HIGHER PLANT CHLOROPHYLL *a*/*b*–PROTEIN COMPLEXES: STUDIES ON THE PHOSPHORYLATED APOPROTEINS

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1. Introduction

The photosynthetic membrane of green algal and higher plant chloroplasts contains several phosphoproteins, the most abundant of which are apoproteins of the light-harvesting chlorophyll *a*/*b* protein (apoLHCPs) [1–4]. In peas, for example, there are two phosphorylated apoLHCPs with M_r-values of 26 000 and 24 000 [2]. The LHCPs bind ~1/3 of total chl *a* and most of the chl *b* [5] and transfer absorbed excitation energy principally to photosystem II [6]. Their light-harvesting function is thought to be regulated by protein phosphorylation, since fluorescence analysis has shown that distribution of excitation energy to photosystems I and II is dependent upon the extent of LHCP phosphorylation [7–11]: phosphorylation of LHCPs increases (and dephosphorylation decreases) the proportion of excitation energy that is transferred to photosystem I at the expense of photosystem II.

When chloroplast photosynthetic membranes are solubilized in SDS and fractionated by SDS–polyacrylamide gel electrophoresis in certain buffer systems, 3, sometimes more, pigmented complexes containing both chl *a* and chl *b* can be observed [12–15]. As yet there is no consensus as to whether these complexes represent several multimeric forms of a single monomeric LHCP complex [12–15] or arise from biochemically different types of LHCP [16,17]. Here we examine some properties of the 3 chl *a*/*b* complexes (AB-1, AB-2, AB-3) reported in [12]. We present evidence that:

(i) The commonly observed chl *a*/*b*–protein complex, CPII [5,18], is generated electrophoretically from AB-1, AB-2 and AB-3;
(ii) All 3 AB complexes contain the same two phosphorylated apoLHCPs in approximately the same ratio;
(iii) The two apoLHCPs differ structurally from one another near their surface-exposed phosphorylation sites.

Abbreviations: chl, chlorophyll; LHCP, light-harvesting chlorophyll *a*/*b*-protein; SDS, sodium dodecyl sulphate; CPII, chlorophyll–protein II; M_r, relative molecular mass

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2. Methods

Intact chloroplasts were prepared from pea (*Pisum sativum* L.) and incubated in the light with [^{32}P]orthophosphate to label thylakoid phosphoproteins [1]. From the labelled pea chloroplasts and from unlabelled tobacco (*Nicotiana tabacum* L.) chloroplasts, thylakoids were isolated, washed and solubilized in SDS [12] at the indicated temperatures and for the indicated times. In each case [chl] was 1 mg/ml and the SDS/chl ratio was 10:1 [12]. chl–protein complexes were analyzed by SDS–polyacrylamide gel electrophoresis through polyacrylamide gel slabs, using a Tris–glycine buffer [12] or Tris–HCl buffer [18]. Determination of M_r equivalence for the chl–protein complexes using standard proteins was as in [12]. For the detection of phosphoproteins after electrophoresis, gel slabs were fixed in two changes of 7% acetic acid, dried and autoradiographed.

When excised gel slices containing chl–proteins complexes were to be re-electrophoresed in Tris–glycine buffer or Tris–HCl buffer, they were placed in sample wells on top of a second polyacrylamide gel slab, and electrophoresis was conducted as in [12,18].

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When excised gel slices were to be re-electrophoresed under fully denaturing conditions, they were heated to 70°C for 4 min in buffer A (50 mM $NaHCO_3$, 50 mM dithiothreitol, 2% SDS). The slices were transferred to wells in a 5% polyacrylamide stacking gel that had been formed above a 20% polyacrylamide separating gel. Each gel slice was overlaid with 50 μl buffer A containing 15% glycerol and 0.01% bromophenol blue. All other aspects of the electrophoretic procedure have been described [2]. After overnight electrophoresis, the gel was stained, dried and autoradiographed, and the autoradiogram was scanned with a Joyce-Loebl microdensitometer.

Preparation of apoLHCPs for CNBr cleavage involved the extraction of chloroform/methanol-soluble proteins from ^{32}P-labelled pea thylakoids [19], precipitation of the proteins with an equal volume of ether, solubilization of the proteins in buffer A containing 15% glycerol and 0.01% bromophenol blue, and separation of the proteins by SDS–polyacrylamide gel electrophoresis [2], using a 20% polyacrylamide separating gel. The 24 000 and 26 000 M_r apoLHCPs were located by autoradiography of the wet gel, bands containing one or both of these polypeptides were excised and fragmented by passage through a syringe, and the proteins were eluted into 0.1% SDS, 0.1% 2-mercaptoethanol. After lyophilization, the proteins were taken up in 200 μl water and 700 μl HCOOH and incubated at 4°C for the indicated period in the presence of CNBr (2 mg/ml) [20]. Formic acid and CNBr were removed by lyophilization and the radioactive digestion products were analyzed by SDS–polyacrylamide gel electrophoresis, autoradiography and microdensitometry.

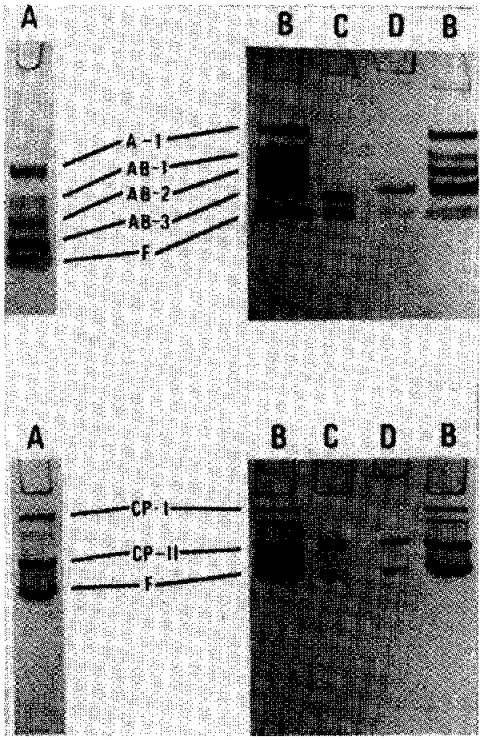

Fig.1. Electrophoretic fractionation of chl–protein complexes from tobacco on polyacrylamide gels. The gels are not stained for protein; the visible bands are due to pigment. Upper panel: Tris glycine gel system containing 5% (left) or 6% (right) acrylamide. Lower panel: Tris–HCl gel system containing 8% acrylamide. Samples: (A,B) thylakoid membranes treated with SDS; (C) an excised gel slice containing CPII; (D) an excised gel slice containing AB-3.

3. Results

Treatment of tobacco thylakoids with SDS at 0°C and fractionation by SDS–polyacrylamide gel electrophoresis in Tris–glycine buffer resulted in the expected [12] resolution of 4 chl–protein complexes (fig.1, upper panel, track A). In the nomenclature introduced in [12], the complexes are A-1 (containing only chl *a*) and AB-1, AB-2 and AB-3 (containing both chl *a* and chl *b*). All of the chl is complexed with protein in the detergent extracts [21], but electrophoretic fractionation liberates a portion of it causing formation of a band of chl–SDS micelles labelled F. The apparent M_r-values of the complexes are: A-1, 110 000; AB-1, 80 000; AB-2, 60 000; and AB-3, 45 000 [12]. When the same preparation was analyzed electrophoretically in a Tris–HCl buffer [18], only two major complexes (CPI and CPII) were observed (fig.1, lower panel, track A). The amount of chl migrating in the F zone owing to electrophoretic denaturation was ~3-fold greater than in the Tris–glycine buffer system [12]. Also visible between CPI and CPII was a complex believed to be a multimeric form of CPII [17]. CPI contained only chl *a* and CPII contained both chl *a* and chl *b*. The app. M_r-values for CPI and CPII (100 000 and 30 000, respectively) were significantly lower than those for the apparently corresponding complexes (A-1, AB-3) resolved with the Tris–glycine system.

Re-electrophoresis of CPII and AB-3 in the Tris–glycine buffer resulted in each complex migrating with the mobility that it showed originally (fig.1, upper panel, tracks C,D) However, re-electrophoresis

in the Tris–HCl buffer system caused the AB-3 complex to migrate approximately as fast as the CPII complex (fig.1, lower panel, tracks C,D). These results establish that the difference in app. M_r between CPII and AB-3 is real and cannot be accounted for by either differences in solubilization procedure (since both complexes were derived from the same sample of solubilized membranes) or differences in mobility of each complex relative to the standard polypeptides in different buffers (since the difference in mobility is maintained during re-electrophoresis in Tris–glycine buffer). It seems that AB-1, AB-2 and AB-3 are present in the solubilized membrane preparation, but that electrophoresis in a Tris–HCl buffer converts them to a single, more rapidly migrating form (CPII) with the concomitant release of an additional fraction of chl.

Since the formation of CPII appears to involve denaturation induced by electrophoresis, we attempted to induce formation of CPII by thermal denaturation. Tobacco thylakoids were solubilized with SDS as above, except that the solubilized membranes were incubated between 0°C and 100°C for 5 min prior to electrophoretic fractionation in Tris–glycine buffer (fig.2). Between 0°C and 40°C the pattern was unchanged from that seen in fig.1. However, at 60°C the AB complexes were completely denatured (i.e., converted to apoproteins and free chl), while complex A-1 was replaced by a slightly more rapidly migrating complex (app. M_r 100 000) termed A-1*. At temperatures above 60°C, A-1* was also completely denatured. The time course for denaturation at 60°C (not shown) indicates that A-1 is converted to A-1* within 4 min and the AB complexes are completely denatured within 8 min. We were unable to detect the formation of a

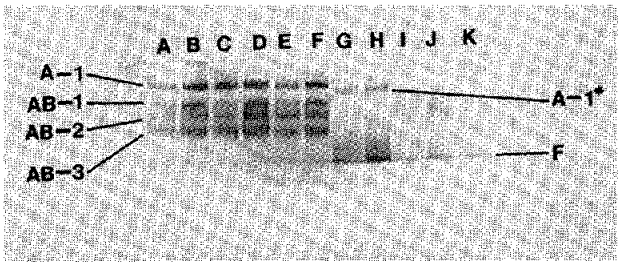

Fig.2. Thermal denaturation of chl–protein complex from tobacco. Thylakoids were solubilized in SDS, incubated for 5 min at the indicated temperatures and analyzed by SDS–polyacrylamide gel electrophoresis in the Tris–glycine buffer system (6% acrylamide). The gel was fixed without staining, dried and photographed. Lanes: (A,C,E,G,I,K), 7.5 μg chl/lane; (B,D,F,H,J), 15 μg chl/lane; (A,B), 0°C; (C,D), 20°C; (E,F), 40°C; (G,H), 60°C; (I,J), 80°C; (K), 100°C.

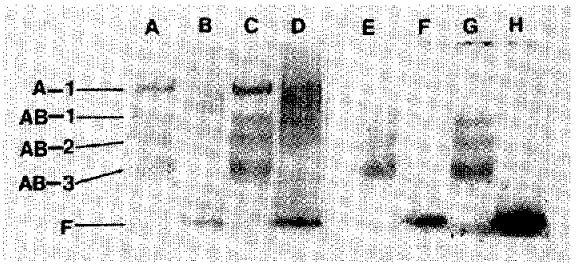

Fig.3. Phosphorylation of chl–protein complexes of pea. Thylakoids from ^{32}P-labelled chloroplasts were solubilized in SDS at 0°C (lanes (A,C,E,G)) or 100°C (lanes B,D,F,H,)) for 10 min and analyzed by SDS–polyacrylamide gel electrophoresis. The gel was fixed without staining, dried, autoradiographed and photographed. Lanes: (A–D), photograph of gel; (E–H), photograph of autoradiogram; (A,B,E,F), 7.5 μg chl/lane; (C,D,G,H), 15 μg chl/lane.

complex with the mobility of CPII in Tris–glycine buffer at any temperature tested.

We wanted to locate apoLHCPs on SDS–polyacrylamide gels by exploiting their phosphorylation. However, when tobacco leaves or chloroplasts were labelled with [^{32}P]orthophosphate the apoLHCPs accounted for <50% of the ^{32}P incorporated into protein, making it difficult to distinguish clearly between apoLHCPs and other phosphoproteins. In contrast, when isolated intact pea chloroplasts were labelled with [^{32}P]orthophosphate, >90% of the label incorporated into thylakoid proteins appeared in the apoLHCPs [1,7,10,11].

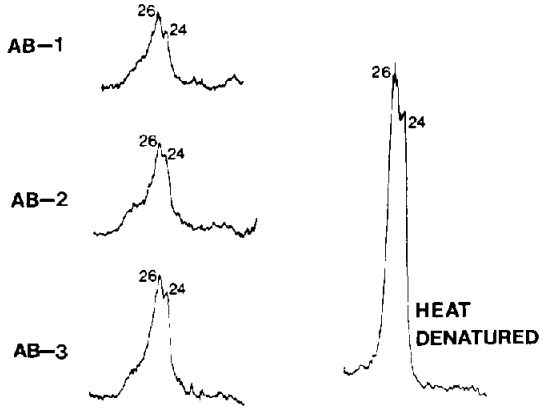

Fig.4. Phosphoprotein composition of chl–protein complexes of pea. Thylakoids were solubilized at 0°C and 100°C and analyzed as in fig.3. Gel slices corresponding to AB-1, AB-2, AB-3 and heat-denatured apoprotein were re-electrophoresed under completely denaturing conditions as in section 2. The gel was dried and autoradiographed and the autoradiogram was scanned to locate the phosphoproteins composing these complexes.

For this reason, the remaining experiments employed pea chloroplasts, which, in all other respects, gave very similar results to those already mentioned for tobacco chloroplasts.

When thylakoids from ^{32}P-labelled pea chloroplasts were solubilized with SDS and fractionated by SDS polyacrylamide gel electrophoresis in Tris–glycine buffer, the radioisotope co-migrated with the AB-1, AB-2 and AB-3 complexes (fig.3). Treatment of this extract at 100°C for 3 min led to the complete denaturation of the 4 chl–protein complexes and to the loss of the 3 bands of ^{32}P, which were replaced by a single band of radioactive apoprotein migrating slightly more slowly than the F band. Note that the unheated sample does not contain free ^{32}P-labelled apoLHCP. This indicates that the membrane has been solubilized without the generation of apoprotein. Excision and re-electrophoresis of the radio-labelled bands under completely denaturing conditons (fig.4) showed that thermally denatured and non-denatured sample contained both the 26 000 M_r and the 24 000 M_r phosphorylated apoLHCPs. The two phosphoproteins were present in approximately the same ratio in each sample.

Amino acid analysis, serological comparisons and proteolytic digestion have established that the 2 apoLHCPs are structurally related [22–24]. What is not clear is the intramolecular location of the structural difference between them that accounts for the difference in their app.M_r-values. To gain some insight into this question, ^{32}P-labelled apoLHCPs were subjected to partial digestion with CNBr which cleaves at methionyl residues [20]. The phosphorylation of the LHCPs is known to take place very close to one end [25]. Partial digestion will generate a series of radioactive fragments containing the phosphorylation site. The number of fragments in this series will be determined by the no. methionyl residues/polypeptide chain, while their M_r-values will depend on the distribution of the methionyl residues along the chain. In addition, digestion with CNBr will generate a series of fragments that lack the phosphorylation site and are therefore non-radioactive. The results of this analysis are reported in fig.5.

The ^{32}P-labelled 26 000 M_r apoLHCP was purified electrophoretically and the single polypeptide was treated with CNBr for 0, 4 or 16 h. Electrophoretic analysis of the cleaved material followed by autoradiography (fig.5(a–c)) revealed 3 labelled products (fragments X,Y,Z) in addition to the undigested 26 000 M_r phosphoprotein. The accumulation of fragment Z with prolonged digestion indicated that this fragment

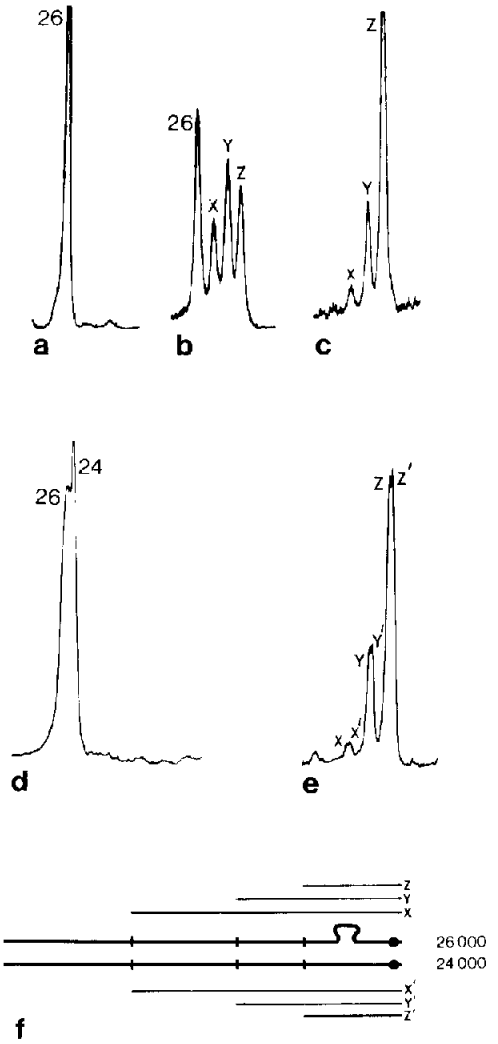

Fig.5. CNBr cleavage of ^{32}P-labelled apoLHCPs. The 26 000 M_r ^{32}P-labelled apoLHCP was digested with CNBr for 0 h (a), 4 h (b) or 16 h (c) and the labelled fragments (X,Y,Z) were detected by autoradiography after SDS polyacrylamide gel electrophoresis. A mixture of the 26 000 M_r and 24 000 M_r ^{32}P-labelled apoLHCPs was digested with CNBr for 0 h (d) or 16 h (e) and the labelled fragments (X,X', Y,Y',Z,Z') were detected as above. In (f) a schematic representation of the 2 phosphorylated apoLHCPs (thick lines) shows the positions of methionines (vertical bars) and the phosphorylation sites (black dots). The loop in the 26 000 M_r apoLHCP represents an additional amino acid sequence that is postulated to be absent from the 24 000 M_r apoLHCP and to be responsible for the difference in electrophoretic mobility of the 2 apoLHCPs.

corresponded to the segment of the polypeptide stretching from the phosphorylated terminus to the nearest methionyl residue. Fragments Y and X corresponded to segments of the polypeptide from the phosphorylated terminus to the second and third methionyl residues, respectively.

When a mixture of the 26 000 and 24 000 M_r apoLHCPs was digested with CNBr for 16 h, 6 labelled fragments were obtained (fig.5(e)). They were present as 3 doublets (X,X′; Y,Y′; Z,Z′). The slower member of each doublet was derived from the 26 000 M_r apoLHCP, whereas the faster member (X′, Y′, Z′) was derived from the 24 000 M_r apoLHCP. Fig.5(f) shows the relationship between these fragments. A major difference between the 2 apoLHCPs would appear to be located in fragments Z and Z′, possibly quite near to the surface-exposed phosphorylation site. Any structural difference between Z and Z′ is bound to be reflected in Y and Y′ and in X and X′ since these larger fragments also contain the phosphorylated end of the polypeptide chains. In fig.5(f), the structural difference between Z and Z′ is assumed to be an additional sequence of amino acids (represented by the loop in the 26 000 M_r apoLHCP). There is, however, no evidence at present to distinguish this model from an alternative model in which differences in the extent of, for example, glycosylation could cause differences in electrophoretic mobility without a change in amino acid sequence. Moreover, the results do not of course eliminate the possibility that other differences in primary structure exist elsewhere along the 2 polypeptides.

4. Discussion

Early studies of chl–protein complexes resolved only one light-harvesting chl *a*/*b*–protein (CPII). It was assumed that this represented the monomeric unit which formed the bulk of the green algal and higher plant antenna system. The development of improved electrophoretic systems which yielded less free chl and multiple chl–protein complexes containing both chl *a* and chl *b*, made it unclear how many LHCPs existed in vivo (cf. [17]). Some groups interpreted the multiple bands as simple multimeric forms of a common monomer [13,14,26] whereas other laboratories suggested that biochemically different LHCPs may give rise to different bands [16]. It was also unclear if any of the newly resolved LHCPs was equivalent to the previously observed CPII (cf. [17]). These data indicate that none of the LHCPs (AB-1, AB-2 or AB-3) resolved in the Tris-glycine electrophoretic system is equivalent to CPII. Rather, the data suggest that CPII is generated during the electrophoretic fractionation in the Tris–HCl system and may not be representative of any particular chl *a*+*b*-containing component within the membrane. Furthermore, fractionation of ^{32}P-labelled thylakoids established the absence of any free apoLHCPs generated during electrophoresis in Tris–glycine (fig.3, track 4) and substantiates earlier evidence [21] that solubilization of the thylakoid membrane with SDS does not itself cause denaturation of chl–protein complexes to free chl and apoproteins. This denaturation occurs during electrophoresis in Tris–HCl buffer (fig.1) or during heating to 60°C or above (fig.2,3).

Because of data derived from the analysis of mutants [27] and from fractionation of intact chloroplasts [16], some of us had postulated that whereas AB-1 is seemingly some form of multimer of AB-3, the AB-2 complex was not. The report in [28] on turnover of labelled LHCPs would corroborate the notion that some larger complexes are not simply multimeric forms of the smaller ones. However, these data show that AB-2 contains apoproteins of the same size and extent of phosphorylation as AB-1 and AB-3. Thus, if AB-2 is distinct from AB-1 and AB-3, the distinction is due to something more subtle than a difference in M_r or degree of phosphorylation. It could be due to another form of covalent modification (e.g., glycosylation [22]), or to the presence of some colourless polypeptide, although results obtained with spinach thylakoids suggest that the 3 chl *a*+*b* complexes in [26] contain only the same 2 apoLHCPs. Moreover, cations transform oligomeric complexes into the monomeric form [29,30]. This effect is reversible [30].

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