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CYTOCHROME b_6 IN REDOX REGULATION OF PHOTOSYNTHESIS AND CHLOROPLAST GENE EXPRESSION

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INTRODUCTION

Cytochrome b_6 is a component of the cytochrome b_6f complex of the chloroplast thylakoid membrane. The recently described structures of cytochrome b_6f (Kurisu et al 2003, Strobel et al 2003) have provided new insight to the function of the complex (Allen 2004). We suggest that cytochrome b_6, apart from being important to both linear and cyclic electron transport, holds another key function, namely signaling in redox regulation of chloroplast function. This suggestion is supported by the observation that cytochrome b_6 is phosphorylated (Gal et al 1992) and by work of other laboratories showing that the occupancy of the Q_o quinol-binding site of the cytochrome b_6f complex is a factor governing the activation of a thylakoid protein kinase. Sequence comparisons reveal homology of an N-terminal segment of cytochrome b_6 with highly conserved phosphorylatable histidine residue, which sensor histidine kinases and receptor phytochromes could act opon (Hwang et al 2002, Grebe & Stock 1999). Recent phylogenetic analysis of Xiong and Bauer (2002) suggests that cytochrome b_6 is homologous with photosynthetic reaction centres, where the non-haem iron of type II centres (e.g., photosystem II) is coordinated by amino-acid side chains originally ligating haem. Electron transfer events in photosynthesis have been of over-riding importance in cell evolution, and in the function of the chloroplast genetic system.

MATERIALS AND METHODS

Chloroplast isolation and blue-native polyacrylamide gel electrophoresis. Leaves were collected from 12 day-old peas (*Pisum sativum*). The leaves were homogenised in grinding buffer (0.33 M sorbitol, 50 mM HEPES pH 7.6, 5 mM $MgCl_2$, 10 mM NaCl, 2 mM EDTA) and the homgenate was filtered through 2 layers, and then 8 layers, of cheese cloth. The filtrate was then layered onto a Percoll cushion and centrifuged at 600g for 7 min. Intact chloroplasts collected as a green pellet in the bottom of the tube were then resuspended in grinding buffer. The chloroplast suspension (0.33 mg Chl/ml) was incubated for 5 min in light with 400 μCi ^{32}P (Amersham) present. To the ^{32}P incubated chloroplast suspension was ferricyanide or dithothreitol DTT (final concentration 2 mM), added, giving the oxidized and reduced chloroplast samples, respectively. These samples were transferred to darkness for 15 min. The remainder of the preparation was carried out under faint green light. To the chemically redox-treated chloroplasts NaF was added to give a final conc of 10 mM. This concentration of NaF was maintained for the remainder of the preparation. Chloroplasts were collected twice with Eppendorf microcentrifuge at 4000 rpm for 5 min, being resuspended and washed once with grinding buffer. The proteins in the final chloroplast preparation were separated using blue-native polyacrylamide gel electrophoresis essentially according to Kügler et al (1997).

Mass spectrometry analysis. Tryptic digests of excised protein spots were analysed with MALDI-TOF MS (Ultraflex, Bruker Daltonics). Protein identification was achieved by peptide mass fingerprinting.

Sequence comparison. The following databases and sequencing programs were used to establish the conserved N-terminal sequence of cyt b_6. Sequences aligned were downloaded from Swissprot Database. Multiple Alignment program used was ClustalW (http://www.ebi.ac.uk/clustalw/).

RESULTS

The Thylakoid Protein Complexes Show Different Levels of Phosphorylation. Figure 1A and 1B show blue-native polyacrylamide gels of thylakoid membranes reduced (A) and oxidized (B). Figure 1C and 1D shows the same gels, visualizing the ^{32}P labeled complexes by autoradiography.

The blue-native polyacrylamide gels are gradient gels (6–12% acrylamide) run without markers. The different protein complexes were identified using MALDI-TOF MS.

Phosphorylation of thylakoid complexes under oxidizing conditions. Figure 1D shows phosphorylation under oxidizing conditions. The major band in the autoradiograph of blue-native polyacrylamide gel representing the Rubisco (1) complex is strongly phosphorylated. The second most conspicuous band on the gel representing a mixture of PSII and ATPsynthase (2) is also strongly phosphorylated. The region of the gel corresponding to the b_6f-complex (3) has very low levels of phosphorylation, and the same is true for the second band, which represents PSII (4) proteins (CP47, D1, D2, cytochrome b_{559}). Finally the band containing LHCII protein (band 5, (containg a mixure of proteins, mostly Lhcb1, Lhcb2 and CP29)) show high levels of phosphorylations.

Phosphorylation of thylakoid complexes under reducing conditions. Figure 1C shows phosphorylation under reducing conditions,

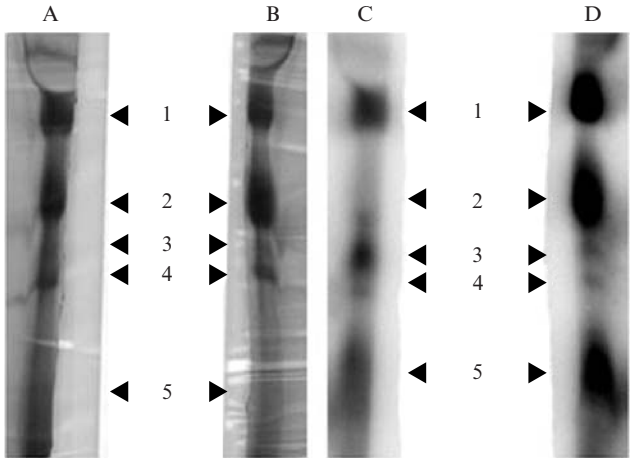

Figure 1: Coomassie stain and Phosphorimage of ^{32}P-labelled protein complexes from a blue native gel of redox treated chloroplasts. (A) and (B) are Coomassie-stained gels, reduced and oxidized. (C) and (D) are phosphoimages, reduced and oxidized. The numbered complex is Rubisco (1), ATPase/PSII (2) , Cytochrome b_6f (3), PSII (4), LHCII (5).

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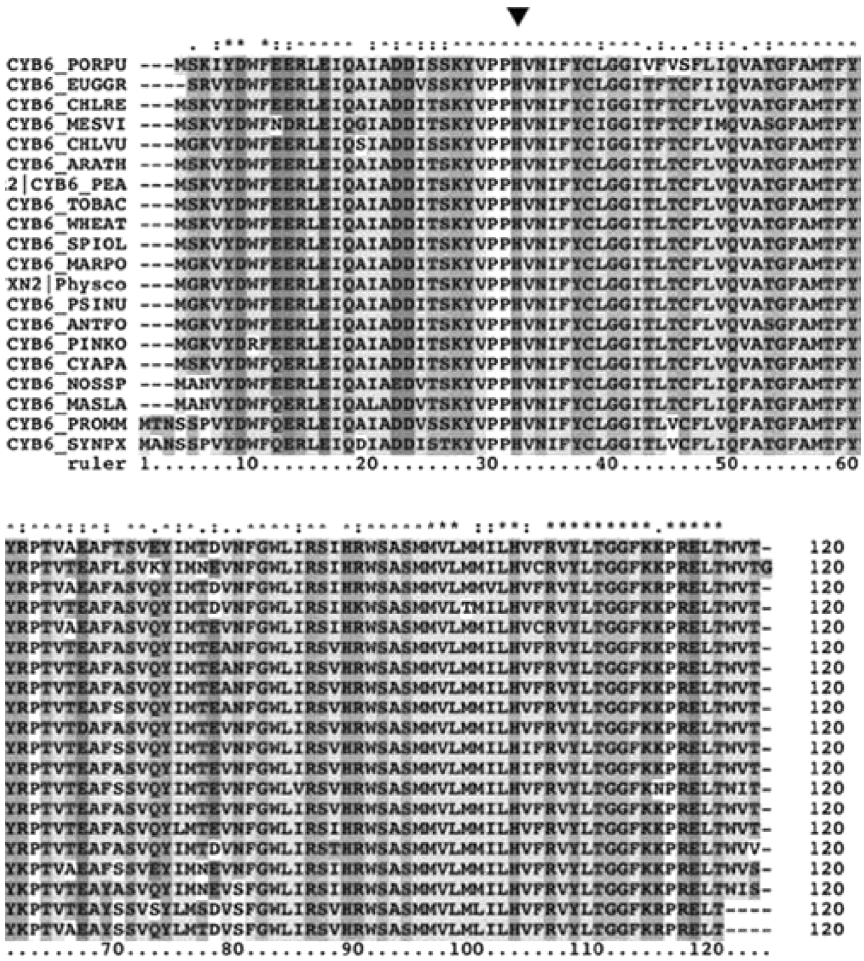

Figure 2: Sequence alignment of cytochromes b_6 from 20 different species (*Chlamydomonas, Arabidopsis, Pisum, Physcomitrella, Nostoc, Synechocystis, Pinus* and more) showing the conserved histidine near the N-terminus.

the major band in the blue-native polyacrylamide gel representing the Rubisco complex is phosphorylated to lesser extent than in Fig. 1D. The second band on the gel, representing a mixture of PS II and ATPsynthase, shows almost no phosphorylation. In contrast, the region of the gel corresponding to the b_6f-complex is now strongly phosphorylated. The second band, of PSII proteins (CP47, D1, D2, cytochrome b_{559}) shows the same low level of phosphorylation as under oxidizing conditions. Finally, the band containing LHCII proteins shows decreased phosphorylation under reducing conditions, but were still strongly phosphorylated.

Sequence Comparison of the N-terminal Region of b_6. We have aligned predicted gene products from *petB* (Fig. 2). The sequences aligned are N-terminal stretch of cyt b_6, length approximately 120 amino acids. A conserved histidine residue lies at the N-terminal part of the alignment (highlighted with a arrow). In the alignment, two of the four histidines, which ligate haem groups) are also seen. The other two haem-ligating histidines lie in the C-terminal part (not included in the alignment).

DISCUSSION

Phosphorylation of the Cytochrome b_6f-Complex. Cytochrome b_6f is involved in redox regulation in the chloroplast, being sensitive to changes in electron flow through the thylakoid membranes. We find that the cytochrome b_6f complex is strongly phosphorylated under reducing conditions. It is known that subunit V of the cytochrome b_6f-complex undergoes reversible phosphorylation (Hamel et al 2000) but subunit V seems to be present only in stroma lamella and not grana (Romanowska & Albertsson 1994). The redox state of the b6f-complex acts as activator of the redox dependent LHCII kinase under reducing conditions (Aro & Ohad 2003). We think that cytochrome b6f is closely involved in both activation of kinases and in regulation of RNA-synthesis. We suggest that the b_6 protein in the complex is phosphorylated (Gal et al 1992) under reducing conditions. This phosphorylation might be the signal initiating changes in rates of RNA synthesis. How this is done is still unclear, but the conserved histidine at the N-terminus of b_6 is a candidate for starting this signal.

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