CIRCULAR DICHROISM OF PROTOCHLOROPHYLLIDE AND CHLOROPHYLLIDE HOLOCHROME SUBUNITS

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1. Introduction

Dark-grown seedlings of angiosperms accumulate protochlorophyll(ide) (Pchl), which upon illumination, is reduced to chlorophyll(ide) *a* (Chl). A photoactive pigment–protein complex, protochlorophyll holochrome (Pchl-H) [1,2] with macromolecules of mol. wt. about 600 000 [3] containing multiple Pchl molecules per particle [4, 5] can be extracted from etiolated bean (*Phaseolus vulgaris* L.) leaves, with the aid of a detergent mixture of saponins, Pchl-H subunits with an apparent mol wt of 63 000 and giving no indication of more than one Pchl molecule per unit have been obtained from dark-grown barley (*Hordeum vulgare* L.) leaves [6,7]. Exciton interaction among closely associated pigment molecules in Pchl-H from bean leaves and its photoproduct, chlorophyll holochrome (Chl-H) has been inferred from circular dichroism (CD) spectra in the red region [8,9]. Previous CD work in the region from 540 to 615 nm on saponin-containing Pchl-H from barley [10] does not allow comparison with the CD results on bean.

This paper gives the results of a comparative study of CD spectra in the red region gained from a number of saponin-containing preparations of Pchl-H and Chl-H from dark-grown barley and bean leaves. In general, we did not detect splitting of Pchl or Chl bands and hence did not find exciton interaction among Pchl or Chl molecules. Exciton interaction among Pchl molecules was detected only in a special case, namely in Pchl-H extracted with saponin from barley leaves which contained a 7-fold increased Pchl content after feeding with δ-aminolevulinate (ALA).

2. Materials and methods

Seedlings of barley (*Hordeum vulgare* L., cultivar Bonus) and bean (*Phaseolus vulgaris* L., cultivar Stella) were grown in darkness as described previously [6]. Dim green safe-light was used for operations requiring vision. The saponin-containing Pchl-H extracts were obtained as described earlier [6], with the following modifications: Saponin Weiss Rein, Art. 7695, Erg. B. 6. E. Merck AG, Darmstadt, Germany, was used. To the whole homogenate of the leaves was added an appropriate volume of either 12% or 4% (w/v) saponin dissolved in the grinding buffer to yield a saponin concentration of 3.6 or 1.2%, respectively. The latter concentrations will be referred to as the extracting concentrations (cf. table 1). Pellets of ammonium sulfate-precipitated Pchl-H were resuspended with and dialyzed overnight against 1.5% or 0.5% saponin (w/v) in 0.1 M tricinate-Na, pH 8.5, with the exception noted below. The 100 000 *g* cen-

Table 1
Descriptions of Pchl and Chl holochromes.

Preparation no.	Source	Saponin concentration	Absorbance (1 cm light path) 642–644 nm[d]	Δ 678 nm[e]
1	barley	*a*	0.56	0.80
2	barley	*a*	0.90	0.10
3	bean	*a*	0.14	0.19
4	bean	*b*	0.20	0.28
5	ALA-fed barley	*c*	0.20	0.08

a Extracting concentration, 1.2% (w/v) saponin; resuspending and dialysis media, 0.5% (w/v) saponin.
b Extracting concentration, 3.6% (w/v) saponin; resuspending and dialysis media, 1.5% (w/v) saponin.
c Same as *a*, but preparation not dialyzed.
d Before illumination of Pchl-H. The absorbances are corrected for scattering [7] and are related to Pchl concentrations.
e After illumination eliciting maximal photoconversion of Pchl to Chl. The absorbances are increases at 678 nm in response to illumination and are related to Chl concentrations [7].

trifugation of the extracts was omitted. To gain exceptionally concentrated Pchl-H one of the preparations (table 1, no. 1) was reprecipitated with ammonium sulfate, and the pellet was resuspended before dialysis with only 0.05 ml resuspending medium per gram leaf material represented.

For one of the Pchl-H preparations from barley (table 1, no. 5) the Pchl content of the leaves was enhanced by feeding ALA [11]. Shoots of 6 day-old seedlings, with the apical 5 mm of their primary leaves removed, were placed with their bases in 5×10^{-3} M ALA in 0.07 M phosphate buffer, pH 6.5. After 20 hr, terminal 5 cm segments of the primary leaves, containing 7 times as much Pchl as corresponding segments from leaves that had not been fed ALA, were ground with a mortar and pestle and processed in the standard way, except for the omission of dialysis.

The preparations were stored at −18°C or lower. Before spectroscopy, large aggregates were removed by centrifuging the solutions for 30 min at 15 000 rpm (Sorvall SS34 rotor).

Maximal photoconversion of Pchl-H to Chl-H was secured by illuminating the Pchl-H in an ice bath for 2 min with a 60 or 75 W tungsten filament lamp at a distance of 10–30 cm.

Absorption spectra were recorded with a Cary 17 spectrophotometer equipped with a scattered transmission accessory. CD spectra were obtained with a JASCO model ORD, CD/UV-5 optical Rotatory Dispersion Recorder. The samples were thermostated at 11 ± 1°C during the CD spectral scans, which were run at 9 nm per minute from the red toward the blue with decreasing slit openings. No spectral change was detected upon promptly rescanning several preparations after completed first scans. The recording of CD spectra of Chl-H solutions began within 6 or 7 min after the beginning of photoconversion. Thus, about 15 min elapsed between the onset of illumination and the CD scan through the Chl band at approx. 680 nm.

3. Results and discussion

No qualitative differences in the spectral properties among the various saponin-containing Pchl-H preparations or among their respective photoproducts, Chl-H, (excepting extracts from ALA-fed leaves) were found (figs. 1 and 2). Thus, holochromes from dark-grown bean leaves, extracted with 3.6 or 1.2% saponin and dialyzed against 1.5 or 0.5% saponin, respectively, cannot be distinguished from one another on the basis of qualitative characters of their absorption or CD spectra. Furthermore, saponin-containing extracts from barley leaves also have the same spectral qualities over a wide range of pigment concentration. The absorption spectrum of Pchl-H extracted from dark-grown bean leaves with 3.6% saponin and dialyzed against 1.5% saponin, as well as the absorption spectrum of the photoproduct, Chl-H are given in fig. 1. Absorption spectra of Pchl-H and Chl-H obtained from barley and bean by extracting leaf homogenates with 1.2% saponin and resuspending the ammonium sulfate-precipitated material with 0.5% saponin are not given here, because examples have been published previously [6,7]. The saponin-containing Pchl-H solutions have an absorption maximum at 642–644 nm, and solutions of the immediate photo-

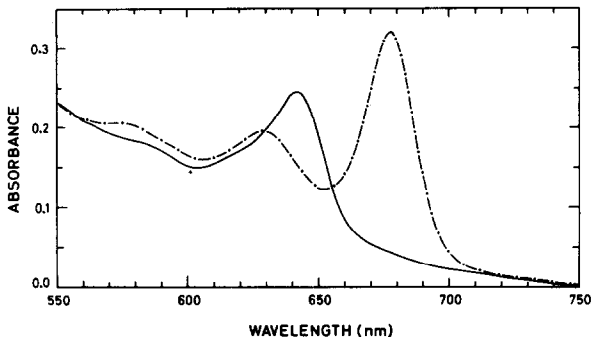

Fig. 1. Absorption spectra of saponin-containing Pchl-H (——) and newly-formed Chl-H (– · –) from bean leaves; preparation No. 4, table 1.

product, Chl-H, have a maximum at 678 nm. These absorption bands are shifted 10–20 nm toward the red in comparison with Pchl and Chl dissolved in ether [cf. 12].

The CD spectra (fig. 2. B–E) of the Pchl-H solutions are characterized by a very broad negative band in the region between 600 and 650 nm, which usually resolves into at least two components with bands at approximately 635 and 615 nm. Since the negative CD signals between 600 and 650 nm disappear upon maximal photoconversion of Pchl to Chl, they clearly belong to the photoactive portion of the Pchl. All of the preparations (except the one from ALA-fed leaves) contained about 35% inactive Pchl absorbing at 630 nm, which seems then to have little or no optical activity in the 600–650 nm region. The turbidity of the Pchl-H and Chl-H did not lead to significant distortion of the CD baselines, which are essentially flat from 750–650 nm before illumination and are close to the zero line, in the absence of CD bands, between 660 and 625 nm after illumination (fig. 2).

The CD spectra of newly-formed Chl-H (fig. 2, B–E) all show a positive signal at approximately 680 nm and a broad trough with maximum negative CD around 585 nm. The negative 585 nm CD band is small for Chl in ether [12] and decays exponentially with time in Chl-H [10]. Because of the known changes in spectral properties of several kinds of Chl-H preparations with time [4, 8–10, 13], the present CD spectra were made at reduced temperature and with as rapid scans as feasible. This necessitated the acceptance of relatively large slit openings and noise levels. To test the effect on the spectra of reducing the slit openings by two-thirds, one of the Chl-H solutions (fig. 2, D) was scanned with smaller slits, as well as in the usual way. While minor differences, in accordance with expectation, are visible in the respective spectra, there is no difference which affects the interpretations or conclusions.

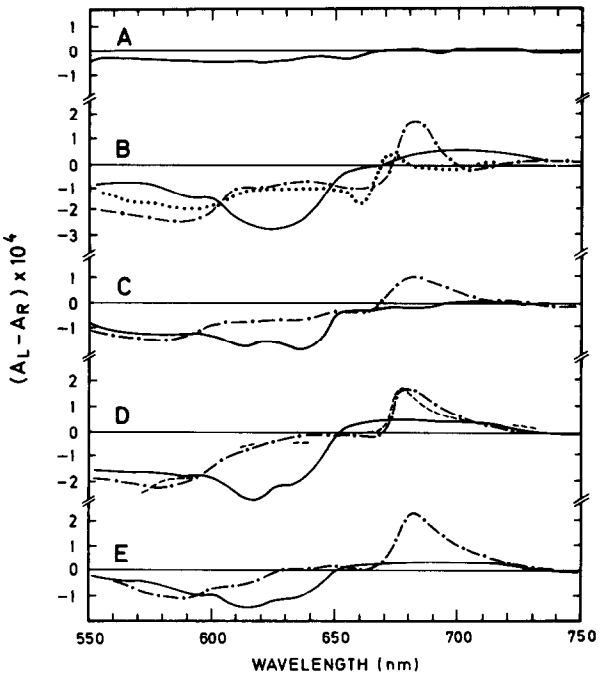

Fig. 2. CD spectra of saponin-containing Pchl-H (——), newly formed Chl-H, (– · –), and Chl-H rescanned after 2 hr in darkness at 20°C, (· · · · ·). A. 0.5% (w/v) saponin, 0.1 M tricinate-Na, pH 8.5, buffer blank; 10 cm light path. B. C, D and E: Preparations 1, 2, 3 and 4 (table 1) respectively, were scanned with 2, 10, 10 and 5 cm light paths, respectively. Spectral band widths at 700, 680 and 620 nm were 15, 9 and 5 nm, respectively, except for the Chl-H spectrum shown in B and the Chl-H designated (- - - - -) in D, where the respective band widths were one-third of those above.

Alternative explanations for spectral changes of Chl in newly-formed Chl-H with time have been offered: i) A conformational change in the protein portion of Chl-H causes the shift of the Chl absorption maximum from 678–672 nm in bean Chl-H [13]. For Chl in saponin-containing Chl-H from barley, the exponential decay of the negative CD signal at 590 nm, as well as the correlated absorption changes at 580, 672 and 678 nm have also been attributed to a conformational change in Chl-H protein

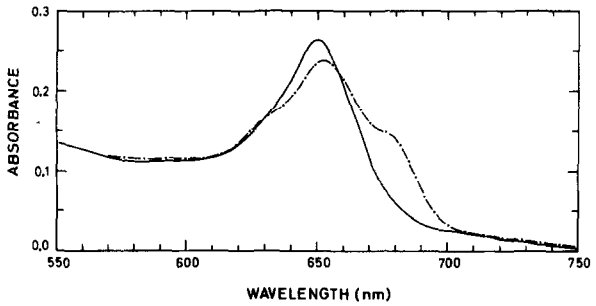

Fig. 3. Absorption spectra of saponin-containing Pchl-H from ALA-fed barley leaves before (——) and after (– · –) illumination; preparation no. 5, table 1.

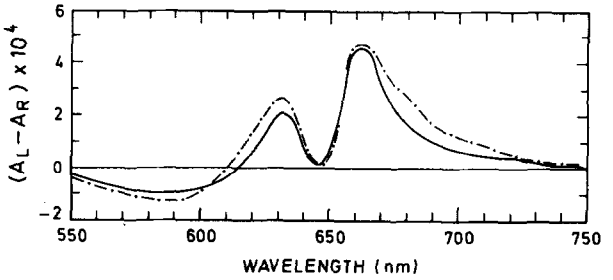

Fig. 4. CD spectra of saponin-containing Pchl-H from ALA-fed barley leaves before (——) and after (– · –) illumination; preparation no. 5, table 1; 10 cm light path; spectral band widths as described for the general case in fig. 2.

[10]; ii) dissociation of aggregated Chl molecules to monomers in bean Chl-H has been taken as the cause of the loss of splitting of the Chl CD band in the red region and a blue shift in the absorption maximum of Chl [9]. Therefore, it was of interest in the present study to look for CD changes with time in saponin-containing Chl-H in the red region. Accordingly, in one case, Chl-H after an initial scan at 11°C was kept in darkness for 2 hr at 20°C before a rescan at 11°C. During the dark interval the 585 nm CD signal decreased and the optical activity in the red region of the spectrum changed markedly (fig. 2, B) in correlation with a shift of the absorption maximum from 678–671 nm. The positive 682 nm CD band was lost and replaced by a positive component at 672 nm plus a negative component at 660 nm. We attribute both components to Qy transitions of Chl molecules and consider it possible that aggregation among particles of Chl-H could contribute to the observed CD changes in the red region. It seems unlikely, however, that Chl molecules which are bonded to separate protein units would en masse couple sufficiently closely and specifically to give rise to detectable exciton interaction. Where exciton interaction among newly-formed Chl molecules in Chl-H has been postulated [8,9], CD components in the red region were negative and positive, respectively, proceeding from red toward blue wavelengths, i.e. opposite from the relation here, and tended to disappear with time. One could think that in our samples Chl was released from the holochrome during the dark interval and subsequently aggregated in the saponin-buffer environment. However, when Chl in acetone was diluted 100-fold into saponin–tricine buffer, entirely different spectral characteristics were observed. The absorption maximum was at 671 nm with a pronounced shoulder at 695 nm, presumably due to aggregates of Chl molecules, since the CD spectrum showed a positive component at 715 nm and a negative component at 670 nm. We infer that the band-splitting found for purified Chl in saponin–tricine buffer is due to Chl aggregates.

While we found no evidence for exciton interaction among Pchl or newly-formed Chl molecules in the Pchl-H and Chl-H considered thus far, strikingly different spectra were obtained with the saponin-containing Pchl-H extracted from ALA-fed leaves of barley (figs. 3 and 4). The absorption maximum of the Pchl-H, with about 90% of the Pchl not photoconvertible to Chl, lies near 650 nm, and after maximal photoconversion, the absorption maximum of the remaining Pchl occurs at 652 nm (fig. 3). Thus, in the red region, the absorption spectrum of Pchl-H from ALA-fed leaves resembles the spectrum of Pchl aggregates in benzene [14]. Chl in the preparation is evidenced by a shoulder near 680 nm. The CD spectrum of the Pchl-H (fig. 4) shows in the red region strong positive maxima at 662 and 632 nm. Neither maximum is lost after maximal photoconversion, showing that they are due to inactive Pchl. The *apparent* increase in the positive signal in the 610–640 nm region after maximal photoconversion is attributed to a *loss* of the negative CD of photoactive Pchl which was converted to Chl. We postulate that the 632 nm band represents the Qy transition of monomeric Pchl; the band now has the same sign as CD of the Qy transition of Pchl monomers in ether [12], but, as in the case of photoactive Pchl, is shifted 10–15 nm to the red.

It is tempting to speculate that the inactive Pchl monomers in Pchl-H from ALA-fed plants, are associated more strongly with membrane lipids than normally, since only in this case is the sign of the CD of the Qy transition the same as for Pchl in ether. On the other hand, the strong negative CD band of the Qx transition of Pchl in ether [12] is very weak or lacking here.

In further analysis of the CD behavior of inactive Pchl in Pchl-H from ALA-fed leaves, we infer that the positive 662 nm component stems from aggregated Pchl molecules. Since this CD maximum is so far to the red of the Pchl absorption maximum (about 10 nm), and a relative minimum in CD in the red region occurs near the absorption maximum, there is a strong suggestion of exciton interaction. The implication then is that a negative CD component representing aggregated Pchl molecules is swallowed by the positive CD band in the 610–640 nm region attributed to Pchl monomers. It is noteworthy that the absorption maximum of the inactive Pchl in vivo in ALA-fed barley leaves occurs at 633 nm. Since, as discussed earlier with reference to Chl-H, we consider it unlikely that aggregation of holochrome particles in solution would form groups of pigment molecules with exciton interaction, the 633 nm-absorbing species of Pchl in vivo may be aggregated Pchl. It is not likely that the 652 nm-absorbing Pchl in vitro became detached from protein and aggregated subsequently, because this Pchl was precipitable with ammonium sulfate.

We return now to the results obtained with saponin-containing Pchl-H and Chl-H from bean and barley leaves that were not fed ALA before extraction. Our failure to detect exciton interaction in the preparations from barley fits well with the prior indications [6, 7] of a single photoactive Pchl molecule per Pchl-H particle. While the Pchl-H subunits from barley have an apparent mol. wt. of 63 000, as determined by Sephadex chromatography, the particle size of bean Pchl-H extracted with 1.2% saponin [6] or 3.6% saponin (K.W. Henningsen, S.W. Thorne and N.K. Boardman, unpublished observations) is substantially larger. Furthermore, whereas the progress of the photoconversion of Pchl to Chl in barley Pchl-H follows first order kinetics, as would be expected for a single photoactive Pchl molecule per subunit [7], the photoconversion departs from first order kinetics in bean Pchl-H extracted with 3.6% saponin (K.W. Henningsen, S.W. Thorne and N.K. Boardman, unpublished observations). Thus it would not have been surprising to find evidence of exciton interaction in saponin-containing Pchl-H from bean leaves. Our failure to detect exciton interaction among pigment molecules in saponin-containing Pchl-H and Chl-H from bean leaves, and also, for that matter, from barley leaves (except from ALA-fed leaves), does not per se rule out the occurrence of more than one pigment molecule per active unit in solution. Multiple chromophores could be oriented or spaced in a manner that does not permit exciton interaction, or exciton interaction among them could give rise to a non-conservative CD spectrum.

Acknowledgements

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