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CHLOROPLAST BIOGENESIS

XXIX. THE OCCURRENCE OF SEVERAL NOVEL CHLOROPHYLL *a* AND *b* CHROMOPHORES IN HIGHER PLANTS

CONSTANTIN A. REBEIZ *, FAITH C. BELANGER, GEORGES FREYSSINET ** and DANIEL G. SAAB

Laboratory of Plant Pigments, Biochemistry and Photobiology, Department of Horticulture, University of Illinois, Urbana, IL 61801 (U.S.A.)

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Summary

With the use of low temperature spectrofluorometry and matrix calculations it was demonstrated that the chlorophyll *a* pool of higher plants is made up of four different chlorophyll *a* chromophores. The latter were segregated by high pressure liquid chromatography on a silica column. They were designated Chl *a* (E432 F664), Chl *a* (E436 F670), Chl *a* (E443 F672) and Chl *a* (E446 F674), where E refers to the Soret excitation maximum and F to the fluorescence emission maximum at 77 K in ether. Likewise the Chl *b* pool was shown to consist of at least four different Chl *b* chromophores which were designated: Chl *b* (E465), Chl *b* (E470), Chl *b* (E475) and Chl *b* (E485). It was proposed that the various chlorophyll chromophores differed by the degree of oxidation of their side chains at the 2 and 4 positions of the macrocycle. It was also suggested that the chemical modifications at the 2 and 4 positions of the macrocycle may play an important role in positioning the different chlorophyll chromophores in the thylakoid membranes.

* To whom correspondence and reprint requests should be addressed.

** Present address: Department de Biologie Générale et Aplliquée, Université Lyon I, 69621, Villeurbanne, France.

Abbreviations: Chl, chlorophyll; Chl(ide), a mixture of chlorophyll and chlorophyllide; E, excitation wavelength; F, emission wavelength.

Introduction

It was recently reported by Rebeiz et al. [1] and by Belanger and Rebeiz [2] that the chlorophyll (Chl) *a* and *b* of higher plants were each made up of at least two distinct chemical species. This conclusion was based on the following observations [2]: (a) That the unesterified protochlorophyll pool of etiolated higher plants was made up of monovinyl protochlorophyllide (i.e. Mg-2-vinyl,4-ethyl- pheoporphyrin a_5 and of divinyl protochlorophyllide i.e. Mg-2,4-divinyl pheoporphyrin a_5). (b) When etiolated tissues were exposed to light, the two protochlorophyllides were photoconverted into two distinct chlorophyllide species. (c) The protochlorophyllide pair as well as the chlorophyllide pair were each characterized by split Soret excitation maxima in ether at 77 K. (d) When etiolated tissues were illuminated with white light, then placed back in darkness, the two newly formed chlorophyllides were converted into two distinct Chl *a* species which also exhibited split Soret excitation maxima. (e) It was also observed that the Chl *a* of green mature tissues consisted of two distinct Chl *a* species with split Soret excitation maxima. (f) Finally the Chl *b* of green mature tissues also appeared to consist of two distinct species which were characterized by split Soret excitation maxima in ether at 77 K.

In more recent work dealing with the details of the conversion of monovinyl and divinyl protochlorophyllide into distinct Chl *a* and *b* species in etiolated tissues (Belanger, F.C. and Rebeiz, C.A., in preparation) it became apparent that more than two different Chl *a* and two different Chl *b* chromophores were formed during greening. In this work, the detection of additional Chl *a* and *b* chromophores in green mature tissues of higher plants is described. The partial segregation of the various Chl *a* chromophores by high pressure liquid chromatography (HPLC) is also reported.

Materials and Methods

Plant materials and growth conditions. Cucumber seeds (*Cucumis sativus* L. cv. Beit Apha MR) were purchased from the Niagara-Chemical Division, FMC Corporation, Modesto, CA. The seeds were germinated under a light (14 h) and dark (10 h) photoperiodic regime at 28°C as previously described [3]. The green fully expanded cotyledons were harvested during the 10th to 14th light cycle. Green spinach (*Spinacia oleracea*) was purchased from local supermarket. *Euglena gracilis* strain 2, was grown on a glutamate/malate medium [4] under constant shaking and continuous illumination. They were harvested at the stationary phase of growth.

Extraction of the chlorophyll. 3 g of green tissue were homogenized in 20 ml acetone/0.1 N NH_4OH (9 : 1, v/v) at 0—4°C for 2 min in a Sorvall Omni-mixer. After centrifugation at 39 000 × *g* for 10 min, the 80% acetone extract was adjusted to 75% acetone with H_2O. Chlorophyll *a* and *b* were transferred to hexane by extraction with 1 volume of hexane, followed by an additional 1/3 volume of hexane [5].

Thin-layer chromatography. Chlorophyll *a* and *b* in the hexane fraction were purified by chromatography on thin layers of cellulose MN 300 developed in ligroin 60—80/*n*-propanol (9.9 : 0.1, v/v) at room temperature [6]. The

segregated Chl *a* and *b* were eluted in peroxide-free ether, dried under N_2 gas, then redissolved in ether for spectrofluorometric measurements.

High pressure liquid chromatography. High pressure liquid chromatography (HPLC) was carried out on a microprocessor controlled Spectra-physics instrument Model SP 8000, equipped with a ternary gradient system, data system with 16K RAM memory, and a dual channel printer/plotter [7]. A Schoeffel F 970 spectrofluorometric monitor fitted with a tungsten-halogen excitation source was used as a detector [7]. The chromatographic segregations were performed either on a 25 cm Spectra physics Spherisorb (5 μm) column, or on a 25 cm Spherisorb ODS (10 μm) column. All separations were performed on the freshly prepared 80% acetone extracts of the green tissues. The eluted fractions were collected, dried under N_2 gas then redissolved in ether for spectrofluorometric monitoring.

Spectrophotometry. Spectrophotometric measurements were performed with an Aminco dual wavelength spectrophotometer model DW-2, operated in the split-beam mode.

Spectrofluorometry. Corrected fluorescence emission and excitation spectra were recorded on a Perkin-Elmer spectrofluorometer Model MPF-3, equipped with a corrected spectra accessory [8]. Purified solutions of Chl *a* and *b* in ether were transferred to cylindrical sample tubes and were monitored at 77 K as described by Cohen and Rebeiz [9]. 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 to 500 nm region, was placed between the excitation monochromator and the sample; a yellow sharp cut-off filter, that excluded light below 520 nm, was interposed between the sample and the emission monochromator. In recording excitation spectra the blue filter was removed from the excitation beam.

Computer analysis of the emission/extraction matrices. Deconvolution of the emission/excitation matrices of the ether solutions of Chl *a* and *b* was performed according to Weber [10]. All computations were programmed on a Controlled Data Corporation computer Cyber 175.

Results

The low temperature spectrofluorometric profile of the purified chlorophyll a pool of green tissues

In studying the photoconversion of monovinyl and divinyl protochlorophyllide to the corresponding chlorophyllides, and the conversion of the latter into Chl *a* [2], it became apparent that the esterification of the monovinyl and divinyl chlorophyllides into chlorophylls was possibly accompanied by chemical modifications of the vinyl groups at position 2 and 4 of the macrocycle (Belanger, F.C. and Rebeiz, C.A., in preparation). It stood to reason to assume that if this were true, it should be possible to detect some new Chl *a* species in addition to the two Chl *a* reported earlier [2]. It was conjectured that the occurrence of additional Chl *a* chromophores may be ascertained from a detailed study of the Soret excitation region of the Chl *a* pool. This approach was particularly promising as a result of two main considerations: (a) Firstly the Soret excitation region of Chl appears to be of

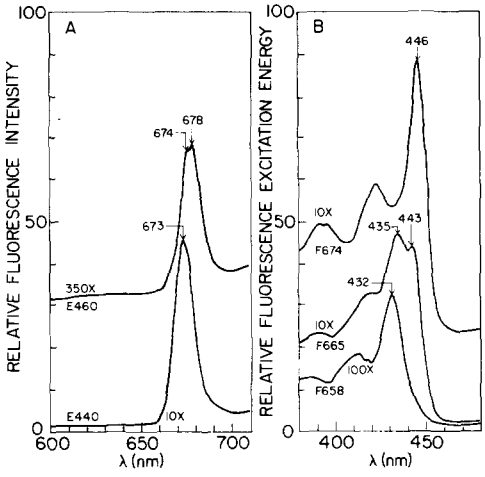


Fig. 1. Fluorescence emission (A) and excitation (B) spectra in ether at 77 K of the Chl *a* pool of green spinach leaves after purification on thin layers of cellulose. Ordinate scale attenuation is indicated on the spectra, 350X being the least attenuation possible. The emission spectra were elicted by the E-wavelength indicated. The excitation spectra were recorded at the F-wavelength indicated. Arrows point to wavelengths of interest.

particular analytical usefulness since it seems to reflect readily, chemical alterations at the 2 and 4 positions of the macrocycle [2]. (b) Secondly it is well documented that the observed relative fluorescence emission and excitation spectra of dilute solutions of a single emitting species is independent of the wavelength of emission and excitation [10]. Furthermore it was recognized that should the various Chl *a* species happen to differ, even slightly, in their emission bandwidth and in their emission maxima, this will facilitate further their detection.

The Chl *a* pool from green spinach leaves was therefore purified on thin layers of cellulose as described in Methods. Two emission spectra of this pool which were recorded at two different excitation wavelengths (E) are depicted in Fig. 1A. At an excitation wavelength of 440 nm, the emission maximum of the Chl *a* pool was observed at 673 nm. Upon excitation at longer wavelength (i.e. at 460 nm), the emission maximum shifted to 678 nm and a distinct emission shoulder became apparent at 674.5 nm (Fig. 1A). This observation suggested that an in-depth examination of the Soret excitation region of the Chl *a* solution may reveal the presence of more than two different Chl *a* species.

Soret excitation spectra of the Chl *a* pool were therefore recorded at 77 K at 2 nm fluorescence emission intervals between 650 and 700 nm. As the Soret excitation spectra were recorded at progressively longer emission wavelengths (F) the Soret excitation maxima shifted from E432 nm (at F658 nm) to E446 nm (at F674 nm). Fig. 1B depicts the Soret excitation spectra recorded at F658, 665 and 674 nm respectively. At these emission wavelengths the Soret excitation maxima were observed at 432, 435, 443 and 446 nm respectively (Fig. 1B). Two of the putative Chl *a* species that were described earlier [2] were apparent in the split Soret excitation spectrum recorded at an emis-

sion wavelength of 665 nm (Fig. 1B). The same profiles were observed in the Chl *a* pool of green photoperiodically grown cucumber cotyledons and in green *Euglena* cultures.

Altogether the above data lent further credence to the contention that the Chl *a* pool of green tissues may be made up of more than two chemically different Chl *a* chromophores. These were tentatively designated as Chl *a* (E432), Chl *a* (E435) Chl *a* (E443) and Chl *a* (E446) where E refers to the Soret excitation maximum at 77 K in ether. The further deconvolution of the Soret excitation profile of the purified Chl *a* pool is described below.

Determination of the minimum number of chlorophyll a species in the chlorophyll a pool of green tissues

At this stage it should be emphasized that although the detection of multiple emission maxima induced by differential excitation of a sample containing two emitting species is highly suggestive, it does not constitute a proof for the presence of more than 2 emitting species in the sample. The same is true for the detection of multiple excitation maxima upon recording excitation spectra at different emission wavelengths. For example the differential excitation of a sample containing two fluorescent species will certainly result in different emission amplitudes of the two emitters, depending, of course, on the wavelength of excitation. Furthermore, the overlap of two fluorescent spectra of changing relative amplitudes will very likely shift the apparent emission maxima of the mixture to shorter or longer wavelength. This in turn will depend on the degree of spectral overlap and on the relative amplitudes of the two overlapping emission spectra. On the other hand, at 77 K, a single fluorescent species may give rise to a continuum of fluorescent species as a result of a variety of structural microlocalizations of an otherwise chemically identical molecule. It was therefore essential to determine by some independent means, the actual number of fluorescent Chl *a* chromophores, in the Chl *a* pool of green tissues, which gave rise to the multiple excitation maxima described in the previous section.

In 1961, Weber stated and solved very elegantly a similar problem [10]. He demonstrated that for an Emission-Excitation Matrix 'M', whose elements $M_{i,j}$, represent the fluorescence intensity measured at wavelength λ_j, for an excitation λ_i, the number of fluorescent components in the matrix was equal to the rank of the minors for which the following inequality was obeyed:

$$\frac{\Delta}{P} \geqslant 3\,\frac{\delta F}{\bar{F}} \tag{1}$$

where

Δ = value of the determinant for a minor of any particular rank,

P = value of the permanent (i.e. the sum of all possible diagonal products in the determinant [11] for the same minor),

δF = noise level or minimum detectable fluorescence,

$\bar{F}$ = mean fluorescence intensity of the $M_{i,j}$, elements of the minor under consideration.

Of course such a treatment of fluorescence data is valid only for samples which fall within the linear response range of the measuring instrument. It

TABLE I

EXCITATION/EMISSION MATRIX OF THE CHLOROPHYLL *a* POOL

The $M_{i,j}$ elements represent the Soret excitation amplitudes measured at wavelength Eλ for an emission Fλ. In this matrix, a column represents an excitation spectrum while a row represents an emission spectrum. The Soret excitation amplitudes were normalized to a 350 x ordinate scale attenuation. See the text for more details.

Soret observation wavelengths (Eλ nm)	Soret excitation amplitudes in arbitrary units; emission wavelengths (Fλ nm)									
	F652	F656	F660	F662	F666	F668	F674	F678	F686	F692
E420	5	25	97	201	568	830	1193	801	164	84
E425	6	31	121	232	551	794	1190	858	171	88
E430	9	47	180	326	655	801	1064	767	161	81
E433	8	49	189	350	714	879	1057	746	156	81
E436	7	37	153	330	750	834	1130	742	161	79
E440	3	17	83	223	750	1081	1410	900	175	94
E443	2	10	60	184	828	1320	1900	1236	234	122
E446	3	7	37	133	770	1333	2258	1550	287	145
E450	1	3	11	42	321	655	1516	1291	256	117
E455	0	2	4	11	47	112	290	360	88	31

should also be emphasized that although this technique will detect all the fluorescent species in a mixture, when they have reasonably well resolved excitation bands, it fails in cases of extreme excitation band overlap [12]. Of course it also fails to differentiate between two fluorescent species if both species possess the same excitation/absorption spectrum [10].

With these limitations in mind, Weber's technique was used for the determination of the minimum number of Chl *a* chromophores in the Chl *a* pool of green tissues. To this effect, 10 Soret excitation spectra were recorded on the same aliquot of the purified Chl *a* pool of green spinach leaves. The excitation spectra were recorded at the following emission wavelengths: F652, 656, 660, 662, 666, 668, 674, 678, 686 and 692 nm. The excitation amplitudes at E420, 425, 430, 433, 436, 440, 443, 446, 450 and 455 nm, were then determined for every excitation spectrum. All excitation amplitudes were normalized to the same attenuation scale. The 10×10 matrix depicted in Table I was then assembled from the normalized Soret excitation amplitudes. In this matrix a row represented an emission spectrum while a column represented an excitation spectrum. The determination of all the minors of a given rank that obeyed Eqn. 1 was achieved by means of the computer. The latter was programmed to report any minor of a given rank 'n' with $\Delta/P \geqslant 3(\delta F/\bar{F})$. According to Minc [11] for a 10×10 matrix, all minors of rank n are given by

$$\left[\frac{10!}{n!(10-n)!}\right]^2$$

All possible 44100 minors of rank 6 and all possible 63504 minors of rank 5 of the Chl *a* pool had Δ/P values that were below the $3(\delta F/\bar{F})$ threshold. However, a considerable number of the 44100 minors of rank 4 had Δ/P values much higher than the $3(\delta F/\bar{F})$ threshold. Fifteen significant Δ/P values for fifteen minors that spanned the emission wavelengths from 652 to 686 nm and

TABLE II

Δ/P VALUES FOR SOME OF THE 4 × 4 MINORS OF THE CHLOROPHYLL *a* MATRIX

For more details consult the text.

Observation wavelengths (Eλ nm)	Δ/P; emission wavelengths (Fλ nm)		
	652, 662, 674, 686	652, 666, 674, 686	656, 662, 674, 686
425, 443, 450, 455	0.041	0.034 *	0.044 *
430, 440, 446, 455	0.041 *	0.029 *	0.042 *
430, 440, 450, 455	0.042	0.039 *	0.047
430, 443, 450, 455	0.044	0.040 *	0.050
433, 443, 450, 455	0.040	0.038 *	0.049

* The corresponding $\delta F/\overline{F}$ values amounted to 0.002, for the others the $\delta F/\overline{F}$ values amounted to 0.003.

the Soret excitation wavelengths from 425 to 455 nm are given in Table II.

Altogether these results indicated that there was not a continuum of fluorescing Chl *a* species in the 77 K aliquot of the Chl *a* pool. Instead there appeared to be present at least four different Chl *a* chromophores. The same results were obtained with the Chl *a* pool of green cucumber cotyledons.

Unsuccessful attempts of separating the different chlorophyll a chromophores by reversed phase high pressure liquid chromatography

It was conjectured that if the different Chl *a* forms, which were detected in the extracted Chl *a* pool indeed represented different chemical species, it should be possible to achieve some sort of chromatographic separation of these species. Since in the past, reversed phase high pressure liquid chromatography on a C-18 bonded column proved very efficient in separating Chl *a* and Chl *b* from pheophytin *a* and pheophytin *b* [7], several attempts were made to adapt this technique for the separation of the putative Chl *a* chromophores.

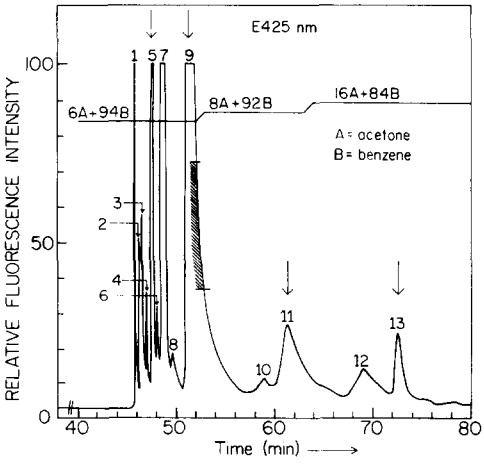
Reversed phase high pressure liquid chromatography with a variety of solvents proved only partially successful in achieving our goal. The best segregations were achieved on a 25 cm Sphersiorb ODS [10 μm] column, eluted isocratically with H_2O/methyl alcohol/acetone (5 : 75 : 20, v/v/v) at room temperature. At a flow rate of 1 ml/min the Chl *a* segregated into two minor peaks with retention times (RT) of about 209 and 263 s respectively and a major peak with an RT of about 311 s. Chlorophyll *b* had an RT of about 221 s. Spectrofluorometry of the eluted Chl *a* fractions revealed that each peak was made up of different proportions of the different putative Chl *a* species. The fast moving Chl *a* peak (RT 209 s) was proportionally more enriched in the Chl *a* (E435) species than the second Chl *a* peak (RT 263 s). The latter was in turn proportionally more enriched in the Chl *a* (E435) species than the slow moving Chl *a* fraction (RT 311 s) which in turn was very enriched in the Chl *a* (E446) species.

In all freshly prepared acetone extracts of green cucumber cotyledons or spinach leaves a certain amount of pheophytin *a* (RT 950 s) was also detected. It amounted to about 0.5% of the total Chl *a* and *b* content.

Partial segregation of the different chlorophyll a chromophores by high pressure liquid chromatography on silica

More successful separations of the putative Chl *a* chromphores were achieved on a silica column (Spherisorb, 5 μm) coupled to a programmed gradient elution. The most promissing separation is depicted in Fig. 2. The first four peaks were mainly Mg-free degradation products of Chl *a* while peak 7 was mainly made up of Chl *b*. Peak 5, 9, 11, and 13 were highly enriched in the Chl *a* species which will be described below.

The highest emission amplitude of peak 11 (Fig. 2) was elicited by excitation at around 430—432 nm. The emission spectrum thus recorded exhibited an emission maximum at 664 nm (Fig. 3A). The Soret excitation spectrum recorded at the emission maximum, i.e. at F664 nm, exhibited a sharp Soret excitation maximum at 432.5 nm (Fig. 3B). This chlorophyll appeared to be very similar to Chl *a* (E432) which was detected in the unsegregated Chl *a* pool depicted in Fig. 1B. It will be therefore referred to as Chl *a* (E432 F664) where E refers to the Soret excitation maximum and F to its emission maximum at 77 K in ether. Peak 11 was also made up of trace amounts of Chl *b* and of small amounts (about 14%) of Chl *a* (E443).



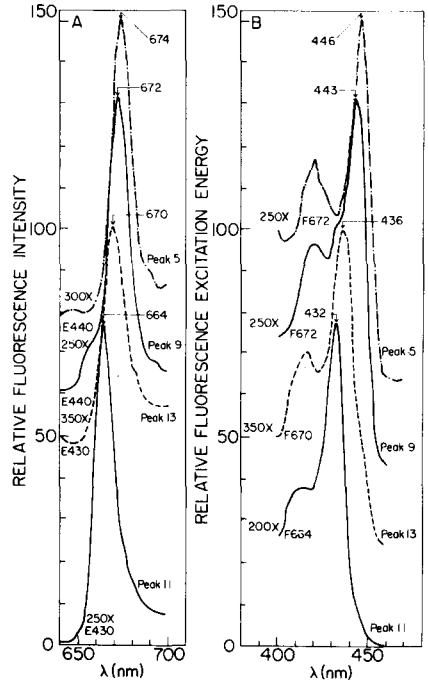


Fig. 2. High pressure liquid chromatography profile of a freshly prepared 80% acetone extract of green spinach leaves. Detection was by fluorescence elicited by excitation at 425 nm. Segregation was on a 25 cm Spherisorb (5 μm) column, eluted at 24°C with the gradient depicted on the figure. For the first 39 min the column was eluted with benzene/hexane (50 : 50, v/v). The ordinate scale is in arbitrary fluorescence units. Arrows point to the four peaks which were discussed in the text. Only the shaded portion of peak 9 was collected.

Fig. 3. Fluorescence emission (A) and excitation (B) spectra in ether at 77 K of four Chl *a* chromophores segregated by high pressure liquid chromatography (see Fig. 2). All symbols are as in Fig. 1.

The main emission maximum of peak 13 (Fig. 2) was observed at 670 nm (Fig. 3A). The Soret excitation spectrum recorded at F670 nm had its excitation maximum at 436—437 nm (Fig. 3B). This chlorophyll was therefore designated Chl *a* (E436 F670) by analogy to the (E435) which was observed in the unsegregated Chl *a* pool (Fig. 1B). In addition to Chl *a* (E436 F670) peak 13 was also made up of about 30% of Chl *a* (E432 F664). The latter became apparent when the Soret excitation spectrum was recorded at F664 nm.

Peak 9 was collected in two separate fractions, which corresponded to the unshaded and shaded areas of peak 9 (Fig. 2). The shaded fraction of peak 9 consisted mainly of a Chl *a* with an emission maximum at 672 nm (Fig. 3A). The Soret excitation spectrum recorded at F672 nm exhibited an excitation maximum at 443.5 nm (Fig. 3B). This chlorophyll was therefore designated Chl *a* (E443 F672) by analogy to the Chl *a* (E443) which was detected in the unsegregated Chl *a* pool (Fig. 1B). Peak 9 was contaminated by Chl *b* and by about 30% of Chl *a* (E432 F664).

Peak 5 (Fig. 2) consisted mainly of a Chl *a* with an emission maximum at 674 nm (Fig. 3A). The Soret excitation maximum recorded at F672 nm exhibited a very sharp maximum at 446.5 nm (Fig. 3B). This chlorophyll was therefore designated Chl *a* (E446 F674) by analogy to the Chl *a* (E446) which was detected in the unsegregated Chl *a* pool (Fig. 1B). Peak 5 was contaminated with about 10% of Chl *a* (E432 F664).

At this stage it should be emphasized that the high pressure liquid chromatography segregation on Silica resulted in some degree of Chl *a* degradation. This was evidenced by the generation of increased amounts of pheophytin *a* (2—4% of the total chlorophyll) during chromatography. As pointed out earlier the amount of pheophytin *a* detected by reversed phase high pressure liquid chromatography on the Sperisorb ODS column amounted to only about 0.5% of the total Chl *a* and *b*. This amount of pheophytin *a* may indeed represent the extent of the endogenous pheophytin *a* pool which is supposedly found in green tissues [13].

Detection of multiple chlorophyll b chromophore in green plants

With the detection of four different Chl *a* species in extracts of green tissues, it was natural to wonder whether multiple Chl *b* species also occurred in green tissues. It may be recalled that it was suggested earlier that at least two different Chl *b* species occurred in nature [2].

The same analytical approach that was successful in eliciting the different Chl *a* species was adopted to detect the different putative Chl *b* chromophores. The Chl *b* pool of green spinach leaves was purified on thin layers of cellulose as described in Methods, and the ether eluate was submitted to detailed spectrofluorometric analysis at 77 K.

The emission spectrum of the Chl *b* pool, elicited by two different excitations at 440 and 475 nm respectively, is depicted in Fig. 4A. Only one emission maximum at 460 to 461 was observed.

By analogy to the Chl *a* pool in which the multiple Chl *a* species were detected by their Soret excitation maxima prior to high pressure liquid chromatography segregation, the Soret excitation region of the Chl *b* pool was sub-

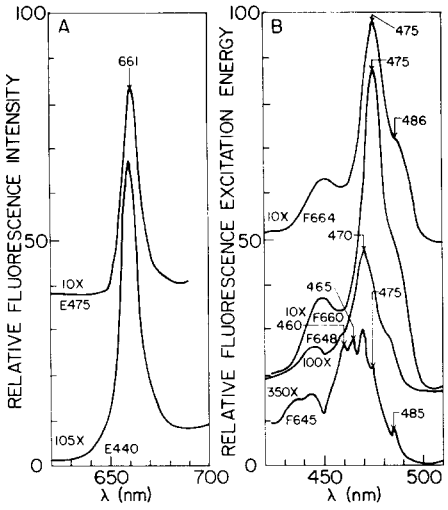


Fig. 4. Fluorescence emission (A) and excitation (B) spectra, in ether at 77 K of the Chl *b* pool of green spinach leaves after purification on thin layers of cellulose. All symbols are as in Fig. 1.

mitted to a detailed spectrofluorometric examination. The Soret excitation spectrum recorded at F645 nm i.e. at the short wavelength tail of the Chl *b* emission band was very revealing. It exhibited distinct Soret excitation maxima at 460, 465, 470 and 485 nm; an excitation shoulder was also detected at about 475 nm (Fig. 4B). These results suggested that probably, multiple Chl *b* chromophores which differed slightly in their emission bandwith and their emission maxima also occurred in green tissues of higher plants.

If this were the case, then the relative amplitudes of the various Soret excitation maxima which were just described should not be constant but should vary or vanish at some particular emission wavelengths. That this was indeed the case is obvious from Fig. 4B. At an emission wavelength of 648 nm, the Soret excitation profile of the Chl *b* pool became dominated by the 470 nm Soret excitation maximum; the Soret excitation maxima at 460 and 465 nm were no longer discernable (Fig. 4B). When the Soret excitation spectrum of the Chl *b* pool was recorded at its emission maximum i.e. at 660 nm, the Soret excitation profile became dominated by the 475 nm Soret excitation maximum and the 485 nm Soret excitation shoulder (Fig. 4B). The Soret excitation maximum at 470 nm was no longer detectable. As reported previously the Soret excitation shoulder observed at 485 nm, became more pronounced when the Soret excitation spectrum was recorded at about 664 nm, i.e. on the long wavelength tail of the Chl *b* emission band (Fig. 4B). Essentially the same results were obtained in the Chl *b* pool of green cucumber cotyledons and green *Euglena* cells.

Altogether the above results suggested that the Chl *b* pool of green plants was made up of multiple Chl *b* chromophores. These are tentatively designated as Chl *b* (E460), Chl *b* (E465), Chl *b* (E470), Chl *b* (E475), and Chl *b* (E485), where as before E refers to the Soret excitation maxima at 77 K in ether. The corresponding emission maxima which can best be determined from the purified individual Chl *b* chromophores are presently under investigation.

TABLE III

EXCITATION/EMISSION MATRIX OF THE CHLOROPHYLL *b* POOL

The $M_{i,j}$ elements represent the Soret excitation amplitudes measured at wavelengths Eλ for an emission Fλ. In this matrix, a column represents an excitation spectrum while a row represents an emission spectrum. The Soret excitation amplitudes were normalized to a 350 X ordinate scale attenuation.

Soret observation wavelengths (Eλ nm)	Soret excitation amplitudes in arbitrary units; emission wavelengths (Fλ nm)									
	F644	F648	F652	F654	F656	F658	F660	F664	F676	F684
E455	19	33	124	250	413	563	627	459	56	26
E459	23	44	140	262	420	557	620	434	51	24
E464	22	65	225	427	630	788	819	532	60	29
E470	20	109	489	916	1400	1715	1764	1099	119	60
E475	13	79	447	916	1575	2198	2481	1704	180	91
E480	6	47	283	531	910	1312	1579	1208	143	62
E485	4	28	202	426	735	1019	1169	837	96	46
E490	1	8	79	204	406	648	795	669	74	29
E495	1	2	19	52	119	210	294	336	39	15
E500	0	0	3	12	31	46	56	67	9	7

Determination of the minimum number of chlorophyll b species in the chlorophyll b pool of green tissues

Since the fluorescence excitation data suggested that the Chl *b* pool of green tissues was probably made up of five different Chl *b* species it was desirable to check this hypothesis by matrix treatment of the data. Keeping the limitation of matrix analysis in mind (vide supra) the emission/excitation profile of the Chl *b* pool was submitted to matrix analysis exactly as was done for the Chl *a* pool. To this effect, 10 Soret excitation spectra were recorded on the same aliquot of the purified Chl *b* pool of green spinach leaves. The excitation spectra were recorded at the following emission wavelengths: F644, 648, 652, 654, 656, 658, 660, 664, 676 and 684 nm. The excitation amplitudes at E455, 459, 464, 470, 475, 480, 485, 490, 495 and 500 nm were then determined for every excitation spectrum. The 10 X 10 matrix depicted in Table III was next assembled from the normalized Soret excitation amplitudes. As for the Chl *a* matrix (Table II) a row represented an emission spectrum while a column represented an excitation spectrum.

TABLE IV

Δ/*P* VALUES FOR SOME OF THE 4 X 4 MINORS OF THE CHLOROPHYLL *b* MATRIX

The $\delta F/\overline{F}$ values were about 0.005.

Observation wavelengths (Eλ nm)	Δ/*P*; emission wavelengths (Fλ nm)		
	644, 648, 658, 684	644, 648, 660, 684	644, 652, 660, 684
459, 470, 490, 500	0.159	0.174	0.135
459, 475, 495, 500	0.089	0.124	0.121
459, 480, 495, 500	0.110	0.145	0.122
459, 485, 490, 500	0.107	0.119	0.118
464, 470, 490, 500	0.092	0.102	0.087

TABLE V

PROPOSED STRUCTURE OF THE DIFFERENT CHLOROPHYLL *a* AND *b* CHROMOPHORES OF HIGHER PLANTS

It is assumed that all modifications reside in the side chain substitutions at the 2 and 4 positions of otherwise conventional Chl *a* and *b* chromophores.

Proposed name of the Chl *a* or Chl *b* chromophore	Substituents at positions 2 or 4 of the macrocycle	
	Position 2	Position 4
2,4-Diethyl Chl	$-CH_2-CH_3$	$-CH_2-CH_3$
2,4-Divinyl Chl	$-CH=CH_2$	$-CH=CH_2$
2,4-Monohydroxy ethyl Chl	$-\underset{\displaystyle OH}{\underset{\displaystyle |}{CH}}-CH_3$	$-\underset{\displaystyle OH}{\underset{\displaystyle |}{CH}}-CH_3$
2-Vinyl, 4-ethyl Chl * or 2-Ethyl, 4-vinyl Chl *	$-CH=CH_2$ / $-CH_2-CH_3$	$-CH_2-CH_3$ / $-CH=CH_2$
2-Monohydroxy ethyl, 4-ethyl Chl * or 2-Ethyl, 4-monohydroxyethyl Chl *	$-\underset{\displaystyle OH}{\underset{\displaystyle |}{CH}}-CH_3$ / $-CH_2-CH_3$	$-CH_2-CH_3$ / $-\underset{\displaystyle OH}{\underset{\displaystyle |}{CH}}-CH_3$
2-Monohydroxy ethyl, 4-vinyl Chl * or 2-Vinyl, 4-monohydroxy ethyl Chl *	$-\underset{\displaystyle OH}{\underset{\displaystyle |}{CH}}-CH_3$ / $-CH=CH_2$	$-CH=CH_2$ / $-\underset{\displaystyle OH}{\underset{\displaystyle |}{CH}}-CH_3$

* These chromophore pairs are geometrical isomers.

All possible 44100 minors of rank 6 and all possible 63504 minors of rank 5 had Δ/P values that were below a $3(\delta F/\bar{F})$ threshold. On the other hand a considerable number of the 44100 minors of rank 4 had Δ/P values much higher than the $3(\delta F/\bar{F})$ threshold. Fifteen significant Δ/P values for fifteen minors that spanned the emission wavelengths from 644 to 684 nm and the Soret excitation wavelengths from 459 to 500 nm are reported in Table IV.

Altogether these results indicated that the Chl *b* pool of green tissues was made up of at least four different Chl *b* chromophores. The failure of matrix analysis to detect unambiguously a fifth Chl *b* chromophore will be examined below.

Discussion

The detailed spectroscopic analysis of the purified Chl *a* pool of green higher plant tissues suggested that this pool was made up of four different Chl *a* chromophores (Fig. 1). Likewise the Chl *b* pool appeared to be made up of at least four different Chl *b* chromophores (Fig. 4). These conclusions were borne out by the matrix treatment of the spectrofluorometric data (Tables II, IV).

Furthermore the spectrofluorometric analysis of the Chl *b* pool also suggested the presence of a fifth Chl *b* chromophore that may have eluded detection by matrix analysis (Fig. 4). This may have been caused by the extreme Soret excitation band overlap of the five putative Chl *b* chromophores within a

wavelength range of 25 nm only (i.e. 460—485 nm). Indeed extreme Soret excitation overlap of this nature has recently been shown to interfere with the detection of all the fluorescent species in reconstituted mixtures of tetraphyrroles [14].

The spectrofluorometric analysis and matrix calculations of the Chl *a* pool were corroborated by the partial purification by high pressure liquid chromatography of four different Chl *a* chromophores (Fig. 2). The latter exhibited distinct emission and excitation maxima (Fig. 3). Their Soret excitation maxima were very similar to those of the unsegregated components of the Chl *a* pool (Figs. 1, 3). In spite of these similarities more structural information is needed before an unambiguous relationship is established between the segregated Chl *a* chromophores and those detected by spectrofluorometry in the unsegregated Chl *a* pool.

At this stage it is very relevant to wonder about the possible chemical differences between the various chlorophyll chromophores just described. Enough information is already available to justify speculation about the possible chemical structures of the different chlorophyll chromophores. Firstly it appears that all these chlorophyll chromophores are derived either from 2,4-divinyl Chl(ide) *a* and *b* or from 2-vinyl, 4-ethyl Chl(ide) *a* and *b* (Ref. 2; Belanger, F.C. and Rebeiz, C.A., in preparation). Secondly by considering that vinyl side chains can be reduced to ethyl groups or can be oxidized to hydroxyethyl groups and by taking into account that these reactions can take place either at position 2 or at position 4 of the macrocycle, as well as at both positions simultaneously, one is lead to a total of six chemically different Chl *a* and six different Chl *b* chromophores (Table V). It is also obvious from Table V that three of the six chlorophyll species, namely the monoethyl monohydroxy ethyl chlorophylls, the monovinyl monoethyl chlorophylls and the monovinyl monohydroxy ethyl chlorophylls can occur as geometrical isomers. This may explain the segregation of monovinyl protochlorophyllide (Rebeiz, C.A. and Belanger, F.C., unpublished) and that of several Chl *a* that differed by their esterifying alcohol at the 7th position of the macrocycle, into spectroscopically idential doublets [15] during reversed phase high pressure liquid chromatography segregations. Of course slight modifications of the cyclopentanone ring or conversion of the substituents at position 2 and 4 of the macrocycle into formyl or acetyl groups is also a possibility.

Finally it is difficult to speculate about the possible structure of the different chlorophyll chromophores without inquiring about the physiological significance of these putative chemical differences. It is possible to visualize the side chain modifications of the chlorophyll chromophores as one way of tagging a particular chlorophyll molecule for a particular site and a particular orientation within its macromolecular environment in the thylakoid membrane. On the other hand modifications of the cyclopentanone ring may result in differences in the basic photochemistry of the chlorophyll chromophore which may be dictated by some specific functional role. The structure and possible function of the different chlorophyll chromophores of higher plants are presently under investigation.

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