Biochimica et Biophysica Acta, 347 (1974) 22–30

BBA 46721

THE RELATIONSHIP BETWEEN CHLOROPHYLL *b* AND PIGMENT–PROTEIN COMPLEX II

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(Received October 8th, 1973)

SUMMARY

1. Sodium dodecyl benzene sulphonate (SDBS) extracts have been prepared from higher plant and *Euglena* chloroplasts having varying chlorophyll *a* : chlorophyll *b* ratios. Chloroplasts from a mutant of barley lacking chlorophyll *b* have also been examined.

2. Discrete chlorophyll–protein complexes were separated by polyacrylamide gel electrophoresis of the extracts. The proportion of chlorophyll associated with pigment–protein complex I was similar whatever the chlorophyll *a* : chlorophyll *b* ratio of the starting material. The proportion of the total chlorophyll bound to pigment–protein complex II varied between 0 and 45%.

3. Loss of chlorophyll from pigment–protein complex II resulted in the formation of the apoprotein which, upon electrophoresis, migrated as a band of lower molecular weight than the pigment–protein complex II.

4. The presence and amount of pigment–protein complex II and of its apoprotein correlated with the chlorophyll *b* content of the starting material.

INTRODUCTION

Washed chloroplast lamellae can be solubilised in the detergents sodium dodecylsulphate or sodium dodecyl benzene sulphonate (SDBS). On polyacrylamide gel electrophoresis, such lamellar extracts can be resolved into three principal chlorophyll-containing bands [1, 2]. These bands (in increasing order of molecular weight) are a free chlorophyll–detergent complex, pigment–protein complex II (chlorophyll *a* : chlorophyll *b*, 1 : 1; molecular weight 35 000) and pigment–protein complex I (chlorophyll *a* : chlorophyll *b*, 9 : 1; molecular weight 100 000) [3].

The appearance of these complexes during greening of leaves [4] has been used as a criterion of development of electron transport driven by Photosystem I and II. Pigment–protein complex II appearance coincided with that of oxygen evolution at 2 h and pigment–protein complex I with light driven P700 oxidation after about

Abbreviations: SDBS, sodium dodecyl benzene sulphonate. DCIP, 2,6-dichlorophenolindophenol. DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

6 h illumination. It is probable, however, that the correlation of pigment–protein complex appearance with certain physiological activities of Photosystems I and II is fortuitous. Chloroplasts extracted from bean leaves exposed to alternate light and dark cycles have chlorophyll *a* : chlorophyll *b* ratios greater than 20 and lack pigment–protein complex II [5, 6]. They have, however, well developed Photosystem II activity, as measured by cytochrome *f* photoreduction and a 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) sensitive, photoreduction of 2,6-dichlorophenolindophenol (DCIP) [6].

Shortening of the dark time between light periods during greening results in the appearance of pigment–protein complex II, but the activity of Photosystem II per chlorophyll molecule remains the same or decreases [6]. Decreased dark time [7] produces not only pigment–protein complex II, but also chlorophyll *b*. We have suggested [6] that the amount of pigment–protein complex II is a reflection of the necessity of chlorophyll *b* for stabilisation of both chlorophylls onto their carrier protein rather than the level of photosystem II activity. This hypothesis has been tested by examining mature photosynthetic systems with normal or above normal Photosystem II activities and above normal chlorophyll *a* : chlorophyll *b* ratios (chl *a* : chl *b* $>$ 3 : 1).

MATERIALS AND METHODS

Euglena was grown autotrophically as described by Scott et al. [8]. Pea and barley plants were grown in the field or in vermiculite at 20 °C under fluorescent lights in a growth chamber. The light intensity at the leaf surface was 0.8 $mW \cdot cm^{-2} \cdot s^{-1}$. A barley mutant [9] lacking chlorophyll *b* and a pea mutant [10, 11] having a variable chlorophyll *a* : chlorophyl *b* ratio were compared with their corresponding wild types grown under identical conditions.

Chloroplasts were prepared from *Euglena* by breaking the cells in a French press and isolating the plastids on a sucrose density gradient [12]. Barley and pea chloroplasts were isolated by blending the leaves in a medium containing 0.3 M sucrose, 0.01 M KCl and 0.05 M phosphate buffer, pH 7.2 (1 g leaves: 10 ml buffer), filtering through two layers of "miracloth" and centrifuging at $2500 \times g$ for 10 min. After washing the pellet once in the above medium the chloroplasts were lysed in a medium containing 0.01 M KCl and 0.05 M phosphate buffer, pH 7.2, and then centrifuged at $15\,000 \times g$ for 15 min. The pellet was washed once by resuspending and centrifuging as described above. SDBS extracts were prepared by homogenising the washed chloroplast membranes in a glass homogeniser fitted with a teflon piston in 0.05 M sodium borate, pH 8.5, containing 0.5% (w/v) SDBS. (SDBS: chlorophyll was not less than 15 : 1, w/w.) The SDBS extract was freed from insoluble material by centrifuging at $25\,000 \times g$ for 20 min. The insoluble residue contained less than 2% of the initial chlorophyll.

Polyacrylamide gel electrophoresis of the extracts was essentially as described by Thornber [13]. The running buffer was 0.05 M sodium borate, pH 8.5, containing 0.15% (w/v) SDBS. To separate the bands for estimation of chlorophyll content the gels were pre-run at 6 mA/gel for 10 min and then after sample application for 15 min at the same current.

For separation and examination of protein bands the pre-run time was 30

min followed by a sample run time of not less than 40 min at 5 mA/gel. To localise protein-containing bands the gels were washed in methanol–glycerol–acetic acid–water (16 : 1 : 2 : 21, by vol.), stained overnight in 0.1% Coomassie blue D and destained in the above mixture [14]. The stained gels were scanned at 608 nm using a Gilford gel scanner.

To estimate the relative proportions of the various chlorophyll-containing bands the gels were scanned at 670 and 650 nm. A planimeter was used to measure the areas under the recorder peaks. The area of each peak was expressed as a percentage of the total area of chlorophyll-containing peaks. The percentage obtained at 670 and 650 nm for each peak was then averaged. Measurement at two wavelengths is necessary because of differing chlorophyll *a* : chlorophyll *b* ratio of the pigment–protein complexes and of the free pigment band. Measurement at e.g. 650 nm alone would bias the result in favour of pigment–protein complex II (chlorophyll *a* : chlorophyll *b*, 1 : 1) and against that of pigment–protein complex I (chlorophyll *a* : chlorophyll *b* > 9 : 1). The wavelengths 670 and 650 nm are the absorbance maxima of chlorophyll *a* and chlorophyll *b*, respectively, in purified [3] pigment–protein complex II. Chlorophyll was determined according to the method of Arnon [15].

RESULTS

The distribution of chlorophyll between the three pigmented bands resolvable by acrylamide gel electrophoresis of SDBS extracts of chlorophyll lamellae is shown in Table I. Pigment–protein complex I contains 10–20% of the total chlorophyll in

TABLE I

DISTRIBUTION OF CHLOROPHYLL BETWEEN THE THREE PIGMENTED BANDS, AFTER SEPARATION BY POLYACRYLAMIDE GEL ELECTROPHORESIS, OF SDBS CHLOROPLAST EXTRACTS

Pigment–protein II includes an unstable minor band (a dimer) which breaks down to pigment–protein II (Hiller, R. G., unpublished). A band containing approx. 5% of the chlorophyll but coinciding with neither pigment–protein I nor pigment–protein II has been included with the *Euglena* free chlorophyll value. A similar minor band was also present in both maize extracts (3.4% mesophyll, 2.8% bundle sheath). At saturating light intensities, the Photosystem II activities (in μmole DCIP reduced · mg chlorophyll^{-1} · min^{-1}) of chloroplasts from both barley and pea mutants exceeded those of their respective wild types (cf. refs 10 and 11).

Organism	Chlorophyll *a*/ chlorophyll *b*	Pigment–protein I (%)	Pigment–protein II (%)	Free chlorophyll (%)
Barley mutant	*	17.5	None	82.5
Barley wild type	2.8	11.0	42.3	46.7
Pea mutant	5.2	12.0	13.2	74.8
Pea wild type	3.1	16.3	37.4	46.3
Euglena	9.3	14.2	4.3	71.2
Maize mesophyll	2.9	14.1	34.5	52.4
Maize bundle sheath	5.5	49.6	5.4	45.0

* Chlorophyll *b* absent.

TABLE II

THE INFLUENCE OF VARYING LIGHT–DARK REGIMES ON THE PROPORTION OF CHLOROPHYLL BOUND TO PIGMENT–PROTEIN COMPLEX II

Etiolated leaves of bean and barley were exposed for 24 h to the light regimes shown.

Light regime	Chlorophyll *a*/ chlorophyll *b*		Pigment–protein complex II (as percentage of total chlorophyll)	
	Bean	Barley	Bean	Barley
Continuous	2.8	3.1	34.0	37.0
2 min light/18 min dark	5.1	5.8	22.8	17.7
2 min light/118 min dark	>10	8.0	0	7.6

all cases except for that of maize bundle sheath. In contrast, the barley mutant lacking chlorophyll *b* has no chlorophyll in pigment –protein complex II, compared with 42.3% in its wild type. It may further be noted that both the pea mutant and *Euglena* have reduced amounts of pigment–protein complex II and reduced amounts of chlorophyll *b*. Since *Euglena*, the pea mutant [10], and the barley mutant [11] lacking chlorophyll *b*, all have normal or above normal Photosystem II activity on a chlorophyll basis, we conclude that the amount of pigment–protein complex II correlates with chlorophyll *b* and not with the activity of Photosystem II.

This correlation is further supported by the data given in Table II. Etiolated leaves of both bean and barley were exposed to varying light–dark cycles. The proportion of the chlorophyll bound to pigment–protein complex II is decreased as the ratio of chlorophyll *a* : chlorophyll *b* increases. Comparison of the data in Tables I and II suggests, however, that chlorophyll *b* content may not be the sole factor in determining this proportion. Both flashed bean and barley leaves having a chlorophyll *a* : chlorophyll *b* ratio of between 5 and 6, have a much higher proportion of chlorophyll in pigment–protein complex II than has the pea mutant or maize bundle sheath although the chlorophyll *a* : chlorophyll *b* ratio is similar. In mature leaves (pooled data for pea, bean, barley and spinach, not less than two determinations for each) the proportion of chlorophyll in pigment–protein complex I was found to be $15.8 \pm 2.3\%$ and in pigment–protein complex II, $41.0 \pm 3.5\%$. Chloroplasts from the mutants of pea and barley and from bean leaves exposed to 24 cycles of 2 min light and 118 min dark had $18.8 \pm 3.5\%$ of the total chlorophyll in pigment–protein complex I. The difference between these values for the chlorophyll content of pigment–protein complex I is not significant, as shown by a *t* test.

In maize, there are two types of chloroplasts; the mesophyll chloroplasts, which have a chlorophyll *a* : chlorophyll *b* ratio close to 3 : 1 and the bundle sheath chloroplasts in which the chlorophyll *a* : chlorophyll *b* ratio is approximately 5 : 1. SDBS extracts of mesophyll chloroplasts show a distribution of chlorophyll between the three bands similar to that of the wild type barley and pea (Table I). Unlike the other situations investigated above, the bundle sheath chloroplasts are either deficient in Photosystem II, or Photosystem II is much less efficient when compared with that in the mesophyll. Bundle sheath chloroplasts have only a small proportion of chlorophyll in pigment–protein complex II, but have more than three times as much in pigment–protein complex I compared with that in the mesophyll.

In all plants investigated, a reduced proportion of pigment–protein complex II was associated with an increase in the proportion of free chlorophyll on the gel. This suggested that pigment–protein complex II might be initially present but was breaking down on electrophoresis at 25 °C. A comparison was made between extracts of the barley mutant run at 4 °C with those run at 25 °C. The results are shown in Fig. 1. At 4 °C, a band between pigment–protein complex I and the free pigment P is clearly visible, but this is scarcely visible at 25 °C. This unstable band is not coincident with pigment–protein complex II. However, a similar but more stable band has been observed in maize and *Euglena* (Table I).

If the role of chlorophyll *b* is to stabilise the binding of itself and chlorophyll *a* to an existing protein as we suggested previously [6], the polyacrylamide gels after staining for protein will look the same in all cases. The results of Fig. 2 clearly that this is not the case. There is probably no protein at all in the pigment–protein complex II position in the barley mutant and a reduced amount of protein in this position in the pea mutant. Similar results (not shown here) to those in the pea mutant were also obtained for *Euglena*.

There is apparently no consistent increase in any band to correlate with the loss of protein from the position of pigment–protein complex II. However, it is possible that in the absence of chlorophyll *b*, pigment–protein complex II loses chlorophyll on extraction and that on electrophoresis the protein runs different position on the gel.

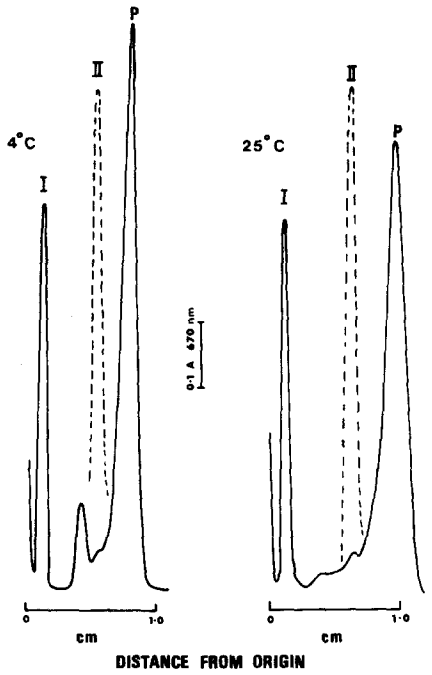

Fig. 1. Densitometer tracing at 670 nm of SDBS pigment–protein complexes obtained from chloroplasts of a barley mutant lacking chlorophyll *b* and separated on polyacrylamide gels run at either 4 or 25 °C. The dashed line shows pigment–protein complex II position and shape in wild-type barley extracts run simultaneously with those of the mutant. (P = free chlorophyll–detergent complex.)

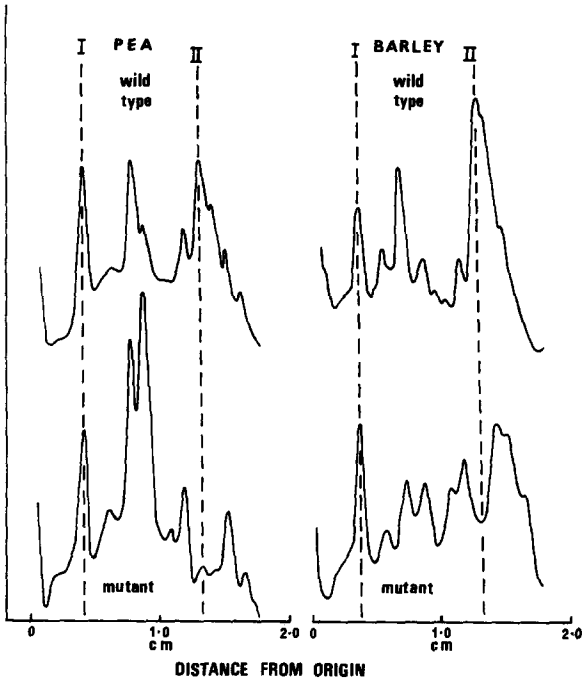

Fig. 2. Densitometer tracing at 608 nm of Coomassie blue stained SDBS chloroplast lamellar proteins of mutant and wild type pea and barley. Dashed vertical lines identify the position of pigment–protein complexes I and II.

We therefore removed the centre of the pigment–protein complex II band from the polyacrylamide gels and incubated it with 0.05 M borate, pH 8.5, containing 0.15% SDBS for 48 h at 4 and 22 °C. After this time, the extracts of pigment–protein complex II were re-run on acrylamide gels and the gels stained with Coomassie blue. The results for bean chloroplasts are shown in Fig. 3. After incubation at 22 °C the proportion of protein in pigment–protein complex II is decreased and that in the band immediately beyond it is increased. A decrease in protein and of chlorophyll in pigment–protein complex II is accompanied by an increase of pigment in the free chlorophyll band. The change in apparent molecular weight of the protein accompanying the loss of chlorophyll is approx. 3000.

DISCUSSION

The results reported in this paper support our original hypothesis [6] that the amount of pigment–protein complex II is related to the chlorophyll *b* content of the chloroplasts from which the SDBS extracts are made rather than to the activity of Photosystem II. We have further shown that when pigment–protein complex II loses chlorophyll, the resulting protein runs on polyacrylamide gels as a discrete band of lower apparent molecular weight. The latter result seems inconsistent with the previous suggestion that chlorophyll *b* permits a stable binding of itself and chlorophyll *a* to a protein which is present in both etioplasts and flashed bean leaves [6]

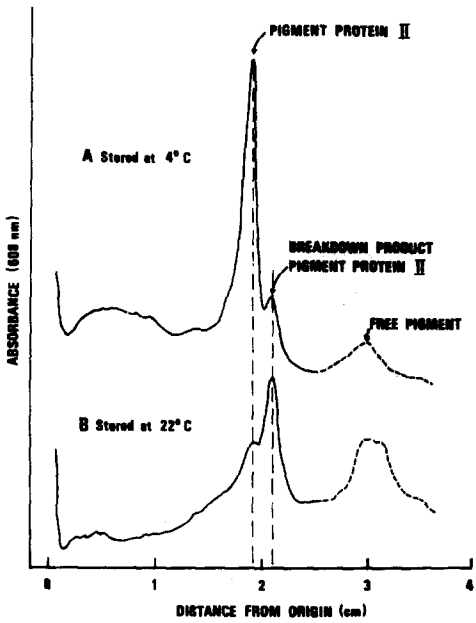

Fig. 3. Densitometer tracings at 608 nm of pigment–protein complex II from bean chloroplasts re-run after incubation for 48 h at 4 and 22 °C. The dashed line shows the position of the chlorophyll–detergent band and was obtained by re-scanning the gels at 430 nm.

and which runs in the position of pigment–protein complex II. Sodium dodecylsulphate extracts of barley etioplasts have little or no protein in the pigment–protein complex II position [16]. The protein with its associated chlorophyll appears after several hours illumination [4, 16]. A re-examination of the proteins from bean etioplasts suggests that they are very similar to those of barley. Our failure to record any marked difference in the pigment–protein II region between extracts of etioplasts and of bean leaves illuminated for 24 h may be attributed to breakdown of pigment–protein complex II whose apoprotein is then moved to a position beneath that of a major etioplast protein. We cannot exclude, however, the possibility that the apoprotein of the pigment–protein complexes are present in etioplasts and only move to their characteristic positions on the polyacrylamide gels after acquisition of chlorophyll.

Chloroplasts which are deficient in chlorophyll *b* may in vivo lack the protein of pigment–protein complex II, or alternatively, the protein may be present but moved to a different position on the polyacrylamide gels. If the latter explanation were correct, the gel patterns of both barley and pea mutants should be identical to those of their corresponding wild types except in the region of pigment–protein complex II. The mutants would be deficient in this position but have a corresponding increase in protein in a band slightly further from the origin of the gel. The results of Fig. 2 for the barley mutant lacking chlorophyll *b* and those from the pea mutant suggest a generally reduced protein content in the region of, and immediately beyond, pigment–protein complex II. In neither mutant, however, is the precise nature of the lesion known. The deficiency in both involves an altered nuclear gene but this might

be one coding for a chloroplast structural protein rather than an enzyme of chlorophyll biosynthesis. We conclude that chloroplasts relatively deficient in chlorophyll *b* are also deficient in the apoprotein of pigment–protein complex II but are uncertain as to whether this is cause or effect at the present time.

Pigment–protein complex I from chloroplasts of a wide variety of plants was found to have 12–20% of the total chlorophyll whereas that of pigment–protein complex II has 0–40% depending on the chlorophyll *b* content. This suggests that the division of light energy between the two photosystems at wavelengths where both absorb is unrelated to the relative proportion of the two pigment–protein complexes. In plants having a chlorophyll *a* : chlorophyll *b* ratio of approx. 3, about half the total chlorophyll occurs in the free pigment zone at the front of the polyacrylamide gels. In plants deficient in chlorophyll *b*, the free pigment band has 60–85% of the total chlorophyll. Kung and Thornber [3] have suggested that most of this free pigment arises by loss from pigment–protein complex II. It is equally possible that it arises by breakdown of other pigment–protein complexes as has been suggested for extracts of Antirrhinum chloroplasts [14]. In the present work we have noted the presence of several chlorophyll-containing bands, besides pigment–protein complex II especially in systems deficient in chlorophyll *b* (see legend to Table I). One such band in the barley mutant lacking chlorophyll *b* runs close to the position where pigment–protein complex II is normally located in the wild type, but slightly nearer to the origin. In extracts of chloroplasts having a normal chlorophyll *a* : chlorophyll *b* ratio of approximately 3 : 1, this unstable band, if present, may not separate from pigment–protein complex II except after a very long electrophoresis by which time it will have lost all its chlorophyll.

Pigment–protein complex II probably functions as the chlorophyll *b* containing antenna for the reaction centre of Photosystem II. When the complex is deficient or absent the efficiency but not the maximal rate of Photosystem II-dependent reactions is reduced. Pigment–protein complex II contains chlorophyll *a* and chlorophyll *b* in approximately equal amount [3]. Our results question of the nature of chlorophyll *a* binding to Photosystem II in those situations where complex II is absent or greatly reduced in amount (cf. also refs 17 and 18).

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