PURIFICATION OF THE 15 kDa PHOSPHOPROTEIN OF *SYNECHOCOCCUS 6301*

B.F., Nore, M.A., Harrison‡ and J.F., Allen*

Dept. of Biochemistry, Arrhenius Laboratories, Stockholm Univ., Sweden, ‡Dept. of Biochem. and Mol. Biol., Univ. of Leeds, U.K., and *Plant Cell Biology, Univ. of Lund, Sweden.

1. INTRODUCTION

In photosynthetic membranes, protein phosphorylation is a regulatory mechanism responding to changes that would otherwise result in damage or reduced efficiency of photosynthetic energy conversion. One mechanism is readjustment of the relative distribution of excitation energy between the two photosystems [1,2,3].

In cyanobacteria the phosphorylation reaction and associated functional changes in energy distribution are under redox control [4], resembling the redox control of the thylakoid LHCII kinase in chloroplasts [5]. The first identification of light-dependent labelling of both membrane-bound and soluble proteins in *Synechococcus 6301* (*Anacystis nidulans*) was observed with cells grown in the presence of [^{32}P] P_i. The most obvious proteins labelled had apparent relative molecular masses 18.5, 15, 13 kDa [6]. The 18.5 kDa and 13 kDa proteins are predominantly in the soluble fraction and the 15 kDa is located exclusively in the thylakoid membrane fraction. From both fractions other labelled bands were seen but their phosphorylation did not appear to be light-dependent. The illumination that produced labelling of the 18.5 and 15 kDa proteins *in vivo* also gave a shift in chlorophyll fluorescence emission spectra, indicating induction of state 2 [7]. This result is consistent with redistribution of excitation energy distribution in favour of PSI at the expense of PSII [7]. In this work, we describe identification and purification procedures for the 15 kDa phophoprotein of the thylakoid membrane of *Synechococcus 6301*.

2. MATERIALS AND METHODS

2.1 Thylakoid Membrane preparation

Cells of *Synechococcus 6301* were grown at 30 °C in the medium BG 11 as described by Rippka et al. [8] and the thylakoid membranes were prepared as described in Ref. [9] with some modifications. Cell suspension, including a few milligrams of DNase and RNase, was disrupted using a bead beater, 20 cycles of 20 sec on 3 min intervals between each cycle, instead of lysozyme treatment. Finally the thylakoid membrane was resuspended to a chlorophyll concentration of 1 mg Chl a. ml^{-1} in 10% glycerol, 25 mM HEPES-NaOH pH 7.8, 10 mM $MgCl_2$ and 20 mM NaF.

2.2 *In vitro* radiolabelling

Thylakoid membranes (100 µg Chl.a) were incubated in Eppendorf tubes with 500 µl of a buffer containing 10% glycerol, 25 mM HEPES-NaOH pH 7.8, and 10 mM $MgCl_2$ in the dark for 5 min. (γ-^{32}P) ATP (4 µM ≈ 20 µC$_i$) was added to the suspension to 40 µM ATP final concentration and then duroquinol (DQH_2) to 0.9 mM final

N. Murata (ed.), Research in Photosynthesis, Vol. IV, 345–348.

concentration. The mixture was transfered to orange light 2 (PS II light)[cinemoid 5] for 20 min. The reaction was stopped with EDTA to 20 mM. Radiolabelled-membranes were centrifuged at 13,000 rpm for 15 min. Samples were washed twice with 80% Acetone at -20 °C, dried under N_2, and resuspended with 100 µl sample buffer for gradient SDS-PAGE (12%-22.5%) analysis [10], on Coomassie blue stained gels.

For protein purification, suspensions of the thylakoid membranes (5 mg Chl. a) were preincubated in the dark for 15 min. (γ-^{32}P) ATP (≈1 mC_i) was added to the suspension to 20 µM final concentration and then duroquinol (DQH_2) to 0.9 mM final concentration. The mixture was incubated further in the dark for 5 min and then cold ATP was added to 35 µM final concentration. The mixture was transfered to orange light 2 for 20 min. The reaction was stopped with EDTA to 20 mM. The mixture was centrifuged at at 100,000 x g for 50 min. Membranes were washed once with 10% glycerol, 10 mM HEPES-NaOH pH 7.8, 20 mM EDTA and 20 mM NaF. Radiolabelled-membranes were pelleted by centrifugation.

2.3 Rotofor running conditions

The thylakoid pellet was solubilised as described earlier [11] in 30 mls of "extraction buffer" containing 10 mM HEPES, pH 7.8, 20 mM NaF, 10% glycerol, 2% Thesit®, 2% Triton X-100 and 0.2 % dodecyl-β-D-maltoside. The solubilised proteins "membrane extract" (≈ 150 mg total protein) was loaded on a Bio-Rad Rotofor (preparative IEF) system with addition of 5 mM dithiothreitol, 5 mM EDTA and 2 ml Bio-Lyte® ampholyte solution (pH range 3-10; 40% w/v). The Rotofor IEF chamber was filled with extraction buffer (total volume 55 ml). Focusing was carried out for 5 hours at 12 W constant power at 4°C. The initial conditions were 240 V and 50 mA. At equilibrium the values were 1020 V and 14 mA. Twenty fractions were collected, their pH values were measured. Aliquots of each fraction were analysed by SDS-PAGE [10], using Coomassie blue stain.

3. RESULTS AND DISCUSSIONS

3.1 Conditions for *in vitro* radiolabelling

A set of incubation conditions for *in vitro* phosphorylation of *Synechococcus 6301* thylakoid membranes were used and the corresponding autoradiographs of SDS-PAGE gels are shown in Fig. 1.

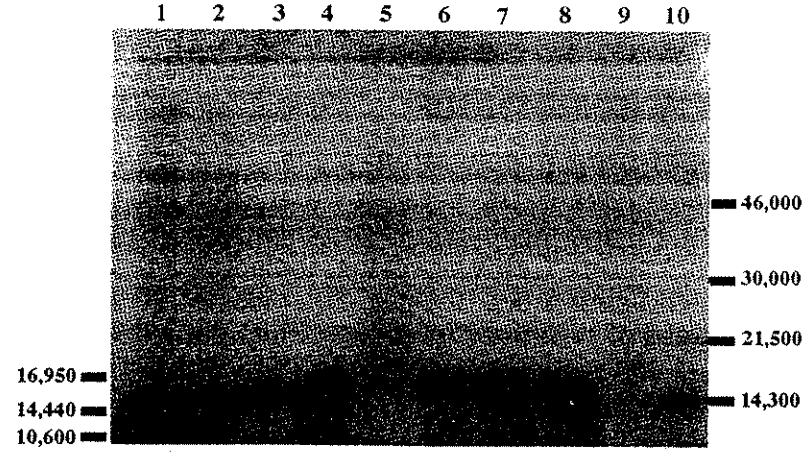

Fig. 1 Autoradiographs of SDS-PAGE analysis of [γ^{32}P]ATP-radiolabelled *Synechococcus 6301* thylakoid membranes under different conditions (for details see under Results and Discussions)

It is seen that the 15 kDa phosphoprotein is labelled specifically in this system and the degree of phosphorylation is affected by a number of reagents. The phosphorylation reaction shows light and NaF–dependence, consistent with the data from Harrison et al. [9]. The presence of 20 mM NaF with light 2 incubation (Lane 1) shows clearly a stimulation of the labelling in comparison to light 2 incubation without NaF (Lane 10). Light 2 incubation (Lane 1) also shows higher labelling than dark incubation (Lane 2) when NaF is present in both conditions. In the presence of 2 mM quinol and under light 2 (Lane 3 and 4) the phosphorylation is elevated, but labelling is much higher when NaF is present (Lane 4). Similar effects are observed when 0.8 mM duroquinol is present, with NaF (lane 7) and without NaF (lane 6). In the presence of 5 mM methyl viologen with 20 mM NaF, the phosphorylation reaction is also stimulated. For control experiments, 5% TCA (lane 5) and 15 mM EDTA (lane 9) final cencentration were added prior to phosphorylation reaction and it is shown that no proteins were labelled. Either quinol or duroquinol with NaF result in maximum labelling (lanes 4 and 7, respectively) compared to other conditions. For subsequent radiolabelling experiments, 0.8-0.9 mM duroquinol and 20 mM NaF under light 2 are used as a standard incubation condition.

3.2 Preparative IEF on the Rotofor System

In order to isolate the 15 kDa phosphoprotein several purification procedures were tested. Initial attempts to fractionate thylakoid membrane proteins with the Rotofor cell were unsuccessful due to protein precipitation at different compartments. These problems were minimised by using 10% glycerol, higher amount of detergents and pre-focussing of the pH gradient before loading the extracted proteins. Still some precipitation in the ends of the compartments was observed. In Fig. 2, a pH gradient of 3.2-12.8 was formed with almost a linear region at pH 3.2 to 10. It is also indicated (Fig. 2) the relative radiolabelling activity of the 15 kDa phosphoprotein. At both ends of the Rotofor compartments (fractions 1-3 and 20), the labelled 15 kDa protein is also observed (Fig. 2). This effect is due to precipitation of proteins at these sites. A single run (4-5 hours) of crude extract (≈150 mg protein) on the Rotofor system produces a good separation of the 15 kDa phosphoprotein (Fig.3). Further analysis of Rotofor fractions by SDS-PAGE (Fig. 3) and autoradiography (not shown) show a purified and radiolabelled 15 kDa band. The 15 kDa phosphoprotein is enriched among fractions 11-14. The fractions 13 and 14 have most purified protein but lower labelling while fractions 11 and 12 less pure protein with high labelling (Fig. 2 and 3).

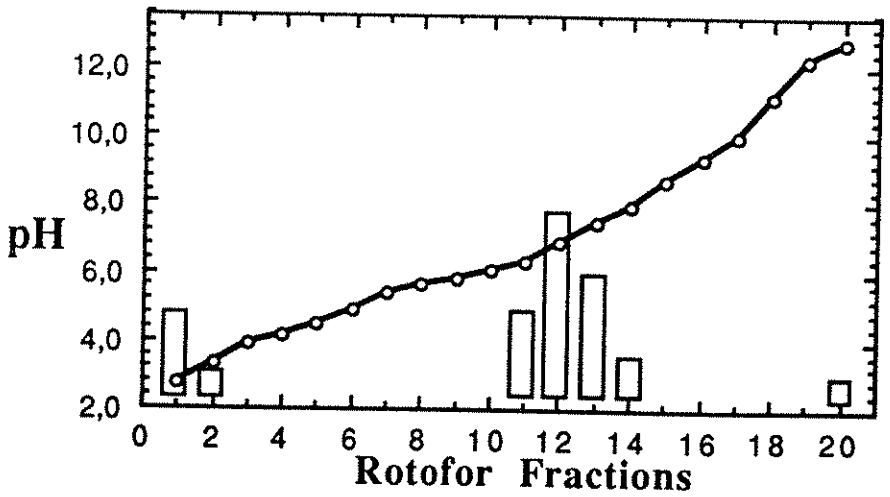

Fig. 2 Illustration of the pH profile of Rotofor separated fractions and relative radiolabelling activity (□) of the 15 kDa phosphoprotein.

This observations suggests that the isoelectric point (Ip) of the phosphorylated protein is shifted toward a lower pH, consistent with the predicted effect of phosphorylation. Therefore, this method can be used for separation between phosphorylated and non-phosphorylated protein forms. Also in these fractions a lower molecular weight protein (≈ 11-12 kDa) is co-purified (Fig. 3). This protein is also a membrane-bound phosphoprotein but it is labelled under different conditions (unpublished results).

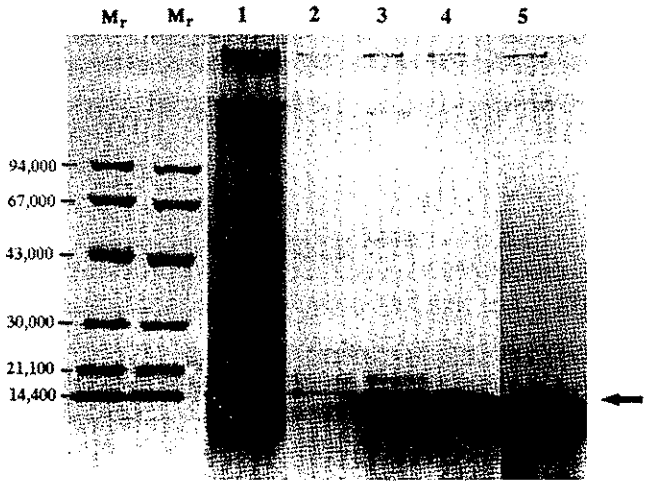

Fig. 3 SDS-PAGE analysis of the Rotofor fractions 11-14 (lane 2-5). Lane 1 is the crude extract of the solubilised thylakoid membrane. The arrow is indicated to the 15 kDa phosphoprotein. Each bands of protein standards (M_r) is equivalent to ≈1.2 μg protein.

4. SUMMARY

Partial purification of the 15 kDa phosphoprotein was achieved using the Rotofor system. This procedure predictes an isoelectric point between 7.0-8.0 for this protein. This technique is also effective for purification of other thylakoid membrane-bound proteins on a preparative scale. The next phase of this work will combine the use of Rotofor purification, SDS-preparative electrophoresis and sequence analysis to determine the identity of this protein.

ACKNOWLEDGEMENTS : BFN and JFA acknowledge Nordic Energiforsknings Program (NLVF) and Carl Tryggers Stiftelse För Vetenskaplig Forskning for support to this project. We thank Prof. Bertil Andersson for providing research facilities and many helpful discussions. BFN thanks Kemila AB (Sollentuna), especially Bo W. Hultgren, for loan of the Bio-Rad Rotofor system.

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