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SPECTRAL ANALYSIS OF CHLOROPHYLL-PROTEIN COMPLEXES FROM HIGHER PLANT CHLOROPLASTS *

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A spectral analysis of chlorophyll-protein complexes was carried out to gain information about the state of chlorophyll in vivo. A light-harvesting chlorophyll *a/b* protein complex and a Photosystem I complex were isolated from pea and from wheat chloroplasts by treatment with 0.5% Triton and centrifugation in a sucrose gradient. Resolution of absorption spectra (89 K) of these fractions showed that their forms of chlorophyll were not altered by the isolation procedure. However, because the sum of the spectra of the fractions had a different shape from the chloroplast spectrum, it may be assumed that a third chlorophyll-protein complex was lost or changed in terms of the state of its chlorophyll. The spectrum of this missing chlorophyll was calculated and found to have a maximum near 683 nm. Circumstantial evidence indicates that this calculated spectrum may represent the native absorption of antenna chlorophyll *a*-protein of Photosystem II. The proportionality between the major absorbing forms of chlorophyll observed by curve analysis of different fractions suggests that the 660 and 678 nm forms may be the result of exciton interaction. The addition of a very small, narrow 675 nm band caused a very large improvement in fitting the spectrum of the antenna chlorophyll *a/b* protein with component bands, but not in Photosystem I spectra. A direct comparison of curve resolution with fourth derivative analysis shows the advantages of the former for studying the states of chlorophyll in vivo.

Introduction

Knowledge of the state of chlorophyll in vivo is essential for our understanding of the mechanism of photosynthesis. Analysis of absorption and fluorescence spectra of Chl *a* in plant membranes have indicated a number (4–8) of states or groups of chlorophyll molecules each with its own electronic transition and absorption maximum [1–4]. Different plant species have approximately the same groups of chlorophyll molecules, but their spectra can vary because of different relative proportions of these groups or absorbing forms [3].

Essentially all of the chlorophyll in plant membranes is closely associated with intrinsic proteins [5]. Recently, there has been considerable progress in separating several chlorophyll-protein complexes which differ in their molecular weights or Chl *a* and *b* content by detergent solubilization and polyacrylamide gel electrophoresis or gradient centrifugation. Many studies of the protein part of various chlorophyll-protein complexes have been made, but only a few of them address the problem of the state of the chlorophyll that is associated with the polypeptide subunits. A major difficulty is that the detergent (usually SDS) used to dissociate the membranes fre-

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Abbreviations: Chl, chlorophyll; SDS, sodium dodecyl sulfate; LHC, light-harvesting chlorophyll *a/b*-complex; PS I, Photosystem I; P-700, primary electron donor of PS I; Tricine, *N*-tris(hydroxymethyl)methylglycine.

quently alters the environment of the chlorophyll molecules as indicated by changes in absorption and fluorescence spectra [6,7].

Criteria need to be developed to ascertain whether or not spectra of isolated chlorophyll-proteins represent the electronic absorption of the same chlorophyll molecular species in their native state. Here we present spectra of the light-harvesting chlorophyll *a/b*-complex (LHC) and a Photosystem I (PS I) complex isolated from pea and wheat chloroplasts and the results of curve analyses of these spectra. The absorption spectrum of a third, hypothetical, antenna chlorophyll *a*-protein has been calculated. This spectrum has a maximum at 683 nm and may represent the original absorption of CPIII [8] or CP*a* [9] before SDS treatment.

Materials and Methods

Isolation of chlorophyll-protein complexes

Chloroplasts were isolated from pea (*Pisum sativum*) and wheat (*Triticum aestivum*) plants which had been grown in a glass house. Leaf pieces were blended with 5 mM $MgCl_2$/10 mM KCl/50 mM Tricine buffer, pH 7.5, for 10 s. The slurry was filtered through miracloth, and the chloroplasts were pelleted by centrifugation at 1000 $\times g$ for 10 min.

The procedure of Burke et al. [10] was followed for isolation of chlorophyll-protein complexes. Chloroplasts were treated in 5 mM EDTA to remove cations and centrifuged at 10000 $\times g$ for 10 min. The pellet was resuspended in 0.5% Nonidet P-40 (Particle Data Laboratories, Ltd., Chicago) or Triton X-100 in deionized H_2O to a chlorophyll concentration of 0.5 mg/ml. Nonidet P-40 is very similar to Triton X-100, but more uniform from one preparation to the next. After incubation for 30 min at 25°C, the mixture was centrifuged at 41 000 $\times g$ for 30 min. 8 ml of the dark green supernatant were layered on each 23 ml of linear 0.1–1.0 M sucrose gradients containing 0.1% Nonidet in H_2O above 2 ml of a 2 M sucrose cushion. The gradient tubes were centrifuged at 178 000 $\times g$ for 4.5 h in a Sorvall vertical rotor, TV-850, at 5°C.

Two major, green bands formed in the gradients. Each was removed with a pipette. The upper, highly fluorescent band fraction was dialyzed overnight against 10 mM $MgCl_2$ in 10 mM Tris buffer, pH 8. The LHC-protein which precipitated was separated by centrifugation at 41 000 $\times g$ for 20 min and homogenized in dilute buffer for spectral measurements. The lower gradient band, formed just above the sucrose cushion, contained the PS I-Chl-complex which usually precipitated in the gradient or upon dialysis against water. This fraction was also sedimented by centrifugation and resuspended in dilute buffer.

Chlorophyll concentrations were determined in 80% acetone/water [11]. P-700 concentration was determined from the light-induced absorbance change at 698 nm [12].

Spectrophotometry

Absorption spectra were measured with a Cary 17 spectrophotometer equipped with a scattering transmission attachment. The base of an aluminum sample holder was suspended in a Dewar flask containing liquid N_2 and placed in the compartment of the spectrophotometer. The temperature of the sample was approx. 89 K. The optical pathlength of the sample was 2 mm and the half-bandwidth of the measuring beam at 680 nm was approx. 1 nm. This instrument measures from 0.5 to 1 nm too high in the red spectral region, and the curves have not been corrected for this small error.

The spectrophotometer was connected on-line to a Hewlett-Packard computer system for processing the data.

Spectral analyses

The original RESOL Program for best, least-squares fitting of component bands to spectral data was obtained from Dr. D.D. Tunnicliff of the Shell Development Company. This program has been used to analyse a large number of absorption spectra of different algae and chloroplast preparations [3]. Now RESOL has been modified slightly by Mr. Glenn Ford to run on our Hewlett-Packard Computer. The wavelength data are no longer transformed to wavenumber [13] and a maximum of nine components can be used. The program works directly on the spectra which have been collected and stored in digital form (2048 points/spectrum). We have estimated the input bands' wavelength position and halfwidth from previous results [3], allowed ten iterations, and a change of 1.0 nm in both parameters for each iteration. Each

spectrum was analysed several times with different input components to find the best fit. We allowed the program to decide the proportions of Gaussian and Lorentzian shaped curve for each band [13]. The error between the spectral data and sum of components is expressed here in two ways. The standard error (S.E.) is given as the percent of a peak which has been normalized to 1000 relative units. The smaller the S.E., the better the fit. The program also plots the difference between the original spectrum and sum of components at each wavelength and calculates an amplification factor for this difference when it is normalized for the largest error. The larger this factor, the better the fit.

Results

Characteristics of chlorophyll-protein complexes. The two chlorophyll-proteins isolated in our experiments are essentially identical to the light-harvesting (LHC) complex characterized in Ref. 10 and to the PS I complex described in Ref. 14. We have repeated this isolation procedure several times with pea, wheat and spinach chloroplasts. The only significant difference between experiments has been in the physical state of the lower gradient band (PS I). This fraction was always enriched in the reaction center (P-700) of Photosystem I (Chl/P-700 = 110–127), but in some experiments it did not precipitate and was depleted in longer wavelength absorption (695–705 nm) and fluorescence (730–740 nm). This variation can be explained by the fact that we did not alter the ratio of detergent to chlorophyll as suggested in Ref. 14 to correct for variations in Nonidet stock solutions and in chloroplast samples.

The ratios of Chl *a* to Chl *b* and P-700 in the pea chloroplasts and two chlorophyll-protein complexes used for our spectral analyses are listed in Table I. The Chl *a*/*b* ratio of 1.3 for the LHC and Chl/P-700 ratio of 127 for PS I are very similar to values reported in Refs. 10 and 14 for their corresponding fractions. Although these fractions no doubt contain a considerable mixture of membrane proteins, these do not interfere with the evaluation of the chlorophyll spectra. The state of chlorophyll in these preparations is less altered by the relatively mild, nonionic Nonidet P-40 than by the anionic SDS used to obtain purer proteins on polyacrylamide gel electrophoresis. SDS has been found to alter the shapes of both the absorption and emission spectra significantly when it has been added to chlorophyll-proteins isolated with low concentrations of Triton [12,15].

However, even Triton can affect the chlorophyll in PS I preparations [6]. Apparently, the longer wavelength-absorbing chlorophyll molecules (greater than 700 nm) which fluoresce at 735–740 nm may be attached to the outer region of PS I complexes [14] and are readily detached by Triton. When our isolated PS I preparations had lower ratios of Chl/P-700 or if they were further purified with respect to P-700 by adding more Triton and passing through hydroxy apatite [16], the chlorophyll absorption maximum stayed near 677 nm but most of the long wavelength absorption and fluorescence disappeared.

Although the yield of LHC which precipitated in the presence of Mg^{2+} varied slightly with different batches of detergent and plant material, this fraction

TABLE I

THE RATIOS OF Chl *a* TO Chl *b* AND P-700 IN PEA CHLOROPLAST FRACTIONS

	Chl *a*/*b*	Chl *a*/P-700
Chloroplasts	2.7	498
LHC complex	1.3	∞
PS I Complex	6.6	127

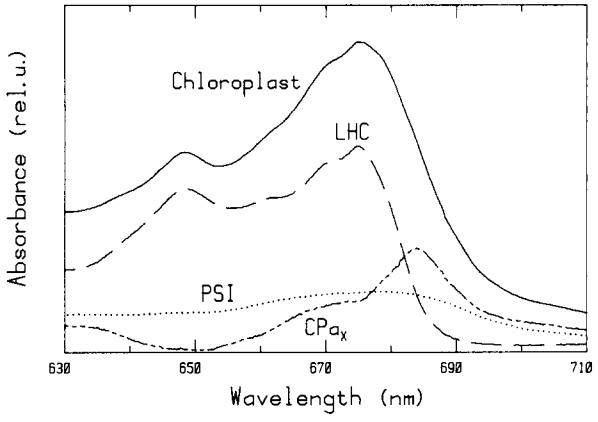

Fig. 1. Absorption spectra at 89 K of chloroplasts (———), PS I complexes (· · · · ·) and LHC-protein (– – –) from peas. See text for explanation of the difference spectrum, CPa_x (– - – - – - –).

contained about 30% of the original plastid chlorophyll in the experiment analysed here. The chlorophyll in the upper gradient band which did not sediment had an *a* to *b* ratio of 3.6 and absorption maximum close to 670 nm, indicating that additional Chl *a* or *a*-*b*-protein complexes were also dissociated by Nonidet action.

Absorption spectra. Absorption spectra at approx. 89 K of pea chloroplast fragments and the LHC and the PS I chlorophyll-protein complexes isolated from them are shown in Fig. 1. The spectrum of PS I is obviously too high below about 650 nm because of a baseline shift. For technical reasons, we did not have a suitable baseline curve stored in the computer memory at the time of the curve analysis. Also, because of uneven freezing of different samples, it is not clear whether a meaningful correction can be made. Other PS I preparations had less absorption in this region, but were depleted in longer wavelength absorption caused by more extensive detergent action. We added approximately two parts of the LHC spectrum to one part of the PS I spectrum, normalized this sum to the height of the Chl *b* peak in the chloroplast spectrum at 650 nm, and subtracted the normalized spectrum from the chloroplast spectrum to obtain a difference spectrum (CPa_x).

We propose that the curve, CPa_x, in Fig. 1 may represent the original absorption of a Chl *a*-protein which has not yet been isolated in its native state. We calculated another difference spectrum in the same way from an experiment in which wheat chloroplasts were fractionated, and it had a similar shape with a main peak between 681 and 685 nm. The shape of the difference spectrum did not vary significantly when the ratio of LHC to PS I was varied between 5 and 2. In making this calculation, we have assumed that normal green chloroplasts contain only three major, native chlorophyll-protein complexes and that CPa_x has no Chl *b*.

TABLE II

AREAS UNDER THE ABSORPTION SPECTRA (660–720 nm) OF THE LHC PS I and CPa_x FRACTIONS EXPRESSED AS A PERCENTAGE OF THE TOTAL AREA

Fraction	Percentage of total	
	Pea	Wheat
LHC	47	58
PS I	25	19
CPa_x	28	23

The areas under the curves in Fig. 1 between 660 and 720 nm were calculated. The area, representing primarily Chl *a* absorption, of each fraction is expressed as a percentage of the corresponding area of the chloroplasts in Table II. Assuming that Chl *a* in all of the chlorophyll-proteins has the same extinction coefficient, it can be seen that about one-half of it is associated with the LHC-protein and one-fourth to one-fifth with each of the other complexes. The percentages of Chl *a* in the PS I samples of peas and wheat in Table II correspond very well with the relative proportions of these same fractions calculated on the basis of their P-700 content which is known to be an integral part of the PS I complex.

Curve resolution. Absorption spectra of pea chloroplasts, the two isolated chlorophyll-protein complexes LHC and PS I) and of the difference between the sum of the two and the chloroplast spectrum (hypothetical CPa_x) have been analysed by computer using the RESOL program (Fig. 2). The essential data obtained from each curve analysis of Fig. 2 and from additional resolutions of spectra of wheat chloroplast fractions are listed in Table III. The peak positions of all the component bands were within 1 nm of the maximum shown at the head of each column except where noted. The area under each component band is shown as the percentage of the total area under the Chl *a* bands (660–707 nm). The numbers in parentheses refer to the bandwidth at half-height.

The percentage of Gaussian shape in a mixture of Gaussian and Lorentzian curves was recorded for each component. All the components were over 89% Gaussian except where noted in Table III. However, when the RESOL program was forced to use only Gaussian-shaped components, the closeness of fit was less.

The second resolutions of pea and wheat chloroplast spectra (Table III) were begun at 650 instead of 630 nm because we wished to test the effect of adding a 676 nm component, and the program allows only nine input bands. However, elimination of a 620-component caused the 640-component to become too large, and both of these spectra show too high a proportion of the latter.

Earlier both the 640 and 649 nm bands were con-

TABLE III

CURVE ANALYSIS OF ABSORPTION SPECTRA OF FRAGMENTS FROM PEA AND WHEAT

The S.E. of each curve, and percentage of each component were calculated from the sum of the areas of Chl *a* components. The bandwidth (nm) for each component is shown in parenthesis.

	S.E. (% of peak)	Peak wavelengths of components (nm)									
		Chlorophyll *b*					Chlorophyll *a*				
	λ(nm):	640	649	660	670	675–676	678	684	693	697–699	703–710
Pea chloroplasts											
(Fig. 3A)	0.35	14 (13)	20 (12)	23 (13)	25 (12)	–	27 (11)	15 (12)	7 (17)	–	2 (13)
	0.30	25 (15)	19 (13)	23 (13)	25 (12)	0.9 (6)	27 (12)	14 (13)	7 (16)	–	3 (14)
Wheat											
chloroplasts	0.32	11 (13)	16 (13)	23 (13)	26 (12)	–	28 (11)	14 (12)	5 (13)	–	2 (15)
	0.24	20 (14)	21 (14)	23 (13)	26 (12)	1.4 (4)	29 (12)	14 (13)	4 (12)	–	3 (17)
Pea LHC											
(Fig. 3B)	0.53	8 (10)	46 (14)	24 (10)	47 (13)	–	28 (10)	1.0 (8) [l]	–	–	–
	0.25	7 (9)	47 (14)	25 (10)	47 (12)	0.7 (3)	27 (9)	0.8 (6) [m]	–	–	–
Wheat LHC	0.33	17 (14)	38 (14)	27 (11)	38 (11)	0.8 (4)	28 (10)	6 (12)	–		–
Pea PS I											
(Fig. 3C)	0.48	53 (33) [a]	19 (18) [c]	16 (13) [f]	23 (13)	–	21 (12)	17 (12)	11 (13)	6 (16)	6 (15)
Wheat PS I	0.29	14 (18) [b]	16 (17) [c,d]	22 (14) [f,g]	26 (13)	–	21 (11)	19 (18)	7 (12) [d]	–	4 (17)
Pea CPa_X											
(Fig. 3D)	0.61		1 (5) [e]	16 (12) [h]	6 (6) [k]	–	18 (9)	19 (7)	18 (10) [m]	11 (14)	14 (20)
Wheat CPa_X	0.48			9 (11) [i,j]	25 (11)	–	31 (11)	19 (8)	9 (10) [n]	6 (17) [o]	–

[a] Peak at 630 nm; [b] 85% Gaussian; [c] Peak at 651 nm; [d] 74% Gaussian; [e] Peak at 657 nm; [f] Peak at 662 nm; [g] 77% Gaussian; [h] Peak at 667 nm; [i] Peak at 663 nm; [j] 79% Gaussian; [k] Peak at 672 nm; [l] Peak at 687 nm; [m] Peak at 688 nm; [n] Peak at 690 nm; [o] 34% Gaussian.

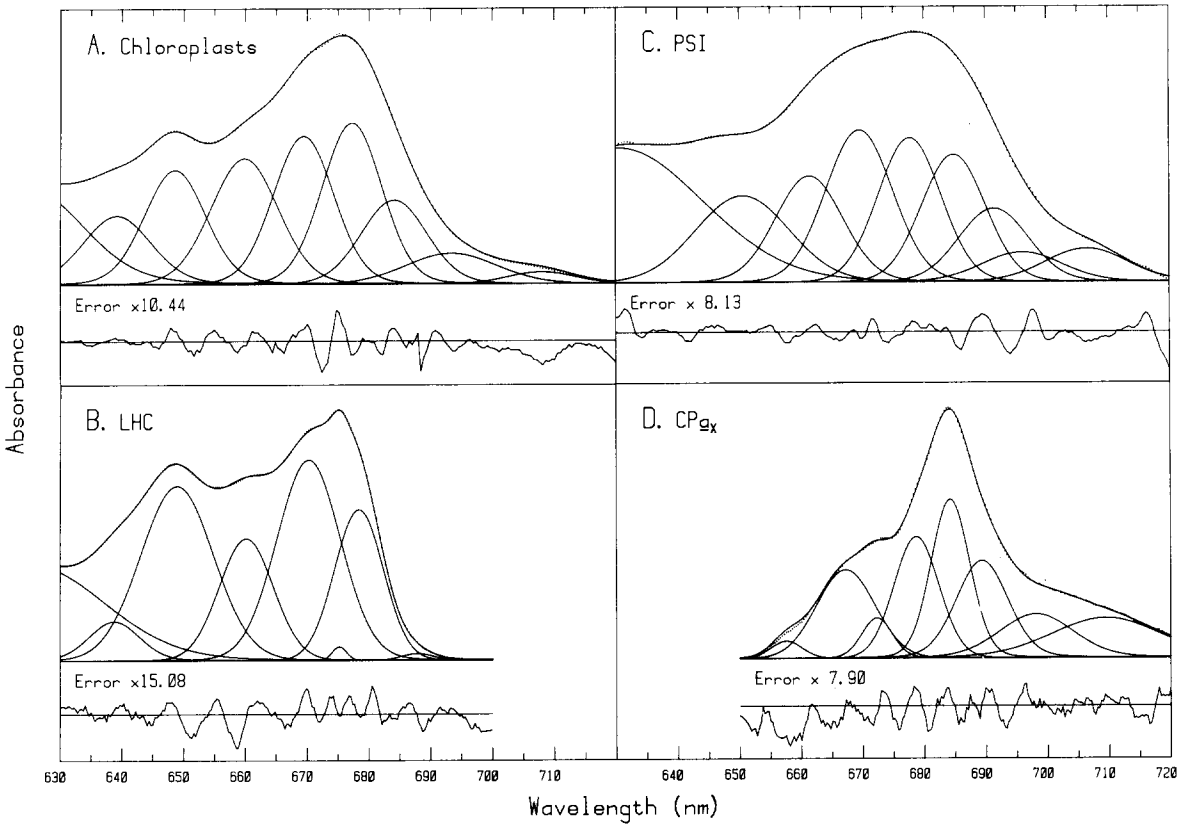

Fig. 2. Absorption spectra at 89 K of pea chloroplasts, two chlorophyll-protein fractions and the derived difference, CPa_x. The measured data are plotted as points, while the line through them is the sum of the component curves the characteristics of which are given in Table III. The error of fit at each point is shown below each spectrum on a scale with the designated magnification.

sidered to represent Chl *b* absorption [3], but in results elsewhere [17] and in our experiments, only the relative amount of the 649-component increased significantly in the LHC fraction which had the lowest Chl *a*/*b* ratio. In PS I fractions which have little Chl *b*, the 640 component peak was always shifted to 651 nm. As mentioned above, the pea PS I spectrum (Fig. 2C) was obviously distorted in the shorter wavelength region which caused the curve resolution to be unreliable below 660 nm.

The relative proportions of the 660- and 678-components were remarkably similar in all of the fractions, but the 670-component was much greater in the LHC than in the chloroplasts or other fractions. Insertion of a very small, narrow component band near 676 nm improved the error factor in the spectral resolution from 10.4 to 12.3 for pea chloroplasts,

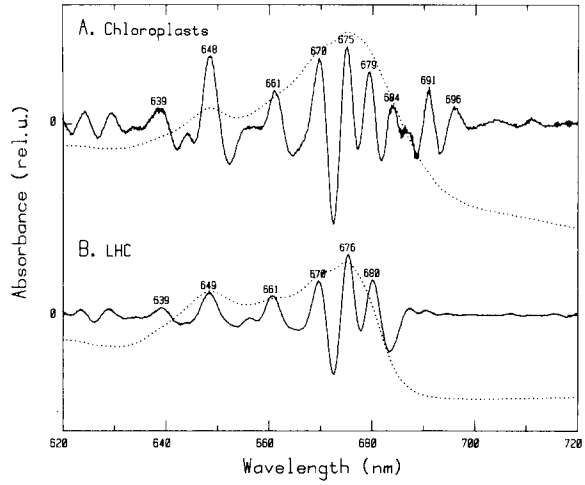

Fig. 3. Absorption spectra (· · · · · ·) at 89 K and fourth derivatives of each spectrum of chloroplasts and LHC-protein from peas. The d*x* increments were 2.0, 2.3, 2.6 and 2.9 nm.

from 6.0 to 15.1 for pea LHC, from 11.2 to 19.2 for wheat chloroplasts, and from 8.9 to 13.8 for wheat LHC. The presence of this 676-band had no effect on the peak positions or proportions of the neighboring components, nor did it improve the error factor when inserted in spectra of PS I or CPa_x.

The 684-component was essentially missing in the LHC fraction and enriched in both PS I and CPa_x. The 693-component was shifted to 690 nm in the CPa_x-difference spectrum and had a narrower bandwidth than in the chloroplasts or isolated fractions. The components at the longest wavelengths are always distorted somewhat, and it may be impractical to try to fit the tails of these spectra with any precision.

Derivative spectra. Fourth derivative spectra of pea chloroplasts and LHC-protein are shown in Fig. 3. Correspondence between the wavelength positions of the derivative peaks and resolved component maxima (Fig. 2) is within 1 or, in one case, 2 nm. Important differences between the two types of analyses will be discussed below.

Discussion

When chloroplasts are fractionated by mild detergent action and sucrose gradient centrifugation, two chlorophyll-protein complexes can be isolated. One aim of the work here was to test whether the chlorophylls in each of these isolated complexes retained their native configuration. To this end, we compared low-temperature absorption spectra of the chloroplasts and fractions and resolved the spectra into component curves which may represent groups of chlorophyll molecules in different environmental states.

One of the fractions studied was a Chl *a*/*b*-protein (LHC) isolated according to Burke et al. [10] and with functional characteristics established by those authors. Gregory et al. [18] decided, on the basis of circular dichroism (CD) measurements, that this LHC 'aggregate' is not representative of the native state of an antenna chlorophyll-protein. However, it is possible that CD spectroscopy cannot be applied in a meaningful way to particulate pigment preparations because of light scattering [19,20]. The emission band near 695 nm which was of variable intensity in different LHC preparations might be related to aggregation of the complex [21]. Comparison of chloroplast and LHC spectra in both Fig. 2A and B and Fig. 3 shows the expected similarity in the Chl *b* and shorter wavelength Chl *a* region.

Previously, it was observed that when SDS (0.1%) was added to a spinach LHC-protein complex, a time-dependent disappearance of the 676 nm peak and 661 nm shoulder occurred and the 670 and 650 nm bands became prominent [15]. In fact, this SDS-modified spectrum of the LHC-protein was nearly identical to the spectrum of the Chl *a*/*b*-protein (designated CPII by various authors) isolated with SDS [17,22,23]. This result is evidence that the chlorophyll molecular arrangement may have been altered in many of the chlorophyll-protein preparations isolated with SDS.

Examination of the resolved LHC spectrum (Fig. 2B) shows that if SDS were to decrease the absorption of the 678 and 660 nm forms of Chl *a*, the observed spectral changes would occur. Table III shows that the relative proportions of the 660- and 678-components are nearly constant in different curve analyses of pea and wheat chloroplast and LHC spectra. Therefore, it is possible that the absorption of these chlorophyll forms reflects exciton interaction leading to band splitting [24]. Because SDS can drastically alter the conformation of protein molecules, its action would be expected to reduce exciton interaction between groups of chlorophyll molecules attached to a protein.

An especially interesting point revealed by curve analysis of the LHC-protein absorption spectrum (Fig. 2B) is the possible existence of a 675 nm form of chlorophyll. It was most unexpected that the presence of this very small, narrow band could cause such a large improvement in overall fit without changing the other bands. It is obvious that with enough component bands any curve can be fitted to a high degree. However, addition of the 675 nm component did not improve the analysis of the PS I or CPa_x spectra. The addition of the 675 band improved the error factor of the chloroplast analysis (Fig. 2A) from 10.4 to 12.3 which is to be expected because LHC-protein comprises such a large fraction of the chloroplast. Whether the small 675-component serves any particular function is unknown.

The results of analyses of pea and wheat PS I spectra (Fig. 2C and Table III) are similar to those of

other, previously analysed, Photosystem I preparations [3]. The longer wavelength-absorbing forms are more prominent, and the bandwidths of all of the components are slightly wider than corresponding components of the LHC spectra.

Because the main spectral forms of Chl *a* observed here in spectra of the LHC and PS I fractions agreed so closely in peak position and bandwidth with those of unfractionated chloroplasts and numerous other species of plants, the state of chlorophyll in these two fractions is probably unaltered. We know from the Chl *a* to *b* and to P-700 ratios that the LHC and PS I fractions comprise approx. 50 and 25%, respectively, of the Chl *a* in the chloroplast. Similar proportions of LHC (CPII) and PS I (CPI) have also been found after SDS-electrophoresis of green algae and higher plant membranes [23]. Recently, procedures have been found which decrease the amount of free pigment separated in polyacrylamide gels and allow for the isolation of additional Chl *a*-protein complexes [5].

Therefore, we calculated a hypothetical spectrum for the missing Chl *a*-protein in our sucrose gradients by subtracting the sum of the LHC and PS I spectra from the chloroplast spectrum. This difference spectrum (CPa_x) is shown in Fig. 1 with spectra of the other fractions and alone in Fig. 2D. Although several assumptions have been made in calculating the spectrum of CPa_x and the S.E. of its curve resolution is fairly great, the large 684 nm component is noteworthy (Table III). The shorter and longer wavelength components differed somewhat in the pea and wheat CPa_x spectra from those components in other fractions. However, the following circumstantial evidence links the spectrum of CPa_x to the Chl *a*-proteins recently isolated by gel electrophoresis; the low temperature fluorescence emission spectrum of freshly isolated chloroplasts and intact algae has a maximum near 695 nm which has been correlated with Photosystem II activity [25]. Both this emission and the activity are very sensitive to detergent action [26]. Either of the 684 or 690 nm absorbing components of CPa_x could logically be a source of 695 nm emission (aggregated LHC could also be the source of 695 nm emission). The Chl *a*-proteins isolated by Delepelaire and Chua [8] (CPIII and CPIV) show a 682 nm absorbing component and have been linked indirectly with Photosystem II activity. Therefore, it is possible that the CPa_x absorption spectrum represents the native state of Chl *a* in the more labile, antenna Chl *a*-protein complexes. We must await more gentle methods of chloroplast fractionation to test our hypothetical spectrum.

Derivative spectrophotometry has frequently been used to identify the absorbing forms of Chl *a* in various chloroplast particles and chlorophyll-protein complexes [27–30]. Advances in computer technology have made such analyses relatively simple. Obviously, plotting the 1st, 2nd or 4th derivative of a curve accurately describes the shape of that curve and indicates the presence and approximate location of component bands. However, the limitations of such derivative spectra are shown by a comparison of Fig. 2A and B with Fig. 3. The 649, 660, 670 and 684 (nm) components are obvious in both types of curve analyses. But the 685–686 fourth-derivative peaks actually show the maximum of the sum of the components and not the very small band in Fig. 2B. The 679 and 680 nm peaks in the two derivative spectra indicate the 678-component band. Thus, curve analysis with a program like RESOL not only suggests the relative amounts of component bands, but also indicates their number and positions with greater precision than derivative analysis.

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