Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 146-152

AMINO ACID COMPOSITION OF THE 9 kDa PHOSPHOPROTEIN OF PEA THYLAKOIDS

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Received May 27, 1986

The 9 kDa phosphoprotein of pea thylakoids was isolated by electroelution from SDS-polyacrylamide gels and its amino acid composition determined. The result is at variance with the amino acid compositions predicted from published nucleotide sequences of the genes for apocytochrome b-559 and for CF_o subunit III. The amino acid composition of the 9 kDa phosphoprotein resembles that of the 25 kDa light-harvesting chlorophyll a/b protein (LHC-II). We propose that the 9 kDa polypeptide is a chlorophyll-binding protein of photosystem II, that it functions as a link in excitation energy transfer between LHC-II and the reaction centre, and that its phosphorylation regulates excitation energy distribution by means of mutual electrostatic repulsion between itself and phosphorylated LHC-II.

A thylakoid membrane polypeptide with an apparent molecular weight of 9 kDa was reported as one of the substrates of chloroplast protein phosphorylation in the original studies of Bennett (1). This 9 kDa polypeptide is phosphorylated in the same way as the 25 kDa LHC-II (2), that is, in a light-dependent reaction (3) catalysed by a membrane-bound kinase (2) and with threonine as the phosphate group acceptor (1). Dephosphorylation of the 9 kDa polypeptide is generally slower than that of LHC-II (4,5) and requires higher Mg^{2+} concentrations (6) but it is nevertheless thought to be catalysed by the same membrane-bound phosphatase (6).

The identity of the 9 kDa phosphoprotein is unresolved. It has been suggested that it is subunit III of CF_o, that is, the "DCCD-binding protein" (7,8) and also that it is the apoprotein of cytochrome b-559 (9,10). Only the latter suggestion is consistent with it being a component of PS II (11).

Here we report the amino acid composition of the 9 kDa phosphoprotein. The result suggests strongly that it is neither CF_o subunit III nor

Abbreviations: CF_o, membrane-spanning complex of chloroplast coupling ATPase; LHC-II, light-harvesting chlorophyll a/b pigment-protein complex; PS, photosystem.

0006-291X/86 $1.50

cytochrome b-559. The result is consistent, however, with the 9 kDa phosphoprotein being a chlorophyll-binding protein structurally related to LHC-II. Some implications of this identification are discussed.

MATERIALS AND METHODS

Intact chloroplasts were isolated in an isotonic sorbitol medium from ten day-old peas as in (12), but before the final resuspension the chloroplasts were lysed by washing the pellet once in a 100 ml of a hypotonic medium containing $MgCl_2$ (5 mM) and Hepes (5 mM) at pH 7.6. For labelling experiments (Fig. 1) thylakoids equivalent to 50 µg chlorophyll were incubated in 0.5 ml of a medium at pH 7.6 containing sorbitol (0.1 M), $MgCl_2$ (5 mM), NaCl (5 mM), Hepes (50 mM), NH_4Cl (5 mM) and [γ-^{32}P]ATP (0.1 mM) at a specific activity of 400 µCi µmol^{-1}. Illumination was provided by a 60 W desk-lamp at 50 cm. The reaction was stopped by addition of trichloroacetic acid to 5%. Pellets were extracted three times in 80% acetone before resuspension in sample buffer, heating and loading on to 10-30% polyacrylamide slab gels for electrophoresis alongside a track of low molecular weight markers (LKB). Slab gels were then stained with Coomassie blue, dried, and autoradiographed. Samples for subsequent electroelution were incubated at higher chlorophyll concentrations, typically 250 µg ml^{-1}.

For electroelution, the 9 kDa polypeptide and LHC-II bands were identified by reference to molecular weight markers as in Fig. 1, then excised from the wet slab gel with a razor blade. Electroelution was performed in a vertical tube gel apparatus through a plug of 5% polyacrylamide in standard Tris-glycine-SDS buffer at 5 mA overnight. The protein was eluted into Spectrapor 3 (18 mm) dialyses tubing, dialysed five times over three days against 5 l 0.1% SDS and then once against 5 l H_2O. The sample was then freeze-dried.

Protein was hydrolysed in 0.1 ml 6 M HCl (containing 1 mg ml^{-1} phenol) at 110°C for 24 h in sealed, evacuated tubes and amino acid compositions determined with a Chromaspek J180 analyser.

RESULTS AND DISCUSSION

Fig. 1 shows an autoradiograph obtained from a single slab gel in which the time-courses of phosphorylation and dephosphorylation of the 9 kDa and LHC-II polypeptides are compared. The 9 kDa phosphoprotein is identified as the most conspicuously labelled band of lower molecular weight than LHC-II, and it is seen that its dark dephosphorylation is slower than that of LHC-II, as previously reported (4,5). The ^{32}P-labelled 9 kDa phosphoprotein is visualised as the lowest of three Coomassie-stained bands near the bottom of the gel, and runs just below the 8.1 kDa molecular weight marker (a myoglobin fragment).

Table 1 presents the amino acid composition of the 9 kDa phosphoprotein from pea together with amino acid compositions predicted from the published

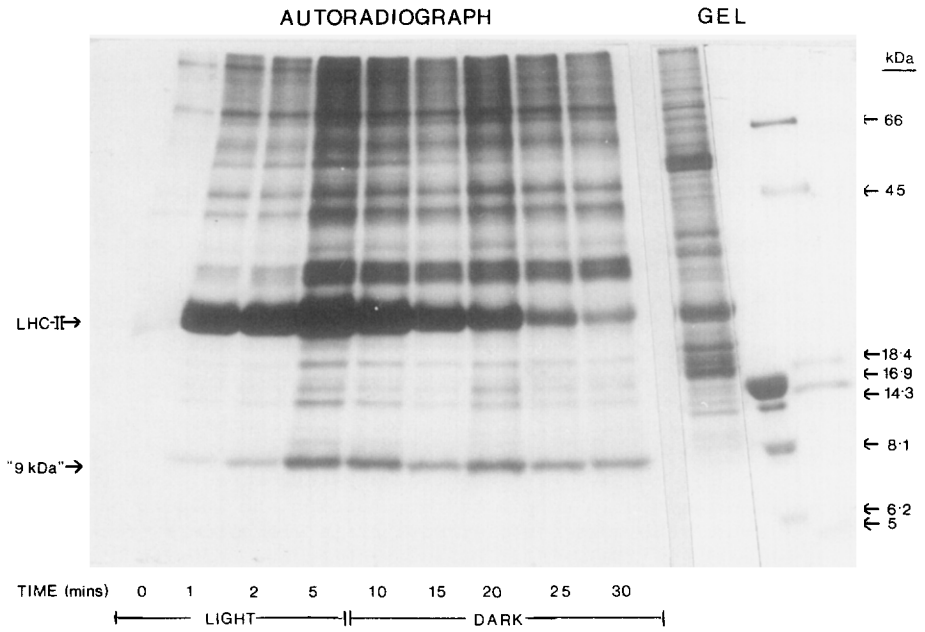

Figure 1. Autoradiograph of ^{32}P-labelled polypeptides from pea thylakoids separated by SDS-polyacrylamide gel electrophoresis. Thylakoids were incubated in the presence of [γ-^{32}P]ATP (100 μM). Increasing protein phosphorylation is seen in a number of bands, including LHC-II and the 9 kDa polypeptide ("9 kDA"), in samples illuminated for 0, 1, 2 and 5 minutes after addition of [γ-^{32}P]ATP. Dephosphorylation of LHC-II is seen in samples illuminated in the same way for 5 min and then incubated in the dark to give 10, 15, 20, 25 and 30 minutes' total incubation time. The Coomassie-stained bands corresponding to LHC-II and to the 9 kDa polypeptide are seen on the right. These were identified by this means for electroelution in a separate experiment. The two tracks on the extreme right show Coomassie-stained molecular weight markers.

Table 1. Amino acid composition in mol% determined for the pea 9 kDa phosphoprotein and deduced from nucleotide sequences of the genes for spinach CF_o subunit III (13) and cytochrome *b*-559 (14)

	9 kDa phosphoprotein	CF_o subunit III	cytochrome *b*-559
Ala	8.03	21.0	3.61
Arg		2.47	8.43
Asx	7.82	2.47	6.02
Cys	1.53	0.00	0.00
Glx	10.42	8.64	7.23
Gly	14.78	13.58	8.43
His	2.93	0.00	1.20
Ile	5.41	7.41	10.84
Leu	8.53	14.81	6.02
Lys	6.75	1.23	0.00
Met	0.74	2.47	1.20
Phe	4.96	3.70	9.64
Pro	5.31	4.94	4.82
Ser	8.98	3.70	14.46
Thr	4.90	3.70	7.23
Trp		0.00	2.41
Tyr	2.68	1.23	3.61
Val	6.24	8.64	3.61

Values for arginine and tryptophan were not determined.

nucleotide sequences of the genes from spinach for CF_o subunit III (13) and for cytochrome b-559 (14). It is seen that CF_o subunit III has neither cysteine nor histidine whereas the 9 kDa phosphoprotein has 1.53 mol% cysteine and 2.93 mol% histidine. Cytochrome b-559 has neither cysteine nor lysine, while lysine is present as 6.75 mol% of the 9 kDa phosphoprotein. Furthermore, CF_o subunit III has a much higher alanine content than the 9 kDa phosphoprotein. We propose that the disparities in amino acid composition (Table 1) are such as to eliminate the possibility that the 9 kDa phosphoprotein is either CF_o subunit III or cytochrome b-559.

Table 2 compares the same amino acid analysis of the 9 kDa phosphoprotein with results obtained from electroeluted LHC-II. The comparison reveals an unexpected similarity between the two proteins. The results obtained with electroeluted LHC-II are also seen (Table 2) to be in close agreement with amino acid compositions predicted from published nucleotide sequences of a pea cDNA clone (15) and of the LHC-II gene of Lemna (16). The same conclusion is supported by predictions from the structures of the LHC-II genes of pea (17) and petunia (18) (results not shown). Table 2 also shows the amino acid compositions of two hypothetical polypeptides consisting of N-terminal segments of pea (15) and Lemna (16) LHC-II. These hypothetical polypeptides were selected to correspond to the first 86 amino acids of pea LHC-II, ending Ser-Arg-Asn-Gly-Val-COOH and giving a molecular weight of 9525.9 Da; and to the first 87 amino acids of Lemna LHC-II, ending Ser-Lys-Asn-Gly-Val-COOH and giving a molecular weight of 9679.4 Da. It is seen that these hypothetical polypeptides show even greater similarity to the 9 kDa phosphoprotein than the LHC-IIs from which they derive. In particular, the decreased mol% values for alanine, leucine and phenylalanine and the increased mol% values for cysteine and histidine are in closer agreement with those determined for the 9 kDa phosphoprotein than are those of each LHC-II.

The values for cysteine may be particularly significant. LHC-II contains a single cysteine residue, at positions 75 in pea (15) and 76 in Lemna (16) and accounting for 0.44 mol% in each case. This agrees with the

Table 2. Amino acid composition in mol% determined for pea LHC-II deduced from the nucleotide sequences of a pea LHC-II cDNA (15) and the Lemna LHC-II gene (16).

					Hypothetical polypeptides from:	
	pea LHC-II	pea LHC-II (cDNA)	Lemna LHC-II (gene)	pea 9 kDA	pea LHC-II	Lemna LHC-II
Ala	10.15	9.65	10.48	8.03	8.14	8.05
Arg		2.63	3.05		4.65	5.75
Asx	10.00	7.90	8.29	7.82	6.96	6.90
Cys	0.43	0.44	0.44	1.53	1.16	1.15
Glx	8.99	8.43	9.17	10.42	6.98	9.20
Gly	13.61	12.28	12.66	14.78	11.63	10.34
His	1.74	1.75	1.31	2.93	2.33	1.15
Ile	3.52	3.07	4.80	5.41	1.16	3.45
Leu	10.31	10.96	9.60	8.53	9.30	9.20
Lys	5.57	3.50	4.37	6.75	3.49	4.60
Met	1.28	1.75	2.18	0.74	1.16	2.30
Phe	6.17	6.14	6.99	4.96	4.65	5.75
Pro	6.50	7.02	6.99	5.31	8.14	8.05
Ser	7.31	7.46	3.93	8.98	12.79	6.90
Thr	3.93	3.50	3.49	4.90	5.81	5.75
Trp		2.63	2.62		3.49	3.45
Tyr	3.42	3.35	2.62	2.68	4.65	3.45
Val	7.06	7.46	6.55	6.24	4.65	4.60

The amino acid conposition of the pea 9 kDa phosphoprotein (Table 1) is repeated in order to facilitate comparison with LHC-II and with the hypothetical polypeptides derived from the first 86 amino acids of pea (15) and Lemna (16) LHC-II. Values for arginine and tryptophan were not determined.

chemically-determined value for pea (Table 2) of 0.43 mol%. The value determined for the 9 kD phosphoprotein (Table 2) is 1.53 mol% cysteine and suggests a single cysteine residue in this shorter polypeptide. The hypothetical polypeptides derived from the LHC-II structures would have cysteine mol% values of 1.16 (pea) and 1.15 (Lemna). In the structure proposed in (16) for LHC-II on the basis of a hydropathy plot, the single cysteine is located in the first of three membrane-spanning α-helices. If the 9 kDa phosphoprotein had a structure similar to that of the part of LHC-II represented by its N-terminal, surface-exposed segment together with the first hydrophobic domain, then the single cysteine residue of the 9 kDa phosphoprotein would be located in a comparable position, that is, in its single membrane-spanning α-helix. The same considerations apply to the first hydrophobic domain's single histidine (LHC-II positions 64 in pea and 65 in Lemna).

We propose on the basis of the results in Table 2 that the 9 kDa phosphoprotein is a chlorophyll-binding protein structurally related to LHC-II, with a single membrane-spanning α-helix containing one cysteine and a chlorophyll-binding histidine. The N-terminal, surface-exposed phosphorylation site would also then be similar to that of LHC-II, with phosphorylation and dephosphorylation of the two polypeptides being catalysed by a single kinase and phosphatase.

This proposal has important implications for the biogenesis and function of chlorophyll-binding proteins. If it is correct, then the 9 kDa phosphoprotein would presumably be a nuclear-encoded protein synthesised cytoplasmically and imported into the chloroplast in precursor form. Furthermore, it would show sequence homology with its evolutionary relative, LHC-II. Sequencing work is in progress.

The functional implications of our proposal are that the 9 kDa polypeptide is a component of an intermediate light-harvesting complex which serves to link excitation energy transfer from LHC-II to the reaction centre of PS II and which may be the origin of the 77 K fluorescence maximum at 695 nm (19). As suggested elsewhere (20), regulation of excitation energy distribution in green plants would then involve mutual electrostatic repulsion between the phosphorylated forms of the 9 kDa polypeptide and LHC-II. This mechanism is proposed as a special case of a more general regulatory phenomenon in which photosynthetic unit function is modified by phosphorylation and hence by mutual electrostatic repulsion of peripheral and intermediate light-harvesting units (20).

A recent paper (21) describes an amino acid composition for the spinach 9 kDa phosphoprotein isolated from PS II particles. The result is in broad agreement with the one presented here (Table 1). The authors (21) rule out CF_o subunit III and cytochrome *b*-559 on the basis of their analysis and on an N-terminal sequence of nine amino acid residues; they draw no comparison with LHC-II such as that in Table 2. They conclude only that the 9 kDa

phosphoprotein is "a unique, as yet unidentified, polypeptide associated with PS II" (21).

ACKNOWLEDGEMENTS

We thank Andrew Cuming, Nigel Holmes, Conrad Mullineaux, Darryl Pappin and Christine Sanders for helpful discussion and Michael Harrison, Tony Thackrah and Martin Thompson for expert assistance with the experimental work. Supported by SERC research grants to J.F.A. and J.B.C.F.

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