Govindjee: Formula [2] need to be corrected as indicated due to an editorial mistake
Trio

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UNEQUAL DISTRIBUTION OF NOVEL CHLOROPHYLL *a* AND *b* CHROMOPHORES IN SUBCHLOROPLAST PARTICLES OF HIGHER PLANTS *

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SUMMARY

The distribution of newly discovered chlorophyll *a* and *b* chemical species [i.e., Chl*a* (*E*432*F*664), (*E*436*F*670), (*E*443*F*672) and (*E*446*F*674) and Chl*b* (*E*465), (*E*470), (*E*475) and (*E*485)] extracted from functional subchloroplast particles prepared from spinach (*Spinacia oleracea*) leaves is described. It is shown that the different chlorophyll *a* chromophores are unevenly distributed among the various subchloroplast particles. This in turn suggests that the different chlorophyll *a* species may have different and distinct functions in photosynthesis.

INTRODUCTION

The photosynthetic membranes of higher plants are presently interpreted in terms of three structural/functional units that appear to cooperate in the conversion of solar energy into chemical energy [1,2]. These have been referred to as core chlorophyll *a* complex of Photosystem I (Chla_I), core chlorophyll *a* complex of Photosystem II (Chla_{II}) and the light-harvesting

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Abbreviations: Chl, chlorophyll; Chla_I, core chlorophyll *a* complex of Photosystem I; Chla_{II}, core chlorophyll *a* complex of Photosystem II; LHC, light harvesting Chl*a*/*b* protein complex; LDAO, lauryl dimethylamine-*N*-oxide.

Chl*a*/*b* protein complex (LHC) [1,2]. Both photosystems (PS) are conceived as containing antenna and a reaction center Chl [2,3]. For example, the reaction center of PS-I is considered to be made up of chlorophyll *P*-700 [2,3]. On the other hand, the LHC is visualized as a separate entity which could transfer excitation energy to either of the two photosystems [2,3].

It was recently shown in our laboratory that the Chl*a* pool of higher plants and of green algae is made up of four different Chl*a* species which exhibit different Soret excitation and red emission maxima in diethyl ether at 77 K. These were designated Chl*a* (*E*432*F*664), Chl*a* (*E*436*F*670), Chl*a* (*E*443*F*672) and Chl*a* (*E*446*F*674), where *E* and *F* refer to the Soret excitation maxima and fluorescence emission maxima respectively in ether at 77 K [4,5]. The four Chl*a* chromophoric species were detected in extracts of green tissues before and after chromatographic separation of the Chl*a* from the Chl*b* pools and from carotenoids, and were partially purified by high pressure liquid chromatography [5]. They were also detected by difference spectrofluorometry at room temperature in ether solutions of the Chl*a* pool (Freyssinet and Rebeiz, in preparation). Most recently the origin of the different Chl*a* chemical species were traced back to monovinyl and divinyl chlorophyllide *a* and Chl*a* which were shown to be formed by the photoreduction of the monovinyl and divinyl protochlorophyllide and protochlorophyllide ester pools of etiolated plants [6—8]. Likewise, the Chl*b* pool of green plants was shown to consist of four different Chl*b* species. These were designated Chl*b* (*E*465), Chl*b* (*E*470), Chl*b* (*E*475) and Chl*b* (*E*485), where *E* again refers to the Soret excitation maximum of the different Chl*b* chromophores at 77 K in ether [5].

In an effort to determine whether all these Chl species played distinct functional roles during photosynthesis, we have undertaken a study of their distribution in subchloroplast preparations enriched in Chla_{I}, Chla_{II} and LHC complexes. It was observed that the Chl*a* species were unequally distributed among the photosystems and the LHC, which suggests that they may have different and distinct functions in photosynthesis.

MATERIALS AND METHODS

Preparation of chloroplast lamellae and subchloroplast particles

Leaves from market spinach were homogenized in a buffer containing 0.05 M potassium phosphate, 0.5 M sucrose, 5 mM $MgCl_2$ and 10 mM KCl at pH 7.4 as described previously [9]. Chloroplasts were sedimented by centrifugation at 4000 *g* and were resuspended in a low osmotic strength solution having the same composition as the homogenization buffer but lacking sucrose, in order to obtain lysed chloroplast membranes. The thylakoid membranes were recentrifuged, then resuspended in the homogenization buffer adjusted to pH 7.8 (1 mg Chl/mg) and treated with digitonin at a Chl/digitonin ratio of 100 : 1 (w/w). Treatment of the PS-I fraction obtained after differential centrifugation was as described previously [9] except for

the following modifications. In the present study the D-144 pellet was incubated for 10 min in a 100 mM NaCl Tris—HCl buffer pH 7.6 containing 1% LDAO. Both the NaCl and LDAO were diluted 10-fold by the addition of 10 mM Tris—HCl buffer pH 7.6 before the sample was run on a DEAE Sephacel column. Finally, in addition to LDAO and Triton X-100, the column was washed with a solution containing 10 mM Tris—HCl, pH 7.6, 50 mM NaCl and 0.1% digitonin. The sample was eluted in the presence of digitonin and 200 mM NaCl. The use of digitonin in these final steps was to minimize the generation of solubilized Chl. The final sample (see ref. 9 for details) was highly enriched in PS-I activity and had a Chl/*P*-700 ratio of about 35.

Particles enriched in LHC were prepared according to the methods of Thornber [1] and those enriched in Chla_{II} were prepared by the methods of Vernon et al. [10]. The spectral characteristics of the Chla_{II} particles were described in [11].

Pigment extractions

One ml of subchloroplast particle suspension was extracted in 5 ml of acetone : 0.1 NH_4OH (9 : 1 v/v) as previously described [12]. The chlorophyll was transferred to ether as follows: an equal volume of ether was added to the acetone extract and the acetone was washed away by slowly introducing the acetone/ether Chl solution, with a transfer pipet, to the bottom of a separatory funnel containing 500 ml of cold H_2O saturated with $MgCO_3$ [13]. The Chl collected in the ether epiphase while most of the acetone dissolved in the aqueous hypophase.

Spectrophotometry

Absorption spectra of suspensions of the subchloroplast particles were recorded at room temperature with an Aminco dual wavelength spectrophotometer model DW-2, operated in the split beam mode. The suspensions were diluted with 0.4 M sorbitol/0.1 M tricine—NaOH buffer, pH 7.8.

Spectrofluorometry

Corrected fluorescence emission and excitation spectra were recorded on a Perkin—Elmer spectrofluorometer Model MPF-3, equipped with a corrected spectra accessory [12]. At 77 K, the spectra were recorded essentially as described by Cohen and Rebeiz [14] on dilute Chl samples (10^{-6} to 10^{-8} M) in order to avoid quenching and aggregation artifacts. The following combination of filters was used to eliminate interference by scattered light: a blue filter pyrex No. 5543 which is transparent in the 350—500 nm region was placed between the excitation monochromator and the sample; a Turner yellow sharp cut-off filter No. 2A-15 that excluded light below 520 nm was interposed between the sample and the emission monochromator. In record-

ing excitation spectra, the blue filter was replaced by a short wavelength cut-off filter (Turner No. MV-35) that excluded light below 370 nm.

Determination of the chlorophyll a *and* b *content*

The amount of Chl*a* and Chl*b* in the ether extracts was determined at room temperature according to Koski [15]. The percentage of Chl*b* in ether solutions containing high amounts of Chl*a* was calculated at 77 K from the following equation:

$$\%\text{Chl}b = \frac{(1.9Y)(100)}{Z + 1.9Y} \tag{1}$$

where Y = fluorescence emission amplitude at 660 nm of the Chl*b* which is elicited by a 470 nm excitation of the Chl*a*/*b* solution; Z = fluorescence emission amplitude at 674 nm of the Chl*a* which is elicited by a 440 nm excitation of the Chl*a*/*b* solution; and 1.9 = ratio of the fluorescence emission amplitudes at their respective emission maxima (i.e., at 674 and 660 nm, respectively) of equimolar concentrations of purified Chl*a* and Chl*b*, when the Chl*a* emission is elicited by excitation at 440 nm and the Chl*b* emission is elicited by excitation at 470 nm.

It was observed that due to the forementioned choice of excitation wavelengths, a Chl solution in ether, containing about 2—3% of Chl*b* and 97—98% Chl*a* exhibited at 77 K, upon excitation at 470 or 475 nm, a distinct Chl*b* emission with little interference by Chl*a* emission as shown later in Fig. 3.

Estimation of the percentage of the different chlorophyll a *and* b *chromophores in the extracts of the subchloroplastic particles*

The percentage of the different Chl*a* and Chl*b* species in the various extracts was estimated from excitation spectra recorded on the ether extract of the subchloroplastic particles at 77 K. It should be emphasized, however, that the procedure described below yields only approximate results for the following reasons. 1. Since the quantum yield of the individual Chl*a* species is unknown, we had to assume that all the Chl*a* species have the same quantum yield of fluorescence. The same assumption was made for the Chl*b* species. 2. In order to minimize the Soret excitation band overlap, the excitation amplitudes of the Chl species were recorded at about 6 nm below their emission maxima. For comparative purposes it was, therefore, assumed that the emission bands of the individual Chls were Gaussian in shape, an assumption that seems to be borne out by experimental evidence for the Chl*a* species [5]. 3. Finally, the Soret excitation amplitudes of the respective Chl species were only partially corrected for the Soret excitation band overlaps, by triangulation. Since the same approximations were made for all the subchloroplast particles, comparison between various particles was, however, possible.

The percentages of Chl*a* (*E*432*F*664), (*E*436*F*670), (*E*443*F*672) and (*E*446*F*674) for a particular preparation was, thus, approximated as follows: the Soret excitation amplitudes at *E*432, *E*436, *E*443 and *E*446 nm were determined from Soret excitation spectra recorded at the following respective emission wavelengths: 658, 664, 666, and 668 nm, unless otherwise indicated. The percentage of Chl*a* (*E*432*F*664) was estimated as follows:

$$\%\text{Chl}a(E432F664) = \frac{(E432)}{(E432)+(E436)+(E443)+(E446)}(100) \qquad (2)$$

where *E*432 = Soret excitation amplitude of Chl*a* (*E*432*F*664) recorded at an emission maximum of 658 nm; *E*436 = Soret excitation amplitude of Chl*a* (*E*436*F*670) recorded at 664 nm, etc.

The percentage of Chl*b* (*E*465), (*E*470), (*E*475) and (*E*485) in the ether extract of a particular particle preparation was estimated, as above, from the Soret excitation amplitudes at *E*465 and *E*470 which were recorded at an emission wavelength of 648 nm and from the Soret excitation amplitudes at *E*475 and *E*485 which were recorded at 654 nm.

RESULTS

Spectrophotometric characteristics of the subchloroplastic particles

The room temperature absorption spectra of the various preparations are compared to the chloroplast membranes in Fig. 1. The light harvesting Chl*a*/*b* protein-enriched fraction exhibited an absorption maximum at 677 nm, pronounced absorption shoulders at about 650 and 470—475 nm and a Soret absorption maximum at 436 nm. These results were similar to those reported by Burke et al. for purified LHC [16]. The $\text{Chl}a_{II}$-enriched particle, i.e. TSF2A, had a red absorption maximum at 672 nm and was contaminated by LHC as evidenced by the Soret absorption maximum at about 465 nm [10]. Finally the *P*-700-enriched particle exhibited the same absorption profile as that reported by Malkin [17]. It contained 1 *P*-700/~35 Chl*a* as shown by Fenton et al. [9].

Distribution of the different chlorophyll a *chromophores among the various subchloroplast particles*

The chlorophyll was extracted from the different subchloroplast particles and was transferred to ether as described in Methods. The distribution of the different Chl*a* chromophores among the various subchloroplastic particles was then determined from the Soret excitation spectra recorded at 77 K (see Methods).

Chlorophyll *a* (*E*446*F*674) appears to be the major Chl species in the chloroplast membranes and in the LHC-enriched fraction (Table I). On the other hand, Chl*a* (*E*436*F*670) appears to be the major Chl species in the

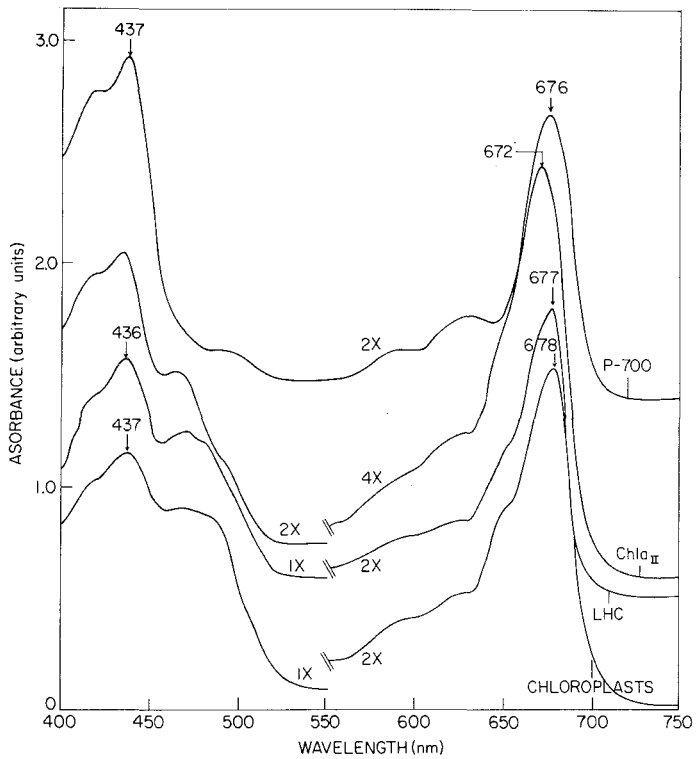

Fig. 1. Room temperature absorption spectra of the different subchloroplast particles. The suspensions were diluted with 0.4 M sorbitol/0.1 M tricine-NaOH buffer, pH 7.8. The spectra were recorded at the sensitivities indicated, 4X being the highest sensitivity. In order to avoid overlapping of the spectra the baselines were arbitrarily adjusted as evident from the absorbance values at 750 nm. The absorbance of the chloroplast membranes and of the LHC was about 1.0 OD units while that of Chla_{II} and *P*-700 was about 0.5 OD units. Arrows point to wavelengths of interest.

TABLE I

Distribution of the Chl*a* chromophores among the various subchloroplast particles

Preparation	Chl*a* species (%)				Chl*a* ratio
	*E*432*F*664	*E*436*F*670	*E*443*F*672	*E*446*F*674	LW/SW [a]
Isolated chloroplasts [b]	7.1	21.2	11.5	60.3	2.5
LHC-enriched [b]	3.5	19.2	23.0	54.5	3.4
P-700-enriched [b]	11.4	31.9	14.8	42.0	1.3
Chla_{II}(TSF2A)-enriched [c]	22.5	36.8	14.7	26.0	0.7

[a] LW Chl = (*E*446*F*674) + (*E*443*F*672); SW Chl = (*E*436*F*670) + (*E*432*F*664).
[b] Freshly prepared and immediately extracted.
[c] Stored at −4° C before extraction.

Chla_{II}-enriched fraction (Table I). It is also most noticeable from Table I that the LHC fraction was enriched in long wavelength (LW) Chl*a* [i.e. Chl*a* (*E*446*F*674) + Chl*a* (*E*443*F*672)] while the *P*-700 and Chla_{II} fractions were proportionally enriched in short wavelength (SW) Chl*a* [i.e. Chl*a* (*E*432*F*664) + Chl*a* (*E*436*F*670)]. For example, the LW/SW Chl*a* ratio was 4.8 and 2.6 folds higher for the LHC than for the *P*-700 and Chla_{II} preparations, respectively (Table I). This was caused by a porportionally higher concentration of Chl*a* (*E*436*F*670) and a lower concentration of Chl*a*

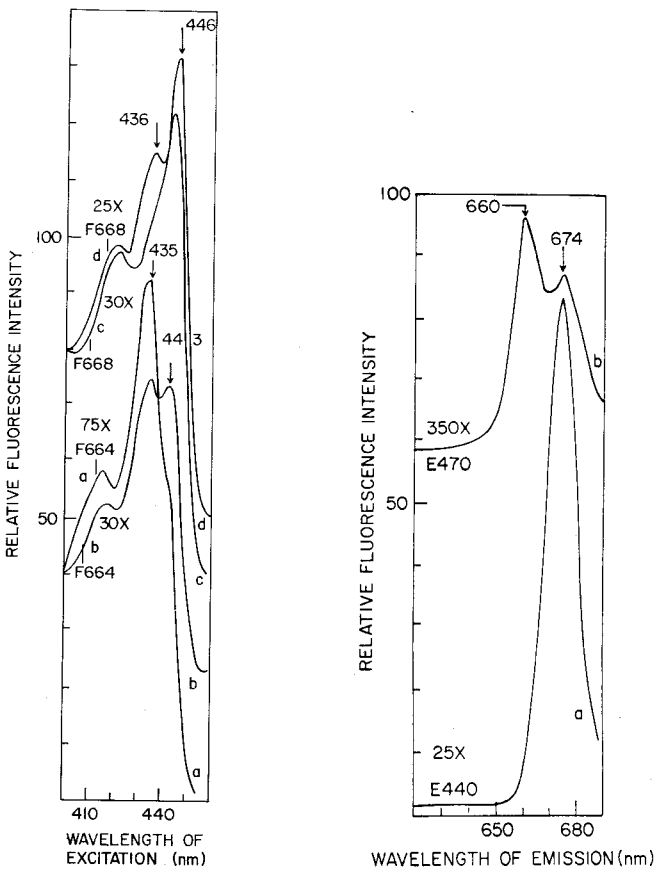

Fig. 2. Excitation spectra of fluorescence of the extracted Chl*a* pool from fractions enriched in LHC (0.5×10^{-6} M) (b, c) and *P*-700 (0.6×10^{-6} M) (a, d), in diethyl ether, at 77 K. Ordinate scale attenuation is indicated on the spectra, 350X being the highest sensitivity used. The excitation spectra in the 400—460 nm region were recorded at the fluorescence (*F*) wavelength indicated. Baselines were arbitrarily adjusted to avoid overlap of spectra. Arrows point to wavelengths of interest.

Fig. 3. Fluorescence emission spectra of the Chl extract of the *P*-700 preparation, in ether at 77 K, elicited by excitation at 440 nm (a) and at 470 nm (b). Baselines were arbitrarily adjusted to avoid overlap of the spectra.

(*E*466*F*674) in the Chla_I and Chla_{II} than in the LHC preparation (Table I). Isolated chloroplasts gave results similar to the LHC-enriched particles. This is depicted pictorially in Fig. 2 which compares two Soret excitation spectra of the Chl*a* pool extracted from *P*-700 to those of the LHC-enriched fractions. The excitation spectra were recorded on the short wavelength side of the Chl*a* pool emission band, i.e. at emission wavelengths of 664 and 668 nm respectively in order to emphasize the difference in the Chl*a* (*E*436*F*670) content between the LHC and the *P*-700 preparations (Fig. 2). The Soret excitation spectrum of the Chl*a* pool of the *P*-700 fraction, recorded at an emission wavelength of 664 nm, was distinctly enriched in Chl*a* (*E*436*F*670) as compared to that of the LHC (Fig. 2a, b). On the other hand, the latter exhibited a more pronounced Chl*a* (*E*443*F*672) content (Fig. 2a, b). In the spectra recorded at an emission wavelength of 668 nm, Chl*a* (*E*435*F*670) was detectable only as a weak shoulder in the Chl*a* pool of the LHC fraction, as the latter became dominated by Chl*a* (*E*446*F*674) (Fig. 2c). In contrast, Chl*a* (*E*436*F*670) was still detectable as a distinct peak in the Chl*a* pool of the *P*-700 preparation (Fig. 2d).

Distribution of the different chlorophyll b *chromophores among the various subchloroplast particles*

The highly purified *P*-700 preparation used in this work (37 Chls/*P*-700) surprisingly contained about 4% of Chl*b*. It was impossible to detect such a low amount of Chl*b* by conventional absorption spectrophotometry or even by spectrofluorometry at room temperature [18]. The extracted Chl*a* pool exhibited an emission maximum at 674 nm (Fig. 3a). Emission spectra of the unextracted *P*-700 particles were reported elsewhere [19]. Upon exciting the Chl extract near the Soret absorption/excitation maximum of Chl*b* (470 nm, in this case) at 77 K, a distinct Chl*b* fluorescence emission appeared at 660 nm (Fig. 3b).

The distribution of the Chl*b* species extracted from the various subchlo-

TABLE II

Distribution of the Chl*b* chromophores among the various subchloroplastic particles

Preparation	Chl*b* species (%)				Chl*b* ratio
	*E*465	*E*470	*E*475	*E*485	LW/SW [a]
Isolated chloroplasts	1.6	8.2	66.9	23.3	9.2
LHC-enriched	2.3	7.9	65.6	24.1	8.8
P-700-enriched	—	6.2 [b]	64.9	28.9	15.1
Chla_{II}(TSF2A)-enriched	2.2	10.3	60.1	27.3	7.0

[a] LW Chl = (*E*485 + *E*475); SW Chl = (*E*470 + *E*465).

[b] Determined from the Soret excitation spectrum recorded at an emission wavelength of 650 nm instead of 648 nm.

roplast particles is shown in Table II. The four newly described Chl*b* [5] were present in all the subchloroplast particles except that the *P*-700 particle appeared to lack Chl*b* (*E*465) (Table II). In all cases the predominant Chl*b* species was Chl*b* (*E*475) followed by Chl*b* (*E*485). The Chl*b* distribution in the various subchloroplast particles appeared to be more uniform than that of Chl*a*. Since Chl*b* is assumed to be present only in the LHC, the uniformity of its distribution among the other particles suggests that these particles were probably slightly contaminated by the LHC.

DISCUSSION

This paper describes the distribution of newly discovered Chl*a* and Chl*b* chromophores [4,5] among various functional/structural entities prepared from mature chloroplasts. Although a better knowledge of the structure of these Chls is needed before their function is fully understood, differences in the distribution of Chl*a* among various subchloroplast preparations strongly suggest that they may play distinct and different roles in photosynthesis (Table I). For example, the *P*-700 and Chla_{II} preparations were relatively enriched in Chl*a* (*E*436*F*670) as compared to the LHC preparation. The latter was in turn relatively enriched in Chl*a* (*E*446*F*674). Both reaction center-enriched preparations were also proportionally enriched in Chl*a* (*E*432*F*664).

The detection of Chl*b* in the highly purified *P*-700 preparation by low temperature spectrofluorometry was unexpected (Fig. 3b). It is not presently clear, however, whether the small amount of Chl*b* associated with the *P*-700 preparation constitutes a structural part of PS-I or is merely a contamination by the LHC, as suggested above.

It was recently shown that the different Chl*a* chromophoric species, the distribution of which is described in this work, were quite stable. Their spectrofluorometric profile in ether at 77 K was shown by Freyssinet and Rebeiz (in preparation) to be independent (a) of the solvent used in their extraction (i.e., petroleum ether vs. acetone vs. ethanol), (b) of the pH of the extraction medium as long as the pH remained above the level at which pheophytinization took place, (c) of the Chl concentration (in 10^{-6} M solutions or less), (d) of the lipid content of the medium from which the Chls were extracted, (e) of the rate of cooling of the sample to 77 K, (f) of the dark-light photoperiodic regime to which a mature green plant may be subjected to. Finally, the same Chl*a* and Chl*b* profile in ether at 77 K was observed before and after chromatographic purification. However, during the purification of the P-700 particle it was observed that treatment with LDAO and subsequent elution of the solubilized Chl from the DEAE column resulted in the conversion of some of the Chl*a* (*E*446*F*674) into a blue shifted Chl*a* degradation product. The reaction of Chl*a* with LDAO will be published elsewhere. It should be pointed out, however, that amines such as LDAO are known to rupture the isocyclic ring of Chl*a* and Chl*b* even at very low concentrations [19,20] although it has been our experience that the NH_4OH added to the

acetone used throughout this work, did not. However, since only the solubilized Chl seemed to be affected by the LDAO treatment, we believe that the Chl that was still attached to the lipoproteins in the *P*-700 particle was unaffected and indeed reflected the native condition of Chla_I. It should also be pointed out that neither digitonin nor Triton X-100, which were used for the preparation of all the particles, had any observable effects on the Chl.

Further work about the structure, function and distribution of the various Chl*a* and Chl*b* chromophores among the pigment-protein complexes of higher plants is in progress.

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