Biochimica et Biophysica Acta, 934 (1988) 118–122
Elsevier

BBA 42776

Separation of phosphorylated and unphosphorylated light-harvesting chlorophyll *a/b*-protein complex by column chromatography

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(Received 9 November 1987)

Key words: Membrane protein; Chlorophyll-protein complex; Protein phosphorylation; Chromatography, column; Light harvesting complex

Ion-exchange column chromatography of ^{32}P-labelled light-harvesting chlorophyll *a/b*-protein complex (LHC II) isolated from spinach or pea chloroplasts yielded in each case several fractions, differing with respect to polypeptide composition and ^{32}P incorporation. Separation of native, phosphorylated and unphosphorylated complex on a preparative scale was achieved for spinach LHC II.

Introduction

The light-harvesting chlorophyll *a/b*-protein complex (LHC II) is the most abundant membrane protein in chloroplast thylakoids where it occurs predominantly in close proximity to the Photosystem II reaction centres in the chloroplast grana. The complex has three important functions in plant photosynthesis: (i) it intercepts solar energy and transmits it mainly to the reaction centres of PS II [1]; (ii) it is chiefly responsible for membrane appression and, hence, grana formation [2]; and (iii) it is involved in the regulation of energy distribution between PS II and PS I [3]. The latter function is intimately linked to the reversible phosphorylation of the complex by a specific kinase [4]. Upon phosphorylation, some of the LHC II redistributes to the non-appressed, stromal regions of the thylakoid membrane, increasing the effective antenna size of PS I [5,6].

Electron microscopy and image analysis of two-dimensional [7] and thin three-dimensional crystals [8,9] of pea LHC II have shown that the complex is a trimer, composed of three apparently identical monomers. Sedimentation equilibrium ultracentrifugation indicated that the complex is normally a trimer in detergent solution [10]. Spectroscopic studies [11] have suggested that this is also the configuration of the functional complex in vivo.

LHC II isolated from pea contains three (one major and two minor) polypeptides [8], at least two of which can become phosphorylated [12]. Spinach LHC II seems to contain only one major and one minor polypeptide, both of which are subject to phosphorylation [13,14]. Each polypeptide binds a total of 15 chlorophyll molecules [10], 8 Chl *a* and 7 Chl *b*. LHC II is the product of a small multi-gene family [15–17]. Two genes coding for the major polypeptide of pea LHC II have been sequenced [15,18], indicating a molecu-

Abbreviations: LHC II, light-harvesting chlorophyll *a/b*-protein complex; PS II, Photosystem II; PS I, Photosystem I; Chl, chlorophyll; OG, *n*-octyl-β,D-glucopyranoside.

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lar weight of 25000 [19]. Partial peptide sequencing has shown that major and minor polypeptides of the pea complex are very similar in terms of their amino acid sequence [20]. Nevertheless, there are significant differences between the minor and the major polypeptide, in particular with respect to phosphorylation kinetics. Islam [13] has shown that the minor polypeptide of spinach LHC II becomes phosphorylated three times faster than the major polypeptide. Larsson et al. [14] confirmed this and reported preferential migration of LHC II containing the phosphorylated minor polypeptide into the non-appressed membrane region.

So far, the biochemical separation of phosphorylated and unphosphorylated LHC II protein has only been achieved analytically by two-dimensional SDS/polyacrylamide gel electrophoresis [21] which denatured the complex, dissociating all chlorophylls from the polypeptides. We report here a simple method of separating the phosphorylated and unphosphorylated forms of the native chlorophyll-protein complex on a preparative scale. To our surprise, we found that the success of the separation depends on the plant species from which the complex had been derived.

Materials and Methods

LHC II was isolated from chloroplasts obtained from pea seedlings grown in the laboratory or from market spinach as described [22]. Broken chloroplasts were phosphorylated biochemically by incubation for 45 min in the dark with ferredoxin [23] or sodium dithionite [24] in the presence of ^{32}P-ATP and 10 mM NaF. LHC II was isolated by solubilization of destacked thylakoids with Triton X-100 and sucrose density centrifugation according to Burke et al. [25] as modified by Kühlbrandt et al. [26]. LHC II was precipitated from the red fluorescent gradient fraction with KCl [8] and resolubilized in 50 mM *n*-octyl-β,D-glucopyranoside or 0.5% (w/v) Triton X-100. Highly labelled LHC II was isolated from phosphorylated chloroplasts and combined with unlabelled LHC II (isolated omitting the incubation step) to a final activity of $(1-2) \cdot 10^5$ decays per min per mg Chl.

A fractogel TSK DEAE-650 (S) anion-exchange column of approximate dimensions of 10 cm × 1 cm was equilibrated with 10 mM sodium/potassium phosphate (pH 7.0) and 35 mM OG or 0.1% (wt./vol.) Triton X-100. ^{32}P-labelled LHC II corresponding to roughly 1 mg Chl was loaded onto the column. Fractions of the complex were released with a linear 50 mM to 150 mM KCl gradient in the same buffer at a flow rate of 0.5 ml/min at 20 °C. The total gradient volume was 100 ml. The absorbance at 280 nm of the eluent was monitored in a flow cell. Fractions of approx. 1 ml were collected. 100 µl aliquots of peak fractions were analyzed by standard SDS/polyacrylamide gel electrophoresis and autoradiography. All fractions were then shaken with 1 ml diethyl ether to extract chlorophyll and counted by liquid scintillation. Ether extracts were taken to dryness and 1 ml 80% aqueous acetone was added. The chlorophyll concentration and the Chl *a*/Chl *b* ratio was determined spectroscopically according to Arnon [27].

Results

Column chromatography of spinach or pea LHC II solubilized in OG yielded seven (Fig. 1a) or eight (Fig. 1b) peaks in the absorbance$_{280}$ and ^{32}P profiles, referred to as S1–S7 (spinach) and P1–P8 (pea). For pea LHC II, qualitatively similar results were obtained using Triton X-100 as a detergent, although the fractions were less well resolved (not shown). The Chl concentration followed the absorbance profile closely. The Chl *a*/Chl *b* ratio of peak fractions was between 1.07 and 1.13 for both species, compared to 1.15 for single crystals of pea LHC II [8], indicating some loss of Chl during column chromatography. The peak Chl concentration was around 30 µg/ml. At a molar Chl/polypeptide ratio of 15, this corresponded to a concentration of LHC II trimers of roughly 0.7 µM. As assessed by sedimentation equilibrium ultracentrifugation, LHC II trimers were stable at this concentration under similar conditions (35 mM OG, 15 °C) but began to dissociate into monomers and dimers below this concentration [10]. LHC II was thus released from the column at a detergent/protein ratio at which the

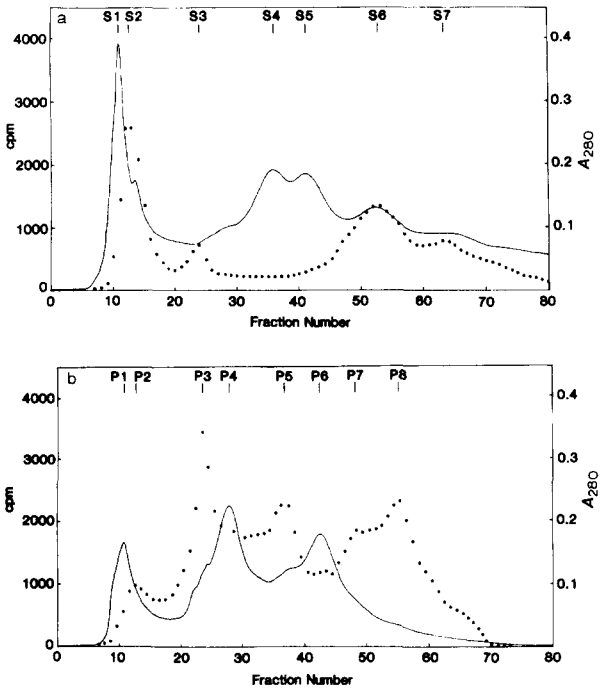

Fig. 1. Elution profile (absorbance$_{280}$) of (a) spinach and (b) pea LHC II phosphorylated with ^{32}P-ATP and fractionated by column chromatography. Radioactive label found in each fraction is indicated (●).

trimer appeared to be at equilibrium with the monomeric and dimeric complexes.

SDS/polyacrylamide gel electrophoresis and autoradiography revealed the polypeptide composition and the ^{32}P content of peak fractions (Fig. 2). Fractions S1 and P1 consisted largely of free Chl as well as some unlabelled LHC II protein eluting with the void volume. Fractions S2 and P2 contained some ^{32}P-labelled LHC II protein. In spinach, low levels of an unlabelled polypeptide of approx. 20 kDa apparent molecular mass were also present, representing a non-LHC II contaminant or a proteolytically cleaved LHC II polypeptide. Fractions S3 and P3 eluted at the same salt concentration, but the amount of radioactive label varied over a wide range in different experiments. Both fractions contained little labelled protein (not shown), suggesting that they represented non-specifically bound ^{32}P-ATP (pK_{a_1} = 6.0–6.9; see Ref. 28) which was expected to elute in this region.

Fractions S4–S7 and P4–P8 accounted for roughly 75% of Chl recovered in each experiment, and hence contained the bulk of the complex. In each profile (Fig. 1), the highest peaks indicated largely unphosphorylated LHC II. The fractions of unphosphorylated spinach LHC II, S4 and S5, eluted close together and were well separated from the phosphorylated fractions, S6 and S7, which eluted at higher salt concentrations, as expected due to their more negative net charge at neutral pH. The polypeptide composition of S4 and S5 appeared to be identical, as judged by one-dimensional SDS/polyacrylamide gel electrophoresis (Fig. 2a), implying that they perhaps represented different oligomeric forms of the complex in which major and minor polypeptides combined non-stoichiometrically. The phosphorylated fractions S6 and S7 were well resolved in the absorbance and ^{32}P profiles. The degree of phosphorylation in terms of specific activity (the percentage ratio of peak areas from the ^{32}P and the absorbance profiles) of both fractions was very similar. Their polypeptide composition resembled that of the unphosphorylated fractions closely, except that an unlabelled component was present (perhaps a proteolytically cleaved form of the major polypeptide), running between the major and minor polypeptide

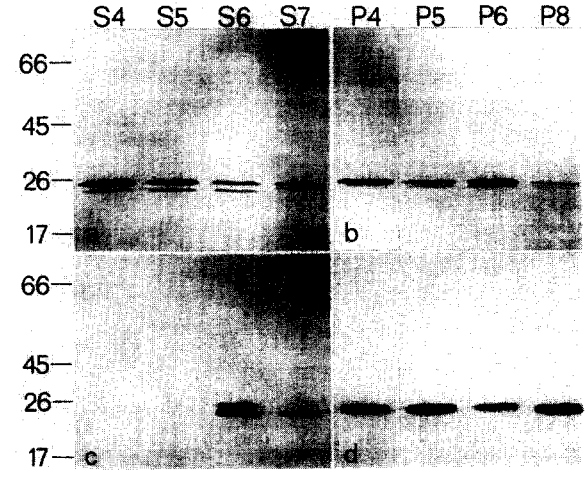

Fig. 2. SDS/polyacrylamide gel electrophoresis of (a) peak fractions S4–S7 (see Fig. 1a) of phosphorylated spinach LHC II and (b) peak fractions P4–P6 and P8 (see Fig. 1b) of phosphorylated pea LHC II. (c) and (d) are the autoradiograms of (a) and (b), respectively.

in fraction S7. Autoradiography (Fig. 2c) showed that the minor polypeptide which accounts for 20%–30% of total LHC II protein in spinach [13,14] carried most of the radioactive label. The relative level of phosphorylation of the minor polypeptide thus appeared to be higher than previously reported [13,14]. The phosphorylated fractions S2, S6 and S7 accounted for approx. 40% of the recovered complex. We estimated from the relative intensities of the bands in Fig. 2a and 2c that about one-third to one-half of the complex in these fractions was phosphorylated. The level of phosphorylation of total spinach LHC II was therefore 15–20%, in agreement with other findings [13].

In the elution profile of pea LHC II (Fig. 1b), phosphorylated and unphosphorylated fractions alternated and were therefore less well separated than spinach fractions. The two major, largely unphosphorylated fractions (P4 and P6) showed highly reproducible differences in terms of one-dimensional SDS/polyacrylamide gels (Fig. 2b). The protein in fraction P4 consisted almost entirely of the major polypeptide, with only a trace of the minor polypeptide present, whereas fraction P6 contained the three polypeptides normally found in isolated pea LHC II. The two minor polypeptides (which are not well resolved in Fig. 2b but clearly distinguished in other gels of identical column fractions) seemed to be somewhat enriched in fraction P6. The phosphorylated fractions P5, P7 and P8 were poorly resolved in the absorbance profile, but evident in the ^{32}P profile. These three fractions contained similar amounts of radioactive label but decreasing amounts of protein and Chl. Their specific activity increased in the order P5/P7/P8 = 1 : 1.7 : 2.8, consistent with LHC II trimers including one, two and three phosphorylated polypeptides, respectively. In total, the major and minor polypeptide bands appeared to carry roughly equal amounts of radioactive label. As in spinach LHC II, this indicated a significantly higher specific labelling of the minor polypeptide relative to the major one. From the autoradiographs it was not possible to decide whether both minor polypeptides were phosphorylated. The phosphorylation level of total pea LHC II, estimated in the same way as for the spinach complex, was 10%–15%.

Discussion

Larsson et al. [14] showed that the preferentially phosphorylated minor polypeptide of spinach LHC II moves into the non-appressed stromal regions at a significantly higher rate than the major polypeptide. They argued that there were two populations of LHC II antennae; one closely associated with PS II, exclusively containing the major polypeptide; and another mobile form, containing all the minor and some major polypeptide. Our results suggest that in spinach LHC II these subpopulations do not form biochemically distinct complexes, but become intermingled during isolation or column chromatography, dissociating and recombining into oligomers of average stoichiometry. In pea, on the other hand, the largely unphosphorylated main fractions (P4 and P6) may correspond to the two types of LHC II postulated by Larsson et al. [14].

We have shown that the phosphorylated and unphosphorylated forms of LHC II can be separated by column chromatography, depending on the species from which the complex has been isolated. None of the column fractions could be induced to form three-dimensional crystals which have been obtained from the unfractionated complex [8], either because a component essential for crystallization was lost or because the structural integrity of the complex was impaired at the high detergent/protein ratio required for column chromatography. Nevertheless, we hope that the fractionation of LHC II on a preparative scale will prove useful in future structural and functional studies of this important membrane protein.

Acknowledgements

We thank Dr. U. Larsson, University of Lund, Sweden, and Dr. K. Islam, University of Milano, Italy, for communicating results prior to publication. This project was supported by a grant from the Agricultural and Food Research Council of Great Britain and a Heisenberg Research Fellowship (Deutsche Forschungsgemeinschaft) to W.K.

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