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RESOLUTION OF THE LIGHT-HARVESTING CHLOROPHYLL *a/b*-PROTEIN OF *VICIA FABA* CHLOROPLASTS INTO TWO DIFFERENT CHLOROPHYLL-PROTEIN COMPLEXES

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Summary

Thylakoids of *Vicia faba* chloroplasts disaggregated by sodium dodecyl sulfate were separated by means of different electrophoretic systems. Under the conditions of a high resolving gel system the chlorophyll containing zone previously termed chlorophyll-protein complex II or light-harvesting chlorophyll *a/b*-protein was found to be inhomogeneous. It represents a mixture of two distinct chlorophyll-proteins characterized by different spectral properties and different apoproteins. One chlorophyll-protein exhibits a chlorophyll *a/b* ratio of 0.9 and is associated with polypeptides of 24 000 and 23 000 daltons. The 24 000 dalton band is proved to bind chlorophyll and has a light-harvesting function. The function of the 23 000 dalton band is unknown. The second chlorophyll-protein has a chlorophyll *a/b* ratio of 2.1 and an additional absorption maximum in the position of 637 nm. It is associated with only one polypeptide which has an apparent molecular weight of 23 000. The two 23 000 dalton polypeptides occurring in both complexes are not identical.

Introduction

Since the fundamental experiments by Ogawa et al. [1] and Thornber et al. [2] concerning electrophoretic separation of detergent-disintegrated thylakoids into chlorophyll-protein complexes, numerous data have been published with

Abbreviations: CP I, CP II′ and CP III, chlorophyll-protein complexes I, II′ and III; AP II′ and AP III, apoproteins of chlorophyll-protein complexes II′ and III; SDS, sodium dodecyl sulfate.

respect to their composition [3—6]. Although there is some agreement that the zone of lowest electrophoretic mobility, termed *P*-700-chlorophyll *a*-protein or alternatively chlorophyll-protein complex I, is characterized by a single polypeptide, some investigators have resolved two or more bands [7—9]. This is also true for the pigment zone of intermediate electrophoretic mobility, referred to as light-harvesting chlorophyll *a/b*-protein or chlorophyll-protein complex II. According to previous experiments this zone was assumed to represent a homogeneous band composed of pigments bound to a single polypeptide chain [2,10]. Recent studies based on re-electrophoresis of isolated light-harvesting chlorophyll *a/b*-protein using high resolving gel systems resulted, however, in more than one polypeptide [9,11—13].

Materials and Methods

Isolation, purification and disintegration of thylakoids

Thylakoids of *Vicia faba* chloroplasts were isolated and purified as previously described [14]. The membranes were disaggregated with 30 mM borate-HCl buffer (pH 8.9) containing 2% SDS by shaking at room temperature for 20 min followed by centrifugation at 150 000 × *g* for 15 min. Concentrations corresponding to 3.5 mg chlorophyll per ml buffer were used. To disrupt disulfide bonds and to achieve a complete denaturation of the polypeptides some of the samples were incubated with 2% dithiothreitol (final concentration) for 24 h at +5°C or for 2 min at 100°C. The samples were stored at −18°C.

Gel electrophoresis

Electrophoresis was performed using three different electrophoretic systems summarized in Table I. For system I, Cyanogum 41 Gelling Agent was used (American Cyanamid Comp.) which is of a constant monomer/comonomer ratio. The ratio of acrylamide to *N,N'*-methylenebisacrylamide was 30 : 0.8 (w/w) for systems II and III. Electrophoresis was carried out either with Ortec cuvettes Type 4214 (I) or with a slab-gel apparatus modified from the design of Studier [17] (II and III). Gels were stained with Coomassie brilliant blue

TABLE I

Electrophoretic systems	I	II	III
Upper reservoir buffer	Borate-HCl (pH 8.0) 0.1% SDS	Tris-Boric acid (pH 8.64) 0.1% SDS	Borate-HCl (pH 8.0) 0.1% SDS
Stacking gel buffer	Tris-HCl (pH 8.9)	Tris-sulfate (pH 6.10)	Tris-HCl (pH 8.9)
Stacking gel	4 mm; 6% Acryl/Bis = 15:08	12 mm; 6%; 0.1% SDS Acryl/Bis = 30 : 0.8	4 mm; 6%; 0.1% SDS Acryl/Bis = 30 : 0.8
Separation gel buffer	Tris-HCl (pH 8.9)	Tris-HCl (pH 9.0)	Tris-HCl (pH 8.9)
Separation gel	Homogenous 10% Acryl/Bis = 15 : 0.8	Gradient 12—18% Acryl/Bis = 30 : 0.8 0.1% SDS	Gradient 12—18% Acryl/Bis = 30 : 0.8 6 M urea + 0.1% SDS
Lower reservoir buffer	Borate-HCl (pH 8.0)	Tris-HCl (pH 9.18)	Borate-HCl (pH 8.0)
Gel length (in cm)	7	22	22
Literature	[14]	[15,16]	

G-250 or R-250. Isolated chlorophyll-protein complexes were obtained by preparative scale gel electrophoresis. The green bands were dissected from the gels by means of a razor blade and homogenized. After elution and centrifugation, the extracts were concentrated for analytical scale gel electrophoresis using an Amicon microultrafiltration system 8 MC.

Absorption measurement

To characterize the spectral properties of the chlorophyll-protein complexes the derivative spectra (second derivative of absorbance with respect to wavelength) of gel slices were measured using the apparatus previously described [18,19]. The approximate chlorophyll *a/b* ratios were estimated from the derivative spectra on the basis of following assumptions. The red region of the absorption spectrum of native chlorophyll consists in the main of two chlorophyll *a* bands with peaks at 673 and 684 nm and of one chlorophyll *b* band with a peak at 650 nm. Furthermore, the derivative spectra of the native chlorophylls as well as the ratio of their absorbance coefficients correspond to those obtained with chlorophyll in solution (diethyl ether) with the exception, however, that differences exist between the positions of the absorption maxima. Under consideration of these differences the relative amounts of the chlorophyll *a*-673, chlorophyll *a*-684 and chlorophyll *a*-650 were estimated [20].

Photographic representation

Orthochromic plates were used to photograph the pigment profiles and panchromic plates to photograph the polypeptide profiles.

Results

Analytical scale gel electrophoresis of SDS-disaggregated thylakoids using system I results in a pigment profile as shown in Fig. 1A. The zones of lowest and intermediate electrophoretic mobility represent chlorophyll-protein complexes, previously termed CP I and CP II [2,21], respectively, while the third zone consists of pigments and lipids complexed to detergent micelles. On the basis of their function CP I and CP II were renamed *P*-700-chlorophyll *a*-protein and light-harvesting chlorophyll *a/b*-protein, respectively [3].

Under the conditions of the high resolving system II, electrophoresis of the same material provides a pigment profile shown in Fig. 1B. In the gel region of the light-harvesting chlorophyll *a/b*-protein two chlorophyll bands appear and are termed CP II′ and CP III. Since, as shown in Fig. 3, CP II′ and CP III are each associated with a protein moiety they must represent chlorophyll-protein complexes. Both complexes form a common band if separated in electrophoretic system I but can be resolved into two distinct bands by means of system II.

To characterize CP II′ and CP III on the basis of their chlorophyll moieties their absorption spectra and derivative absorption spectra were analysed. The derivative spectroscopical data shown in Fig. 1C support previous results [10] indicating different absorption maxima for chlorophyll *a* in the three chlorophyll containing zones. Contrary to this, the two bands (CP II′ and CP III)

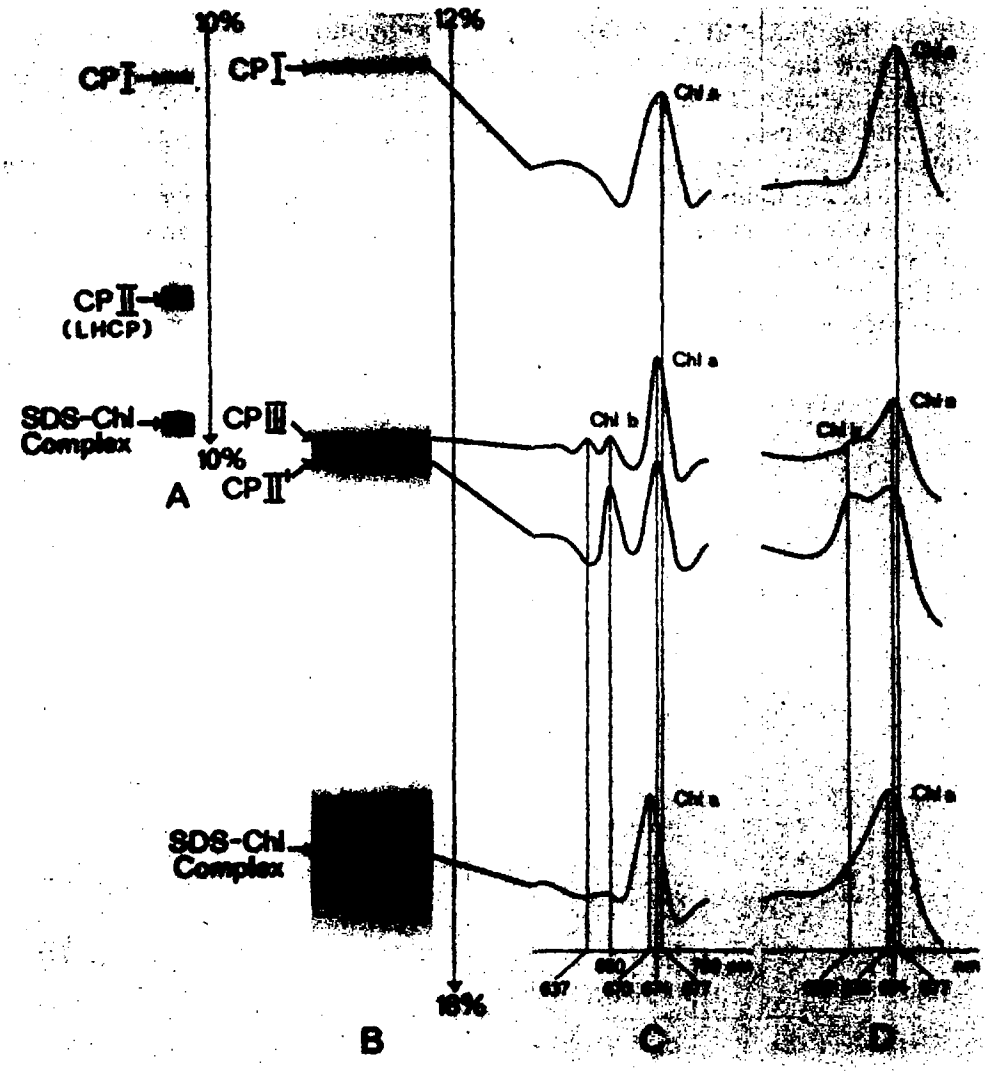

Fig. 1. Pigment profiles obtained with electrophoretic systems I and II. System I provides a single chlorophyll zone of intermediate mobility previously termed chlorophyll-protein complex II (CP II) or light-harvesting chlorophyll *a/b*-protein as shown by gel A. This zone is not homogeneous and, under the conditions of electrophoretic system II, is separated into two bands termed CP II′ and CP III (B). The derivative spectra of the two bands are shown in C and the normal absorption spectra shown in D.

which appear after resolution of CP II exhibit the same maxima for chlorophyll *a* as well as for chlorophyll *b*, however, significant differences exist between the chlorophyll *a/b* ratios which on the basis of their molar extinction coefficients were found to be approximately 0.9 for CP II′ and 2.1 for CP III. CP III shows as a further characteristic feature an additional peak in the position of 637 nm where CP II′ reveals a minimum. The normal spectra shown in Fig. 1D support the derivative spectroscopic data.

The position of the two complexes after staining the polypeptides is indicated in Fig. 2. CP II′ is located above the 26 000 dalton polypeptide and CP III below the 30 000 dalton polypeptide. Since it can be expected that the complexes retain at least part of their tertiary structure and would not necessarily bind the usual 1.4 g SDS per gram protein [22] it was not possible to estimate the true molecular weights by electrophoretic methods. In contrast to former results obtained with system I [21], electrophoresis with system II provides a lower electrophoretic mobility of CP II′ and CP III as compared with their apoproteins. This demonstrates the dependence of the electrophoretic mobility on the system being used.

For further characterization of both complexes, CP II′ and CP III were

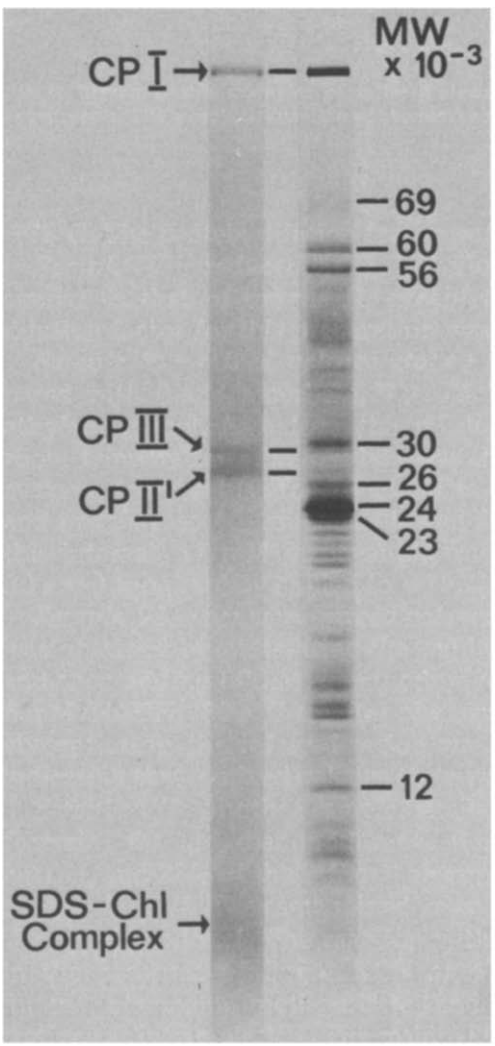

Fig. 2. Pigment and polypeptide profiles obtained by electrophoretic system II. Both chlorophyll bands CP II′ and CP III are each associated with a protein moiety and, therefore, represent chlorophyll-protein complexes. They are located between the 26 000 and 30 000 dalton polypeptides.

isolated by preparative scale gel electrophoresis (a section of a preparative slab gel is shown in Fig. 1B) and re-electrophoresed using electrophoretic systems II and III. The processes of elution and concentration prior to re-electrophoresis resulted in the removal of most of the chlorophyll molecules attached to the carrier protein.

Under the conditions of system II, re-electrophoresis of CP II′ reveals two major bands of 24 000 and 23 000 daltons as well as some minor bands (Fig. 3A). The minor bands are very probably contaminations originating from other polypeptides contained in the excised gel section, especially the 26 000 dalton polypeptide. Re-electrophoresis of CP III shows a major band in the position of 23 000 daltons. The additional minor band exactly corresponds to the 24 000 dalton polypeptide and, therefore, may be a contamination by CP II′.

As compared with system II, re-electrophoresis of the same material using system III results in denaturation of CP II′ and CP III, respectively. Both bands are completely missing on the pigment profile before staining as well as on the polypeptide profile after staining. CP I is, at least partially, preserved (Fig. 3B). This result supports previous findings which indicated that the light-harvesting chlorophyll *a*/*b*-protein is extremely sensitive to the action of urea [23]. On the other hand, system III provides a better resolution of the apoproteins of CP II′ and CP III since urea molecules compete with dodecyl sulfate molecules which are responsible for the extrinsic charge of the polypeptides and, as its results, for their electrophoretic mobility. As a consequence, the minor polypeptide of CP II′, termed AP II′b, and the apoprotein of CP III, termed AP III, exhibit different electrophoretic mobilities if separated with system III although their apparent molecular weights are identical. Therefore, the application of electrophoretic system III allows rather good inferences with respect to the polypeptide composition of the two complexes. CP II′ is associated with two polypeptides of 24 000 (AP II′a) and 23 000 daltons (AP II′b) of which only AP II′a is known to bind chlorophyll. The function of AP II′b is still unknown. CP III has one polypeptide of 23 000 daltons (AP III) which is also a chlorophyll carrier protein.

Beside the intact complexes CP II′ and CP III, the control samples reveal rather large amounts of the 24 000 and 23 000 dalton polypeptides representing the fully denatured apoproteins free of chlorophyll as shown in Figs. 2 and 3 (sample 1 of gel A). This can be assumed to be due to the action of the detergent used for thylakoid membrane disaggregation as well as to the action of long-time electrophoresis, both of which may already dissociate large portions of CP II′ and CP III.

From the results it can be concluded that the chlorophyll-protein zone of intermediate electrophoretic mobility previously termed CP II and later on light-harvesting chlorophyll *a*/*b*-protein is not homogeneous in its composition and can be resolved into two sharp chlorophyll containing bands. Both bands are not only differing in their spectral properties but are also characterized by distinct apoproteins and, therefore, represent two chlorophyll-protein complexes which may differ with respect to their physiological functions. On the basis of results obtained with chlorophyll *b*-less mutants of barley [13,24,25] only the chlorophyll associated with AP II′a is up to now exactly known to have a light-harvesting function.

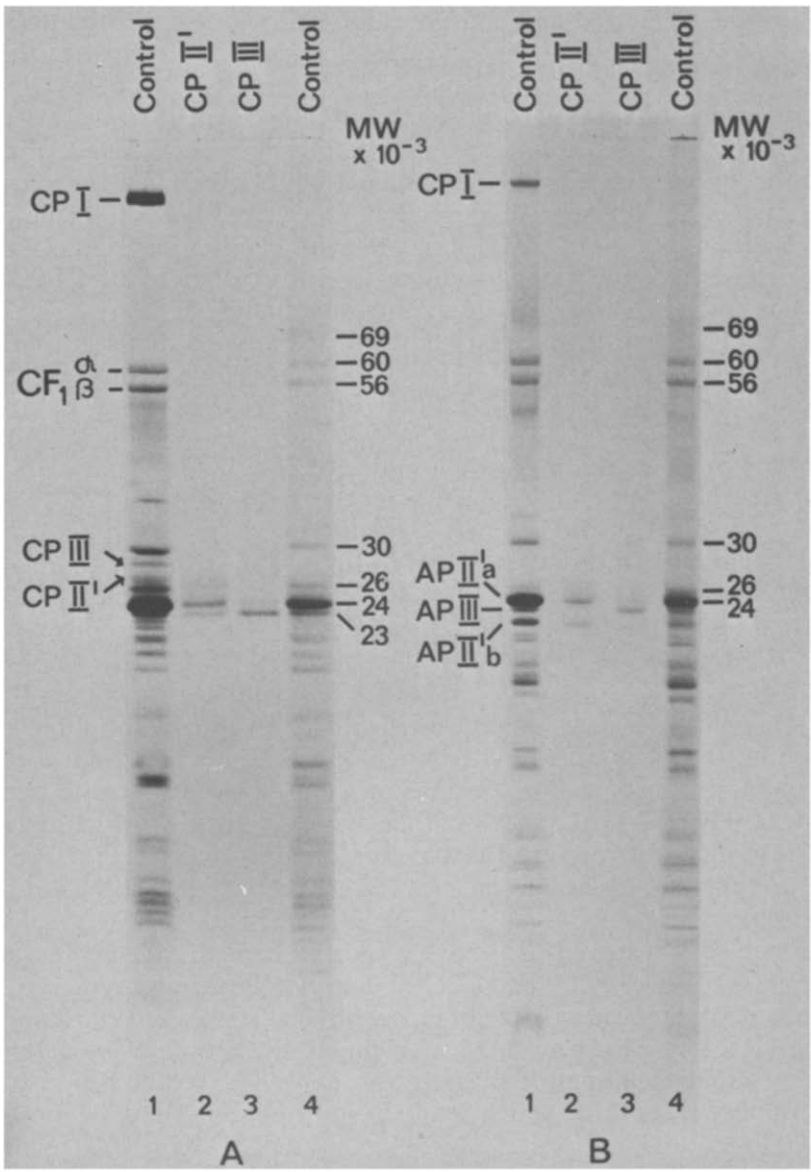

Fig. 3. Re-electrophoresis of isolated and concentrated CP II' and CP III under the condition of electrophoretic system II (A) and system III (B). A better resolution of the polypeptides was obtained with system III. Due to the influence of urea, the polypeptides do not move in conformity with their molecular weights. Gel B shows that the two polypeptides associated with CP II' and termed AP II'a and AP II'b are differently positioned than in system II. In correspondence with the results obtained for system II (A) re-electrophoresis of CP III using system III (B) also yields only one major polypeptide (AP III). Under these conditions, however, the electrophoretic mobility of AP III differs significantly from that of AP II'b. Although being of similar molecular weights, AP III and AP II'b are not identical. The minor band present in the sample of CP II' is a contamination by the 26 000 dalton polypeptide. The two minor bands of CP III very probably represent small amounts of CP II'. Samples No. 2, 3 and 4 were incubated with 2% (final concentration) dithiothreitol prior to electrophoresis.

Discussion

The first information that the chlorophyll zone of chlorophyll-protein complex II extends over two polypeptides of 26 000 and 24 000 daltons was provided by Anderson and Levin [26]. Apel et al. [11] isolated a chlorophyll-protein complex from the Photosystem II-active fraction of *Acetabularia meditarranea* exhibiting an apparent molecular weight of 67 000. The complex was found to contain two subunits of 23 000 and 21 000 daltons of which only the small subunit could be identified as a chlorophyll-binding protein. Further evidence that isolated chlorophyll-protein complex II can be separated into two polypeptides by means of urea-containing polyacrylamide gels was provided by Süss et al. [12] and Machold et al. [13] for *Vicia faba* and *Hordeum vulgare*, respectively. The existence of two chlorophyll-binding polypeptides of 24 000 and 22 000 daltons was postulated by Bar-Nun et al. [9] from indirect evidence. Henriques and Park [27] found three bands of 27 000, 25 000 and 23 000 daltons after electrophoresis of chromatographically isolated complex II of romaine lettuce. Three polypeptides of similar molecular weights were also found by Burke et al. [28] after separation of a purified light-harvesting complex of pea.

The present experiments support the findings that the chlorophyll-protein band termed CP II or light-harvesting chlorophyll *a/b*-protein is composed of more than one polypeptide and provide evidence that it is a mixture of two distinct chlorophyll-protein complexes. One of the two complexes is associated with two polypeptides (AP II'a and AP II'b) of which one polypeptide represents the major 24 000 dalton band of the complete polypeptide pattern. Since this polypeptide was previously found to be a constituent of the zone termed light-harvesting *a/b*-protein or CP II [13,24,25], the new complex was designated as CP II'. It is evident that the 24 000 dalton polypeptide is associated with chlorophyll and that it has a light-harvesting function. Whether or not the 23 000 dalton polypeptide (AP II'b) of CP II' represents also a chlorophyll carrier protein is still unknown. The two polypeptides associated with CP II' correspond to the bands previously termed IIa and IIb [12,13].

The second complex is characterized by only one polypeptide but a high chlorophyll *a/b* ratio. Since the denaturing processes which occur during longtime electrophoresis are not exactly known the estimated ratio might not necessarily represent the native state. If, however, chlorophyll *a* really predominates it is justified to assume that the complex is identical with a third chlorophyll *a*-protein as postulated by Thornber et al. [29] on the basis of theoretical and experimental considerations. Since a uniform nomenclature is presently lacking the complex was, therefore, termed CP III. Besides its high chlorophyll *a/b* ratio CP III has an additional absorption peak in the region of 637 nm. This peak has not yet been characterized. In this connection, a 640 nm band described by Kleinen Hammans and Thomas [30] and correlated with a chlorophyll *a* band at 682 nm in the sense that the former one represents the first vibrational band of chlorophyll *a*-682 might be of interest.

The present results count for the existence of at least three chlorophyll-proteins; it remains uncertain, however, whether or not CP II' represents a single complex. Apart from this a fourth complex representing the reaction

center of Photosystem II may be expected in the range of 45 000 daltons [3,13]. Based on its electrophoretic mobility a chlorophyll *a*-protein found by Hayden and Hopkins [31] and termed complex IV can be assumed to be identical with the 45 000 dalton complex. The other minor chlorophyll-containing bands electrophoresing between the major complexes and described by several authors [32—34] very probably represent dimers or trimers of known chlorophyll-proteins.

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