tein complexes is in good agreement with that derived from other sources.

Fluorescence excitation spectra

To probe the functional associations between the chlorophyll-protein complexes, fluorescence excitation spectra were measured at 77 K (Fig. 6). Because of the variable optical properties of the sample following freezing, the excitation spectra have all been normalized to 1 at 400 nm. Large differences can be seen between the excitation spectra of the chlorophyll-protein complexes. Since the most dramatic differences between the two photosystems occur near 470 nm (Fig. 6B), the ratios of fluorescence emission due to excitation at 470 to that at 400 nm, and the ratio of absorptance ($100 - \%T$) at 470 to that at 400 nm are presented (Table II)). Note that for LHC_1, LHC_3 and CPa, the fluorescence emission ratios are in good agreement with the absorptance ratios. For CP1 and CP1a, however, the ratio of fluorescence emission due to excitation at 470/400 nm is much greater than expected from the ratio of absorptance at 470/400 nm.

The reason underlying the disagreement between the absorptance and fluorescence is unclear. Consequently, it is difficult to manipulate quantitatively the fluorescence excitation spectra of the chlorophyll-protein complexes. While there is reasonable agreement between the fluorescence excitation spectra of the two photosystems in spinach thylakoids (Fig. 6B) with the calculated absorption spectra (Fig. 4B), we conclude that fluorescence emission is an insensitive method for assessing changes in the association of light-harvesting chlorophyll *a*/*b*-protein complexes with PS II or PS I during State 1–State 2 transitions.

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