Separation of phosphorylated from non-phosphorylated LHCP polypeptides by two-dimensional electrophoresis

R. Rémy, F. Ambard-Bretteville and G. Dubertret*

Laboratoire de Photosynthèse and **Laboratoire de Cytophysiologie de la Photosynthèse, CNRS, 91190 Gif-sur-Yvette, France*

Received 21 May 1985

'In vitro' phosphorylated thylakoid polypeptides were studied by means of different electrophoretic techniques. A highly resolving two-dimensional electrophoresis method, recently developed in the laboratory using CHAPS and SDS as detergent for electrofocusing, allows the separation of each of the LHCP apoproteins into several molecular species. Those having more acidic isoelectric points correspond to the phosphorylated forms.

Two-dimensional electrophoresis Electrofocusing Light-harvesting complex Phosphorylation Polypeptide Pea

1. INTRODUCTION

Higher plants and algae are able to regulate light energy distribution between their two photosystems. This regulation is thought to involve the reversible phosphorylation of LHCP polypeptides. According to the actual model of regulation, a mobile part of LHCP, when phosphorylated, migrates from appressed thylakoid regions (rich in PS II) to non-appressed regions (rich in PS I). The reverse movement occurs when LHCP is dephosphorylated. The abundant literature published on this topic in the last decade can be found in recent reviews [1–4]. At present, among the many questions raised by LHCP phosphorylation, a major problem is knowledge of the exact amount of LHCP which can be phosphorylated and, to this aim, to achieve the biochemical separation of phosphorylated and non-phosphorylated LHCP forms. Conventional methods such as SDS-PAGE do not allow this separation. Several years ago, we developed 2-D electrophoresis to characterize different lines of higher plants, especially *Brassica*, by their mitochondrial or chloroplast polypeptides [5]. Unfortunately, as mentioned in [5–7], LHCP polypeptides generally run as streaks in 2-D separations due to their hydrophobicity. Recently, using CHAPS as detergent, we greatly improved the resolution of our 2-D analyses. From total thylakoid polypeptides or purified LHCP, we succeeded in the separation of LHCP polypeptides into several molecular species differing in isoelectric point [7]. Here, we demonstrate that phosphorylated LHCP polypeptides can be separated from non-phosphorylated ones.

Abbreviations: CF1, chloroplast coupling factor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; chl, chlorophyll; DCMU, 3-(3′,4′-dichlorodiphenyl)-1,1-dimethylurea; IEF, isoelectrofocusing; LHCP, light-harvesting chlorophyll *a/b*-protein complex; L1, L2, L3, LHCP1, LHCP2, LHCP3; Lx, LHCPx – the light-harvesting complex of photosystem I; PS, photosystem; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Plant material and chloroplast isolation*

The investigations were carried out with pea (*Pisum sativum* L. cv. Kelvedon Wonder) grown in a greenhouse at 22°C and 15 h day length.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30 © 1985 Federation of European Biochemical Societies

Chloroplast isolation was performed according to [8].

2.2. *Thylakoid phosphorylation*

The method in [8] was followed except that chlorophyll concentration in the incubation medium was 150 μg/ml and ATP was 100 μM. Illumination was provided by a white light (500 $\mu E/m^2$ per s). When required, 10 μCi [γ-^{32}P]ATP (Amersham, spec. act. 3000 Ci/mM) were added to the reaction mixture. Phosphorylated samples were defined as those illuminated for 15 min followed by 15 min in the dark in the presence of 20 mM NaF. Dephosphorylated samples differed in that NaF was added only after the dark period. Non-phosphorylated samples were defined as having 30 min dark period with ATP before adding NaF.

2.3. *Fluorescence measurements*

Room-temperature fluorescence induction transients were performed on a laboratory-built device [9] after dilution of the samples at 5 μg chl/ml and in the presence of 2×10^{-5} M DCMU.

Fluorescence emission spectra were recorded at 77 K as in [10] with samples containing about 10 μg chl/ml.

2.4. *Two-dimensional electrophoresis*

Two kinds of 2-D electrophoresis were performed differing in the nature of the 1-D. We used either SDS-PAGE of undenatured chlorophyll-protein complexes as in [10,11] or IEF performed with acetone-depigmented thylakoids. This IEF technique using CHAPS and SDS as detergent has been detailed in [7]. The second dimensions were performed in 12% acrylamide gels according to [12].

3. RESULTS

3.1. *Fluorescence controls of the phosphorylation state of thylakoids*

Fig.1 presents the fluorescence induction transients. As demonstrated in [8], the magnitude of the variable component is reduced in the phosphorylated sample compared to the non-phosphorylated one. Moreover, the induction curve is rather exponential instead of sigmoidal. The dephosphorylated sample shows an in-

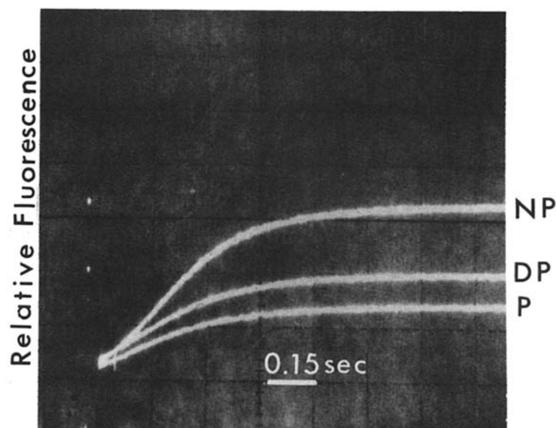

Fig.1. Room-temperature induction transients in presence of 2 μM DCMU of phosphorylated (P), dephosphorylated (DP) and non-phosphorylated (NP) thylakoids.

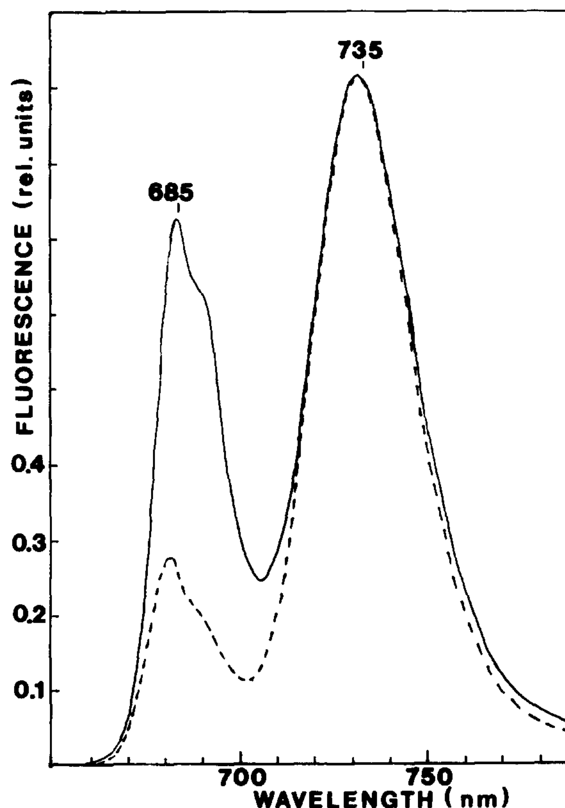

Fig.2. Low-temperature (77 K) fluorescence emission spectra of phosphorylated (---) and non-phosphorylated (——) thylakoids. Spectra are normalized to 735 nm. Excitation, 470 nm.

termediate curve where the maximum level of fluorescence observed with the non-phosphorylated sample is not reached. This is probably due to insufficient dephosphorylation, the occurrence of photodestruction being unlikely, since pigment photobleaching is not observed. Under the 3 conditions investigated, the initial level of fluorescence is constant. Fig.2 compares the fluorescence emission of phosphorylated and non-phosphorylated samples. The short-wavelength PS II fluorescence emissions decrease relative to the PS I 735 nm emission indicating that, as in [13], an increased proportion of the absorbed excitation energy is partitioned to PS I in the phosphorylated sample.

3.2. *Two-dimensional analysis of phosphorylated chlorophyll-protein complexes*

Comparison of fig.3A and B confirms that the major [γ-^{32}P]ATP-labelled polypeptides are those of LHCP, either in its oligomeric forms (LHCP1 and LHCP2) or monomeric form (LHCP3). The 1-D separations presented on the right-hand side of fig.3A and B and in fig.4 show that the 2 constitutive LHCP polypeptides of 29 and 27 kDa are labelled. Other polypeptides about 43, 33, 20 and 11 kDa show faint labelling (figs 3B and 4). It is noteworthy that a 24 kDa polypeptide (arrow in fig.3A) that we mentioned earlier as constitutive of a light-harvesting complex corresponding to the peripheral antenna of PS I [10] is non-phosphorylated (fig.3A,B).

3.3. *Two-dimensional analysis of phosphorylated thylakoid polypeptides*

Fig.5A shows a typical 2-D electrophoresis of phosphorylated thylakoid polypeptides. Up to 90 polypeptides are detected. As reported in [7], the location of LHCP spots was performed from the 2-D analysis of purified LHCP. Here, LHCP is resolved into at least 5 polypeptides. Two major

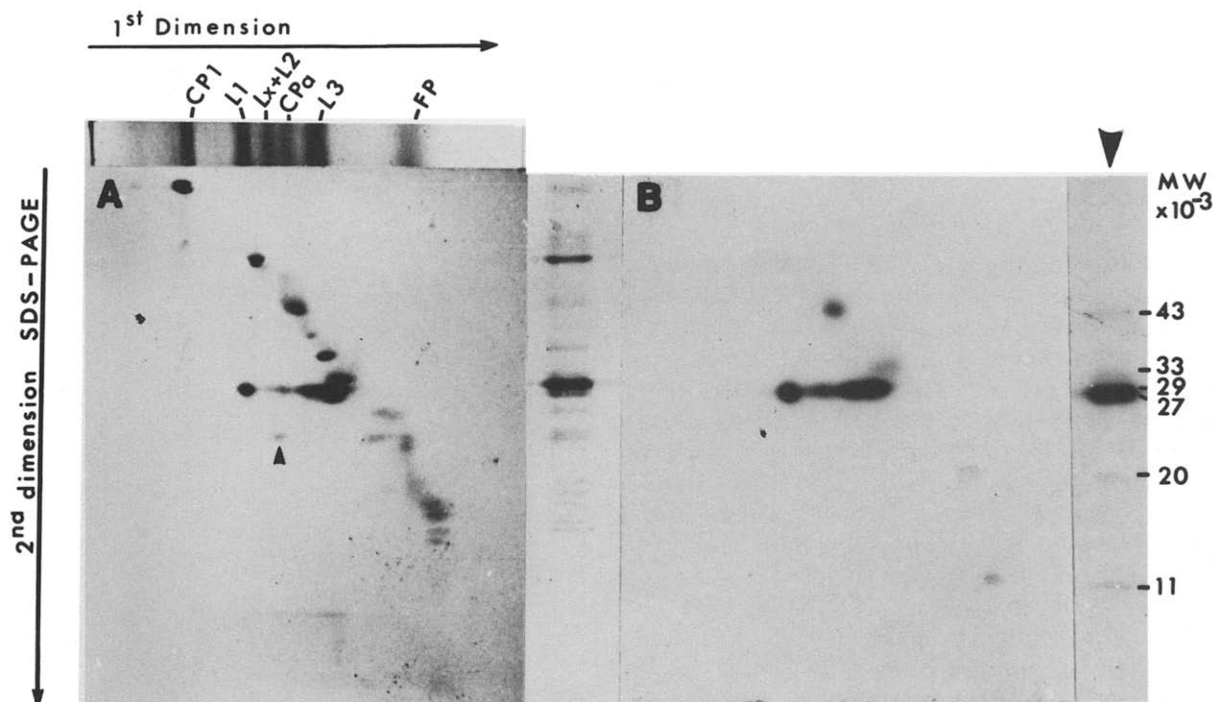

Fig.3. Two-dimensional electrophoresis of phosphorylated pea thylakoids. First dimension, chlorophyll-protein complexes resolved by mild electrophoresis. Second dimension, polypeptides resolved by SDS-PAGE. (A) Coomassie blue-stained gel. (B) Autoradiogram of the gel in A. Large arrowheads to the right of A and B show, as control, a 1-D electrophoresis of thylakoid polypeptides. The small arrowhead in A indicates the 24 kDa polypeptide constitutive of the light-harvesting complex of the peripheral antenna of PS I.

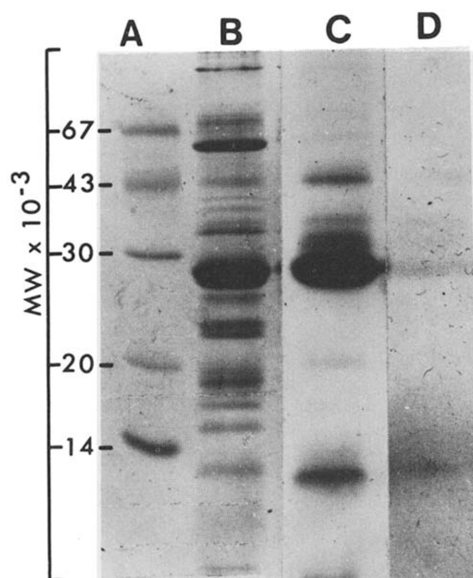

Fig.4. SDS-PAGE of phosphorylated thylakoid polypeptides. (A) Protein markers in order of decreasing M_r: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin. (B) [^{32}P]ATP light-labelled thylakoid polypeptides stained with Coomassie blue. (C) Autoradiogram of the gel in B. (D) Autoradiogram of [^{32}P]ATP dark-labelled polypeptides.

spots correspond to the 29 kDa apoproteins and two minor spots belong to those of 27 kDa. One additional spot is present at 26 kDa as is more obvious from the details in fig.5C taken from a better 2-D separation of this region. From the autoradiogram of gel A shown in fig.5B as well as in the details in fig.5D, it is clear that labelled LHCP polypeptides are those which possess more acidic isoelectric points. These phosphorylated polypeptides are indicated by the open arrows in fig.5A. By comparing in fig.5A and B the intensity of staining and radiolabelling, it appears that the 29 and 27 kDa polypeptides seem equally labelled relative to their protein content.

Apart from LHCP polypeptides, the other phosphorylated polypeptides previously observed are no longer detected. We explain their disappearance by their low labelling and by the more drastic solubilization process performed for 2-D analysis.

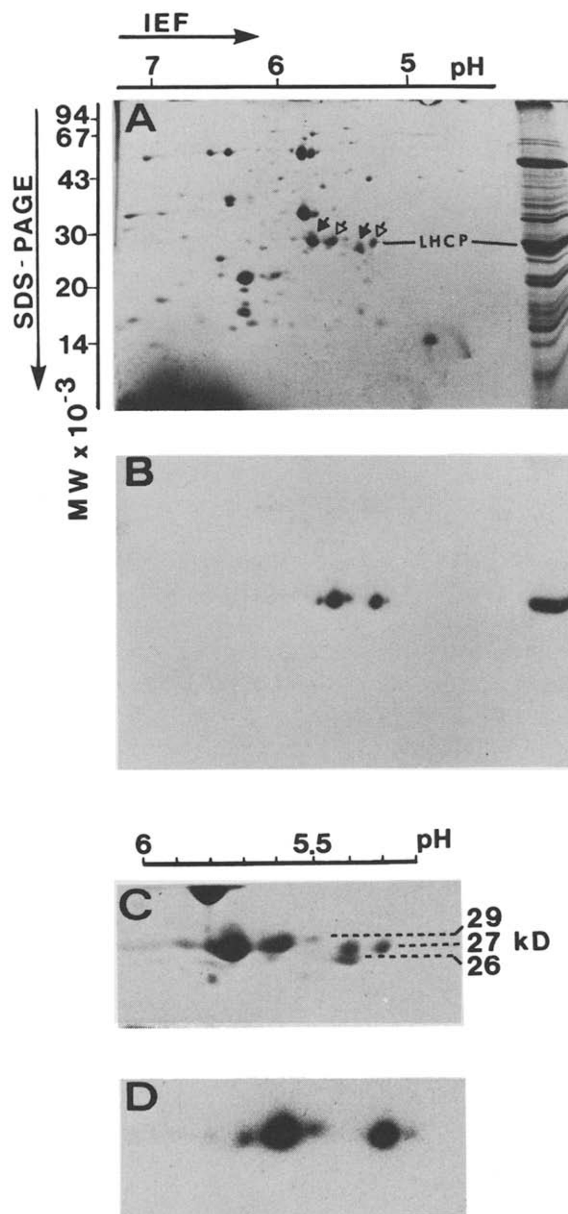

Fig.5. Two-dimensional gel electrophoresis of [^{32}P]ATP-labelled thylakoid polypeptides. IEF in the first dimension, SDS-PAGE in the second dimension. (A) Coomassie blue-stained gel. Filled arrows, non-phosphorylated LHCP polypeptides; open arrows, phosphorylated ones. To the right is a 1-D control with the same sample. (B) Autoradiogram of the gel in A. (C) Details of LHCP spots resolved by 2-D electrophoresis from a gel similar to that in A. Polypeptides stained with Coomassie blue. (D) Details of the autoradiogram corresponding to C.

4. DISCUSSION AND CONCLUSION

Here, we have studied the phosphorylation of thylakoid chlorophyll-protein complexes and polypeptides. The fluorescence data were given as control of the phosphorylation state and will thus not be discussed. Among the chlorophyll-protein complexes, only monomeric and oligomeric forms of LHCP were highly phosphorylated. The chlorophyll-protein complex representing the peripherical light-harvesting antenna of PS I first demonstrated and named LHCPx by us in non-denaturing gels [10,14] and then LHC1 by other groups [15–19] is non-phosphorylated. In addition, in agreement with other data, minor polypeptides of 43, 35, 33, 20 and 11 kDa were also found to be phosphorylated. The original result which is presented here comes from the application of a highly resolving 2-D electrophoretic separation we recently developed [7] which, for the first time, allows discrimination between phosphorylated and non-phosphorylated LHCP apoproteins. It was recently reported that in barley, the high-M_r apoprotein of LHCP consists of at least 3 peptides with the same M_r but different isoelectric points [20]. We confirm this finding for the larger apoprotein of LHCP (29 kDa) with more resolving analyses and show that forms having more acidic isoelectric points are phosphorylated. The isoelectric point difference between phosphorylated and non-phosphorylated 29 kDa forms is estimated at less than 0.1 pH unit. These observations can also be applied to the 27 kDa polypeptide.

It is noteworthy that we are at present unable to prove that phosphorylation induces an acidic shift of the 29 and 27 kDa polypeptides. All attempts we have made to obtain full labelling of LHCP apoproteins have failed. Conversely, under dephosphorylating conditions, each of the 29 and 27 kDa polypeptides always remains composed of at least 2 different electrofocusing spots detected by Coomassie blue or silver staining even if all ^{32}P labelling disappears from acidic LHCP forms (unpublished). If we keep in mind that quantitative variations in the relative amounts of stained polypeptides are difficult to estimate, the possibility of a molecular difference between phosphorylated and non-phosphorylated polypeptides cannot be excluded. To support this suggestion, LHCP is now known to be encoded in the nucleus by multiple genes [21]. To clarify this, we believe that 2-D analysis and purification of these LHCP polypeptides could, in the future, bring forth new information about this subject.

ACKNOWLEDGEMENTS

The authors are grateful to A. de Courcel for improvement of the English paper and thank Mrs Montaggioni for typing the manuscript and R. Boyer for the photographic work.

REFERENCES

[1] Barber, J. (1983) Photobiochem. Photobiophys. 5, 181–190.
[2] Bennet, J. (1983) Biochem. J. 212, 1–13.
[3] Staehelin, L.A. and Arntzen, C.J. (1983) J. Cell Biol. 97, 1327–1337.
[4] Bennet, J. (1984) Physiol. Plant. 60, 583–590.
[5] Rémy, R. and Ambard-Bretteville, F. (1983) Theor. Appl. Genet. 64, 249–253.
[6] Ellis, R.J. (1981) Annu. Rev. Plant Physiol. 32, 117–137.
[7] Rémy, R. and Ambard-Bretteville, F. (1985) Physiol. Vég. 23, in press.
[8] Kyle, D.J., Haworth, P. and Arntzen, C.J. (1982) Biochim. Biophys. Acta 680, 336–342.
[9] Dubertret, G. and Lefort-Tran, M. (1978) Biochim. Biophys. Acta 503, 316–332.
[10] Rémy, R. and Ambard-Bretteville, F. (1984) FEBS Lett. 170, 174–180.
[11] Rémy, R., Hoarau, J. and Leclerc, J.C. (1977) Photochem. Photobiol. 26, 151–158.
[12] Laemmli, V. (1970) Nature 227, 680–685.
[13] Bennet, J., Steinback, K.E. and Arntzen, C.J. (1980) Proc. Natl. Acad. Sci. USA 77, 5253–5257.
[14] Rémy, R., Ambard-Bretteville, F., Dubacq, J.P. and Trémolières (1984) in: Advances in Photosynthesis Research (Sybesma, C. ed.) vol.2, pp.133–136, Martinus Nijhoff/Junk, The Hague.
[15] Lam, E., Ortiz, W. and Malkin, R. (1984) FEBS Lett. 168, 10–14.
[16] Kuang, T.Y., Argyroudi-Akoyunoglou, J.H., Nakatani, H.Y., Watson, J. and Arntzen, C.J. (1984) Arch. Biochem. Biophys. 235, 618–627.
[17] Argyroudi-Akoyunoglou, J. (1984) FEBS Lett. 171, 47–53.
[18] Anderson, J.M. (1984) Photobiochem. Photobiophys. 8, 221–228.
[19] Sarvari, E., Nyitrai, P. and Gyure, K. (1984) Photobiochem. Photobiophys. 8, 229–237.
[20] Li, J.G., Li, Y.X., Gen, Y.X. and Li, J.Y. (1984) Theor. Appl. Genet. 68, 381–384.
[21] Dunsmuir, P. and Bedbrook, J. (1983) Life Sci. Ser.A 63, 221–230.