Chiroptical properties of chlorophyll–protein complexes separated on Deriphat/polyacrylamide gel

R. P. F. GREGORY

Department of Biochemistry, University of Manchester, Manchester M13 9PL, U.K.

and G. BORBÉLY, S. DEMETER and Á. FALUDI-DÁNIEL

Institute of Plant Physiology, Biological Research Center, Hungarian Academy of Sciences, Szeged 6701, Hungary

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Circular dichroism (c.d.) was measured for four chlorophyll–protein complexes, resolved from sodium dodecyl sulphate extracts of chloroplasts by electrophoresis in polyacrylamide gel containing Deriphat 160 (disodium *N*-dodecyl β -imidopropionate), a zwitterionic detergent. The slowest-band (1) complex was found to be identical with the complex CP1 as found on electrophoresis in the presence of anionic detergent, but it was in a much higher yield (30% of the chlorophyll *a*). In band-2 and -3 protein complexes a c.d. pattern described for the complex CP2 could be recognized. Another c.d. component of a split-exciton type with extrema at 680 (–) and 669 (+) nm, together with evidence of disorganized chlorophyll, was found in band-2, -3 and -4 complexes. When a barley (*Hordeum vulgare*) mutant lacking chlorophyll *b* was examined, only bands 1 and 4 were obtained, and the c.d. of the band-4 complex was much less affected by disorganized chlorophyll. C.d. spectra resembling that of this band-4 complex could be generated by subtracting the c.d. of complex CP1 from the c.d. of photochemically active mutant chloroplast fragments, or by subtracting the c.d. of complexes CP1 and CP2 from pea (*Pisum sativum*) chloroplast fragments. The Deriphat appears to have preserved at least to some extent a new type of chlorophyll *a*–protein complex.

C.d. (circular-dichroism) measurements have been used to characterize various pigment–protein complexes with the ultimate goal of elucidating the molecular architecture of thylakoid membranes (see Gregory, 1977). Chloroplast membranes fragmented by detergent treatment or ultrasonication show a three-banded c.d. spectrum indicating interactions between pigment molecules, and between pigment and its protein environment (Gregory *et al.*, 1972; Demeter *et al.*, 1976). From such fragments, gel filtration and electrophoresis resolve two or three major chlorophyll–protein complexes that originate from the photosystems and from the light-harvesting chlorophyll *a/b* protein (Dietrich & Thornber, 1971; Machold *et al.*, 1977; Siefermann-Harms & Ninnemann, 1979). Solubilized pigments referred to as ‘free chlorophylls’ appear in a separate band. In the presence of dodecyl sulphate or dodecyl benzene-sulphonate, polyacrylamide-gel electrophoresis yielded complexes named CP1 and CP2 (Thornber *et al.*, 1967). In complex CP1 (M_r 100000), chlorophyll *a* molecules exhibit a split-exciton c.d. signal

with a minimum at 689 nm and a maximum at 672 nm (Koenig *et al.*, 1972; Scott & Gregory, 1975). In complex CP2 (M_r 35000) the c.d. spectrum shows chlorophyll *a* (positive maximum at 671 nm) and chlorophyll *b* (negative maximum at 650 nm), as found by Scott & Gregory (1975).

The negative maximum in the c.d. spectrum of complex CP1 can be recognized as a shoulder in the c.d. of lamellar fragments, and the negative maximum of complex CP2 fits very well with the c.d. of the fragments at wavelengths below 660 nm. This is taken to indicate that the complexes CP1 and CP2 pre-exist in the membrane fragments. There is, however, other circularly dichroic material present in the fragments and detergent extracts (negative at 680 nm) that is not preserved during electrophoresis in sodium dodecyl sulphate systems.

There are two processes that destroy the characteristic c.d. spectra of the complexes. The first is a disorganization of the chlorophyll, presumably disrupting chlorophyll–chlorophyll interactions. This occurs with aging, or with heat treatment of the

complexes, and eventually leads to a c.d. spectrum very similar in kind and magnitude to dilute solutions of the corresponding pigments in aqueous acetone. The second effect appears the same by c.d., but on electrophoresis the pigment is seen to be detached from the protein.

The amount of 'free pigments' depends on the method of solubilization and electrophoresis, and can be considerably decreased by mild procedures (Markwell *et al.*, 1978; Délépelaire & Chua, 1979; Siefermann-Harms & Ninnemann, 1979). One of the recent developments was the use of a zwitterionic detergent, Deriphat 160 (disodium *N*-dodecyl β -imidopropionate), in the gel and electrophoresis buffer (Markwell *et al.*, 1979). Although the solubilization of thylakoids was performed by a conventional procedure using sodium dodecyl sulphate, the amount of 'free chlorophyll' observed during fractionation was negligible. All the chlorophyll was found in pigment-protein complexes, and four green bands were resolved. The compounds in these bands were studied in detail, but it was suggested that they may be representative of structural and functional units of the photosynthetic apparatus *in vivo* (Markwell *et al.*, 1979). (It is possible, however, that spurious bands could arise by pigment exchange between proteins.) The present paper describes the c.d. signals of these chlorophyll-protein complexes obtained from normal and chlorophyll *b*-less chloroplasts, and assesses the extent to which the complexes assist our understanding of the intact thylakoid.

Materials and methods

Chloroplasts were isolated from maize (*Zea mays* L., var. KSC 360), tobacco (*Nicotiana tabacum* L., var. Samsun) and barley (*Hordeum vulgare* L., var. Donaria) leaves and from a barley mutant lacking chlorophyll *b* (*chlorina* 3613 of Gatersleben). The plants were grown in a greenhouse. Washed thylakoid membranes were prepared and solubilized in 1% sodium dodecyl sulphate by the method of Markwell *et al.* (1979). Their procedure was used when disc and slab gels and electrophoresis buffer containing Deriphat 160 (General Mills, Kankakee, IL, U.S.A.) were prepared. The electrophoresis was performed at 15°C at 100 V for 45–60 min. After the run the gels were sliced perpendicularly to the electrophoretic movement, and the chlorophyll-containing bands were used for spectrophotometric and c.d. measurements.

Absorption spectra of the green bands in the gel were recorded in a Unicam SP.1800 spectrophotometer fitted with a special sample holder. The gel pieces had smooth parallel surfaces, and scattering and reflection effects were small and were compensated for by using a piece of similar but

unloaded gel in the reference beam and by placing the sample holder close (25 mm) to the photomultiplier. C.d. spectra were obtained in a spectropolarimeter constructed as described by Breeze & Ke (1972). The 50 kHz-modulated component of the photomultiplier signal was selected by an ORTEC-Brookdeal 9501 lock-in amplifier and the c.d. signal was plotted on an *x-y* recorder. A Bausch and Lomb high-intensity monochromator was used with a slit width of 0.2 mm (spectral band width 1.2 nm), and the scanning speeds were 0.2 or 0.5 mm/s. The time constants of the lock-in amplifier were: input, 0.01 s; output, 1 s. It was established that unloaded gel had no c.d. properties in the visible range, and that the c.d. of the bands resolved by electrophoresis (in sodium dodecyl sulphate/polyacrylamide-gel systems) were the same before and after extraction from the gel. Care was taken to ensure that the beam traversed the same region as in the spectrophotometer. Since absorption coefficients are uncertain for the pigment-protein complexes, the c.d. is reported as the observed signal in millidegrees ellipticity.

For extraction of chlorophyll-protein complexes, the green bands were cut from the gel and homogenized in 50 mM-Tris/HCl buffer, pH 7.8. The resulting solutions were separated from the gel material by centrifugation. Chlorophyll was determined spectrophotometrically in samples after extraction in 80% (v/v) acetone and transfer into diethyl ether (French, 1960).

Results and discussion

Electrophoresis of thylakoid extracts prepared from maize, tobacco and normal barley chloroplasts resulted in four green bands of chlorophyll-protein complexes, a resolution pattern similar to that previously described by Markwell *et al.* (1979). 'Free chlorophylls' were hardly detectable unless thylakoid extracts had been heat-denatured before electrophoresis. The complex of lowest mobility (numbered here '1' and corresponding to 'N' of Markwell *et al.*, 1979) was devoid of chlorophyll *b*, in contrast with the bands 2 and 3 with high chlorophyll *b* content (Table 1).

When an extract from the barley mutant lacking chlorophyll *b* was subjected to electrophoresis in the Deriphat system, bands corresponding to bands 1 and 4 ('N' and 'Q') were clearly resolved, but bands 2 and 3 ('O' and 'P') were not seen. At the same time a greater quantity of free pigment was observed.

C.d. spectra of the complexes separated from normal thylakoids are shown in Fig. 1.

Band-1 protein complex has a spectrum closely similar to that of complex CP1 reported by Scott & Gregory (1975). The c.d. spectrum measured on the gel is a split-exciton signal; after extraction some

Fig. 1. C.d. spectra of the chlorophyll–protein complexes resolved by Deriphat 160/polyacrylamide-gel electrophoresis

C.d. on the gel (—), or after elution (---) in 50 mM-Tris/HCl, pH 7.8. The thylakoid extract was prepared from maize chloroplasts. C.d. is shown as ellipticity. All curves are normalized to absorbance 1.0 at the main red maximum (672–674 nm).

Table 1. Chlorophyll distribution of chlorophyll–protein complexes separated from maize thylakoids by Deriphat-160/polyacrylamide-gel electrophoresis

Band*	Relative chlorophyll content (%)	Chlorophyll a/b (molecules/molecules)
1	27	6.9
2	16	2.2
3	20	2.6
4	27	2.9
5	2	3.2
Sum	92	3.3
Thylakoid extract	100	3.2

* Numbers in order of increasing mobility; band 5: chlorophyll content extracted from that region of the gel where 'free chlorophyll' migrates.

somewhat after extraction (see Figs. 1–3). This degradation did not occur with anionic detergents such as dodecyl sulphate in the electrophoretic system (Gregory *et al.*, 1972), and suggests that bands 1–3 in the Deriphat system are labile in their chlorophyll-related structure after extraction.

Bands 2 and 3 probably represent light-harvesting chlorophyll–protein complexes, as judged from their chlorophyll *b* content. These are the complexes that are missing from the chlorophyll *b*-less thylakoids and can probably be related to the 25 000-*M_r* polypeptide shown to be absent in the mutant barley (Machold & Meister, 1979; Burke *et al.*, 1979). An important characteristic of the band-2 component resolved by Deriphat/polyacrylamide-gel electrophoresis is its relatively high chlorophyll *a* content. In this complex the ratio chlorophyll *a*/chlorophyll *b* molecules is about 2:1, in contrast with 1:1 for the corresponding band component on sodium dodecyl sulphate/polyacrylamide gel (Kan & Thornber, 1976). The c.d. spectrum of these band components shows CP2 characteristics similar to those obtained on sodium dodecyl benzenesulphonate/polyacrylamide-gel electrophoresis (negative band at 650 nm, positive at 671 nm) as reported by Scott & Gregory (1975). In addition, this complex exhibits a negative c.d. at 680 nm that can also be recognized in the spectra of faster-moving complexes. This signal is probably derived from a chlorophyll *a* split-exciton c.d. shown in its pure form by the band-4 protein complex of the chlorophyll *b*-less mutant.

The band-4 protein complex in the normal plant contains chlorophylls *a* and *b* (approx. 3:1) and has negative c.d. peaks at 680 nm and 660 nm. In the case of the mutant possessing no chlorophyll *b*, the band-4 complex shows only the 680 nm peak, in a simple split-exciton form (Fig. 2). This split-exciton signal, exhibited here free from other signals, belongs, we suggest, to a new kind of chlorophyll *a* centre. This centre is presumably also present in

'free' or 'loosely bound' chlorophyll *a* can also be detected. The magnitude of the c.d. is much larger on the gel than in the eluate and continues changing

Fig. 2. C.d. spectra of a chlorophyll-protein complex resolved from thylakoids lacking chlorophyll *b*

Spectra measured on the gel, for the faster-moving complex (position corresponding to band 4 of normal chloroplasts), before (—) and after (---) elution in 50 mM-Tris/HCl, pH 7.8. C.d. of band 1 was identical with the corresponding c.d. of normal thylakoids.

band-2-4 complexes of the normal chloroplasts, and is responsible for their 680 nm (–ve) c.d. signals. Furthermore, if this centre belongs to a specific chlorophyll-protein complex, then in the presence of Deriphat this new complex combines with other complexes containing chlorophylls *a* and *b*, forming the supercomplexes in bands 2-4 and accounting both for the resistance to destruction during electrophoresis, and the higher-than-expected M_r reported by Markwell *et al.* (1979). In the mutant band-4 complex the 680 nm chlorophyll *a* complex may be combined with CP2 apoprotein, or it may be self-aggregated.

Scott & Gregory (1975) reported that the c.d. of fresh detergent extracts of chloroplasts contained a 680 nm component that could not be accounted for by the presence of complexes CP1 and CP2. We have calculated the c.d. spectrum that is required to meet the discrepancy by subtracting the sum of complex-CP1 and -CP2 c.d. signals from that of a fresh detergent extract from normal chloroplasts, and also by subtracting the c.d. of complex CP1 from the c.d. of an extract of mutant chloroplasts. In this calculation it was assumed that the fragments contained 20% of complex CP1 and 50% of complex CP2 on a chlorophyll basis. Small adjustments were made until the resulting difference spectrum no longer showed the 690 nm c.d. shoulder of complex CP1, nor the 650 nm negative peak of complex CP2, on a trial-and-error basis. The proportion of the CP1

and CP2 components remained close to the original estimates, and the magnitude and position of the negative peak at 680 nm were not sensitive to these manipulations, since both complexes CP1 and CP2 have very little c.d. at this wavelength. The two spectra obtained, shown in Fig. 3, are very similar in form and position (669 nm, 680 nm) to the c.d. of the band-4 complex of the mutant (Fig. 2).

We conclude that chloroplasts contain a proportion of their chlorophyll *a* organized in a form that generates a c.d. signal at 669–680 nm, and that it is likely that it is a new and specific chlorophyll *a*-protein complex. Deriphat electrophoresis results in supercomplexes with chlorophyll (*a* + *b*)-protein complexes. The function of the new chlorophyll form has yet to be established.

The organization of the chlorophyll-protein complex is not very stable, since under harsher conditions (such as sodium dodecyl sulphate/polyacrylamide-gel electrophoresis), most of the chlorophyll *a* molecules responsible for the 669–680 nm signal become detached. Thus this signal has not been observed previously in isolated complexes (Scott & Gregory, 1975). Decomposition of the complexes contained in bands 2 and 3 occurred also

Fig. 3. To show that a c.d. component, positive at 668 nm and negative at 680 nm is required to account for the c.d. of chloroplast fragments

The c.d. spectrum for CP1, from peas, was subtracted graphically from the c.d. spectrum of chloroplast fragments of the barley mutant lacking chlorophyll *b* (—). C.d. spectra of CP1 and CP2 were subtracted from the c.d. spectrum of pea chloroplast fragments (---). The ratio CP1/CP2 (in terms of chlorophyll *a* content) was approx. 3, adjusted to give the minimum c.d. at 650 and 690 nm. (The 680 nm c.d. is little affected by such adjustments.)

Fig. 4. Comparison between (i) the c.d. of an extract of thylakoids (—) and (ii) the algebraic sum of c.d. measured on the gel for the bands containing chlorophyll-protein complexes resolved from (i) by Deriphat 160/polyacrylamide-gel electrophoresis (-----)

The summation was weighted in terms of the abundance of the four bands in terms of chlorophyll content.

during their elution from the gel, in contrast with band 4, which did not change any further (Fig. 2, broken line). It may be noted that in Fig. 1, band-2 and -3 complexes have a c.d. from 650 to 700 nm that is predominantly negative. This would be consistent with disorganized chlorophyll, still attached to the protein, resulting from the above-mentioned decomposition.

The effect of the electrophoresis *per se* was checked by comparing the c.d. of the thylakoid extract with the sum of the c.d. measured in the complexes (Fig. 4), weighting each in proportion to its abundance (Table 1). It is expected that in a dilute solution the c.d. of the extract should be the sum of the c.d. of its component chlorophyll complexes. It is, however, clear that there is a loss of c.d.-active chlorophyll in the 650–670 nm region. From this we conclude that, during Deriphat/polyacrylamide-gel electrophoresis, organization of complex CP2 is altered to some extent. The reasonable fit at the long wavelengths, the high yield and optical activity of complex CP1, as well as the 669–680 nm c.d. in the other complexes, suggest that the organization of chlorophyll *a* is better preserved by Deriphat/

polyacrylamide-gel electrophoresis than in the other systems previously described.

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