# REDOX DEPENDENT PROTEIN PHOSPHORYLATION AS A FUNDAMENTAL FEATURE OF BIOENERGETIC MEMBRANES IN CYANOBACTERIAL THYLAKOIDS, PURPLE BACTERIAL CHROMATOPHORES AND MITOCHONDRIAL INNER MEMBRANES

André Struglics and John F. Allen, Department of Plant Cell Biology, Lund University, Box 7007, S-220 07 LUND, Sweden

## 1. Introduction

In both eukaryotes and prokaryotes, protein phosphorylation is thought to play a central role in cellular regulation. Protein phosphorylation in regulation of photosynthesis has been intensively investigated in chloroplasts and bacteria (1). Protein phosphorylation in chromatophores from purple bacteria and in thylakoid membranes from cyanobacteria and plants is thought to regulate light-harvesting and electron transport during photosynthesis (1). Evidence for redox-dependent protein phosphorylation in *Rhodospirillum rubrum* chromatophores and in *Synechococcus* 6301 and *Synechocystis* 6803 thylakoids as well as higher plants has been published (2,3,4,5).
Mitochondrial protein phosphorylation reactions previously described in plants are of both soluble proteins such as the $\alpha$ subunit of pyruvate dehydrogenase and of proteins from the outer membrane and inner membrane, but no redox regulatory control has so far been reported (6). Here we describe redox dependent protein phosphorylation from three different membrane systems; thylakoid membranes from *Synechocystis* 6803, chromatophores from *Rhodospirillum rubrum* and inner mitochondrial membranes from potato tubers.

## 2. Procedure

*Synechocystis* sp. PCC 6803 was grown for 3 days in BG11 medium (7) with 10 mM HEPES (pH 8.0) and with 10 mM $HCO_3^-$. The cells were grown in a light intensity of 50 µE and bubbled with 5% $CO_2$ in air. Crude thylakoid membranes were prepared using the method of Murata and Omata (8). The phosphorylation of crude thylakoid membranes started with a preincubation in darkness for 15 min at 30°C, prior to phosphorylation in darkness for 30 min at 30°C. The assays contained (final concentration) 20 mM TES-NaOH and 5 mM $MgCl_2$, pH 7.0. The ATP concentration was 10 µM and the specific activity was 5 µCi/nmol ATP. After 30 min the reaction was stopped by TCA precipitation. The samples were then washed and precipitated by 80% acetone at -20°C and run on 10-15 % SDS-PAGE gel.

Potato tuber mitochondria were prepared as in Struglics et al. (9) and the inner membranes were prepared as in Pical et al. (5). Protein phosphorylation of the inner membrane was carried out in room temperature for 2 min at an ATP concentration of 200 µM. The specific activity was 0.8 µCi/nmol ATP. The assay also contained (final concentration) 50 mM HEPES-KOH, 0.3 M sucrose, 5 mM $MgCl_2$ and 100 µM $CaCl_2$,



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pH 7.5. The reaction was stopped by TCA precipitation and washed with 50 mM HEPES-KOH, pH 7.5 and run on 10-15 % SDS-PAGE gel.

*R. rubrum* was grown anaerobically in the light using a modified Sistrom medium (10). The chromatophores were prepared as in Ghosh et. al. (11). Both the phosphorylation (in the dark) and the sample preparation for SDS-PAGE was carried out as in Ghosh et al. (11).

The gels from the three membrane systems were stained with Coomassie blue, destained, dried and put on an x-ray film for autoradiography.

In the different experiments the membranes were poised by redox reagents such as dithionite ($E_{m7} < -800$ mV), dithiothreitol ($E_{m7} = -330$ mV), ascorbic acid ($E_{m7} = +60$ mV) and ferricyanide ($E_{m7} = +430$ mV), the final concentration of each redox reagents was 5 mM.

## 3. Results and Discussion

Figure 1a shows an autoradiograph of phosphoproteins from the thylakoid membrane of the cyanobacterium *Synechocystis* 6803. 20 µg chlorophyll a was loaded in each track and the Coomassie-stained gel showed equal loading. About 17 proteins were found to be phosphorylated in the dark. The following proteins showed redox dependent protein phosphorylation: 8 kDa, 14 kDa, 15 kDa, 28 kDa, 30 kDa, 37 kDa and 61 kDa. The $^{32}P$ incorporation was higher in samples treated with the reducing reagent dithionite compared with samples treated with the oxidising reagent ferricyanide or with no redox reagent at all. The 8 kDa phosphoprotein could be the same as the one suggested by Race and Gounaris (4) to be psbH. Race and Gounaris (4) also showed that under oxidising conditions the phosphorylation of psbH was inhibited compared with phosphorylation under reducing condition. The redox dependent 15 kDa phosphoprotein could be the same as showed by Harrison et al. (3) to be phosphorylated under plastoquinone-reducing conditions in *Synechococcus* 6301 thylakoids. Harrison et al. (3) also showed an 18 kDa phosphoprotein which was believed to be a phycobiliprotein (12) and has previously been shown to undergo reversible light-dependent phosphorylation in vitro (3). This protein has been identified as phycocyanin-β (13) by sequencing, though it could not be detected in our experiment with *Synechocystis* 6803.

In figure 1b, the autoradiograph shows inner mitochondrial membrane proteins which has been phosphorylated in the presence of different redox reagents and run on SDS-PAGE gel. About 20 phosphoproteins are seen on the autoradiograph and eight phosphoproteins, 10 kDa, 14 kDa, 16 kDa, 17 kDa, 25 kDa, 26 kDa, 28 kDa and 35 kDa show clear redox-dependent protein phosphorylation. All of the redox dependent phosphoproteins except the 28 kDa phosphoprotein had $^{32}P$ incorporation stimulated by oxidising conditions while reducing conditions (in the presence of dithionite) inhibits $^{32}P$ incorporation. The 28 kDa phosphoprotein had $^{32}P$ incorporation stimulated both under oxidising and reducing conditions compared with the control (no redox agents added).

Figure 2 shows an autoradiograph of *Rhodospirillum rubrum* chromatophore proteins, phosphorylated in darkness and in the presence of different redox reagents, and run on SDS-PAGE gel. Five phosphoproteins are seen on the autoradiograph. The α and β polypeptides of the light harvesting complex became more phosphorylated with ferricyanide (oxidising conditions) and ascorbic acid (mildly reducing conditions) then under strongly reducing conditions (dithionite or dithiothreitol). This has also been reported by Ghosh et al. (11) in B873 complexes and by Holmes and Allen (14) in chromatophores. The phosphorylation of a low molecular weight protein (4 kDa), called Ω by Ghosh et al. (11) were slightly less phosphorylated with dithionite compared with the

other conditions. A protein with a molecular weight of 30 kDa was more phosphorylated in the presence of ascorbic acid or dithionite then in the presence of the other redox agents. A protein of the molecular weight of 43 kDa was not phosphorylated in the presence of dithionite but showed phosphorylation under the other conditions.

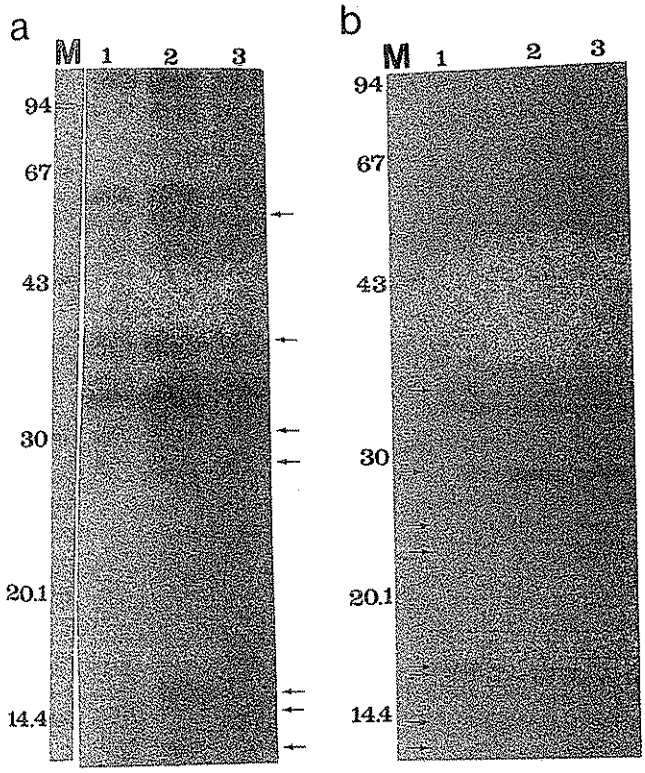


*Figure 1.* Autoradiograph of (a) *Synechocystis* 6803 thylakoid proteins and (b) mitochondrial inner membrane proteins, phosphorylated, with [$\gamma$-$^{32}$ATP] in presence of, lane 1; no redox reagents, lane 2; 5 mM dithionite and lane 3; 5 mM ferricyanide. Lane M shows $^{32}$P labelled molecular weight markers in kDa. The arrows indicate phosphoproteins mentioned in the text.

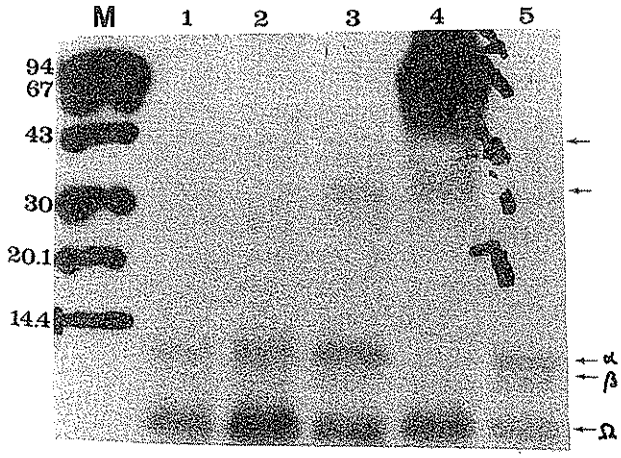


*Figure 2.* Autoradiograph of *Rhodospirillum rubrum* chromatophore proteins, phosphorylated with [$\gamma$-$^{32}$ATP] in the presence of, lane 1; no redox reagents, lane 2; 5 mM ferricyanide, lane 3; 5 mM ascorbic acid, lane 4; 5 mM dithiothreitol and lane 5; 5 mM dithionite. Lane M shows $^{32}$P labelled molecular weight markers in kDa. The arrows indicate phosphoproteins mentioned in the text.

The membranes we have looked at are all coupling membranes of energy-transducing bioenergetic systems. One way of controlling energy transduction may be to have redox-sensitive kinases and/or phosphatases which phosphorylate/dephosphorylate proteins that are directly involved in energy conversion. Besides purely post-translational control of the structure and function of components of bioenergetic systems, redox controlled protein phosphorylation may participate in regulation of gene expression at a number of different levels (15). Our next step is to identify redox dependent phosphoproteins and also to determine the midpoint potentials (Em7). This will help us to find the control point(s) for energy conversion in these different systems.

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