CHAPTER ONE

Redox Effects on Chloroplast Protein Synthesis and Phosphorylation

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Abstract Chloroplasts and mitochondria have retained their own genomes and genetic systems, distinct from those of the cell nucleus and cytosol. It is proposed that the reason for this retention is that chloroplast and mitochondrial genetic systems enable them to respond quickly and directly to changes in their internal redox state (Allen 1993a). This response is necessary to minimise the production of free radicals, which have the potential to damage and even destroy the cell. By controlling which proteins are synthesised, and how quickly, the individual chloroplast or mitochondrion can ensure the safest and most effective operation of its electron transport chain. The results shown here indicate that if the redox poise of isolated chloroplasts is altered, giving reducing or oxidising conditions, the proteins synthesised become different subsets of the whole. The redox state of specific electron carriers may therefore regulate expression of specific genes in chloroplasts, a conclusion consistent with the hypothesis of co-location of gene and gene product for redox regulation (CORR) (Allen 1993a, 2003). We show that phosphorylation of proteins is also influenced by the redox potential of the organelles. Under reducing or oxidising conditions, different proteins are phosphorylated, indicating levels of post translational control. Some proteins are phosphorylated only under oxidising conditions, and some only under reducing conditions, indicating a response to altered internal redox state, or redox stress. Components of the intra-chloroplast redox signalling pathway may themselves be chloroplast phosphoproteins.

Keywords Redox regulation, redox stress, chloroplasts, evolution, cytoplasmic genomes, protein synthesis, protein phosphorylation, photosynthesis

Introduction

Chloroplasts and mitochondria were once free living organisms with their own independent genetic systems (Gray 1992). Over time, the majority of their genes were transferred to the nucleus, but both organelles retained a few genes within their own "cytoplasm" of chloroplast stroma or mitochondrial matrix. These genes now mostly encode proteins of the electron transport chains, and the necessary

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support genes. Keeping an independent genetic system within the organelles is costly to the organism. Their DNA is in a place where it is most open to attack by oxygen free-radicals generated by electron transport. It is thought that this damage to mitochondrial DNA is at least part of the reason that organisms age and die. The existence of chloroplast and mitochondrial genetic systems continues to pose evolutionary questions. Why are any genes at all retained in the cytoplasm? Would it not be selectively advantageous to cells and organisms for chloroplasts and mitochondria to abdicate all genes and gene expression to the nucleus and to cytosolic translation? Why not let chloroplasts and mitochondria concentrate on what they are good at, namely energy transduction in photosynthesis and respiration? And what might chloroplast and mitochondrially encoded proteins have in common that confers a selective advantage on the cytoplasmic location of their genes?

One proposal is that the size of the proteins to be imported, or their hydrophobicity, makes them difficult to get across the chloroplast or mitochondrial outer membrane (Palmer 1997;Von Heijne 1986). The evidence for this hypothesis is not strong, as many large and/or hydrophobic proteins are in fact imported into the organelles. It is also possible experimentally to take organelle-encoded genes, transfer them to the nucleus, express them there and then import the proteins back into the organelle, where they appear to function in the same way as the native proteins (Kanevski and Maliga 1994; Manfredi et al. 2002). This evidence suggests that any gene could have been transferred to the nucleus, and those that remain have done so for a reason. The debate into this continues, and new ideas are still being published.

Another proposal is that chloroplast and mitochondrial genomes exist to provide a location for certain genes that is close to, and in the same compartment as, their gene products (Allen 1993a, b, 2003; Allen and Raven 1996; Race et al. 1999). In looking at the question of why a set of genes has been retained in the organelles, it would be useful to look at the regulation of the synthesis of the proteins encoded by these genes. It is known that the redox state of the electron transport chain in chloroplasts (specifically the plastoquinone pool) is responsible for post-translational modification, which allows regulation and optimisation of light-harvesting in photosynthesis (Allen et al. 1981). It is possible to study protein synthesis by chloroplasts and mitochondria isolated in vitro (Mullet et al. 1986), so it is possible to measure the effects of various external conditions, such as changes in redox potential, on synthesis of these proteins.

Materials and methods

Chloroplasts were isolated from shoots of 8 days old pea (*Pisum sativum* L.) plants, grown at 20°C with a 12 h day. Protein synthesis assays were based on the method of Mullet et al. (Mullet et al. 1986). The isolated chloroplasts were pre-incubated for 15 min, at room temperature with HEPES/KOH buffer, pH 8, ATP-$MgCl_2$ (1 mM), amino acids (each at 25 µM; all protein amino acids except methionine) and additional redox agents and inhibitors as indicated (Allen et al. 1995). Labelling of synthesised proteins was initiated by addition of 30 µCi of ^{35}S-methionine (>1,000 Ci/mmol). Illumination, where present, was provided by a 40 W fluorescent strip lamp at 50 cm from the sample tubes. After 45 min, intact, labelled chloroplasts were broken by brief osmotic lysis, thylakoids (photosynthetic membranes) and stroma (chloroplast soluble phase) were separated by centrifugation, and samples were dissolved in electrophoresis buffer. An equal amount of protein was added to each lane of the gel. ^{32}P-labelling of intact chloroplasts was carried out in the same way, but without added amino acids, and following the method of Allen and Bennett (Allen and Bennett 1981) except that the incubation time was 10 min.

Results

Redox control of protein synthesis in isolated chloroplasts

In chloroplasts isolated from pea leaves, ^{35}S-methionine incorporation reveals that different

subsets of proteins are selected for synthesis in the presence of the external redox reagents ferricyanide, ascorbate, duroquinol, dithiothreitol and dithionite, and in the presence of different electron transport inhibitors in the light (Fig. 1). The redox state of specific electron carriers may therefore regulate expression of specific genes in chloroplasts (and mitochondria; results not shown). The results are consistent with the hypothesis that chloroplast genomes encode proteins whose synthesis must be regulated by electron transport in photosynthesis or respiration.

Redox control of protein phosphorylation in isolated chloroplasts

Regulation of protein structure and function by phosphorylation of amino acid side chains is universal in living cells. Chloroplast protein phosphorylation is strongly influenced by the redox potential of the isolated organelle (Fig. 2). The 39 kDa stromal protein which is strongly labelled at very oxidising potentials (track 2) but not at reducing potentials (tracks 1, 6–9) we have identified as phosphoribulokinase, which catalyses the conversion of ribulose-5-phosphate to ribulose-1,5-bisphosphate in the Calvin cycle (C. A. Allen, J. F. Allen and J. Ström, 2004). A thylakoid membrane phosphoprotein at 55 kDa may be the chloroplast sensor protein kinase (CSK) reported by Puthiyaveetil and Allen (Puthiyaveetil and Allen 2007) and may respond to plastoquinone redox state in order to determine the phosphorylation state of its substrates and to control chloroplast gene transcription.

Discussion

The chloroplast and the mitochondrion both have electron transport chains which need to operate in a very precisely controlled way, and we have shown that altering the redox potential within the intact organelles changes both the proteins synthesised

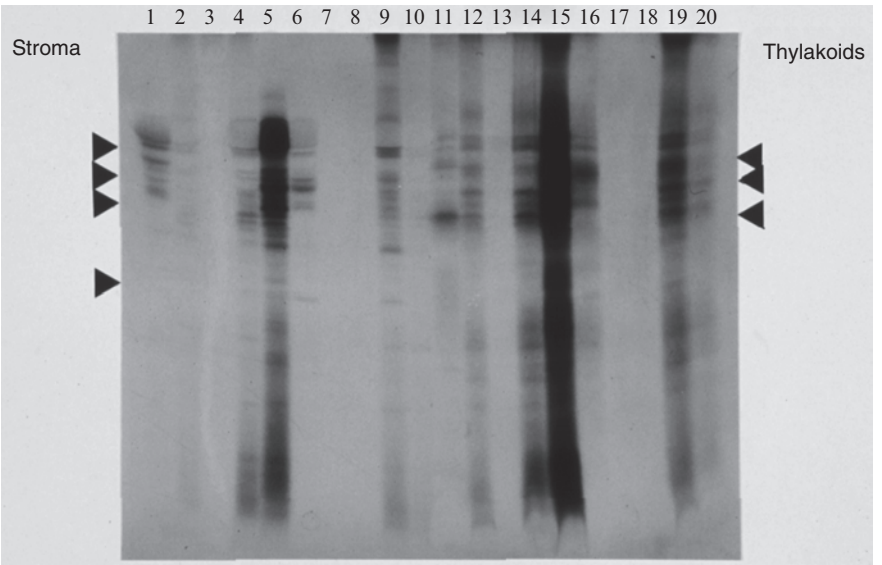

Fig. 1 Autoradiograph of SDS-PAGE gel (15%) showing ^{35}S methionine incorporation into newly-synthesised proteins of isolated, intact pea chloroplasts separated subsequently into soluble (Stroma) and membrane (Thylakoid) fractions. Treatments (1 + 11) Dark (2 + 12) Light (3 + 13) Dark + Ferricyanide (20 mM) (4 + 14) Dark + Ascorbate (20 mM) (5 + 15) Dark + Dithiothreitol (20 mM) (6 + 16) Dark + Dithionite (20 mM) (7 + 17) Light + DCMU (10 µM) (8 + 18) Dark, anaerobic (9 + 19) Light + DBMIB (2 µM) (10 + 20) Dark + Duroquinol (1 mM). Triangles indicate the positions of bands whose labelling is most strongly redox-dependent (Adapted from Allen et al. 1995)

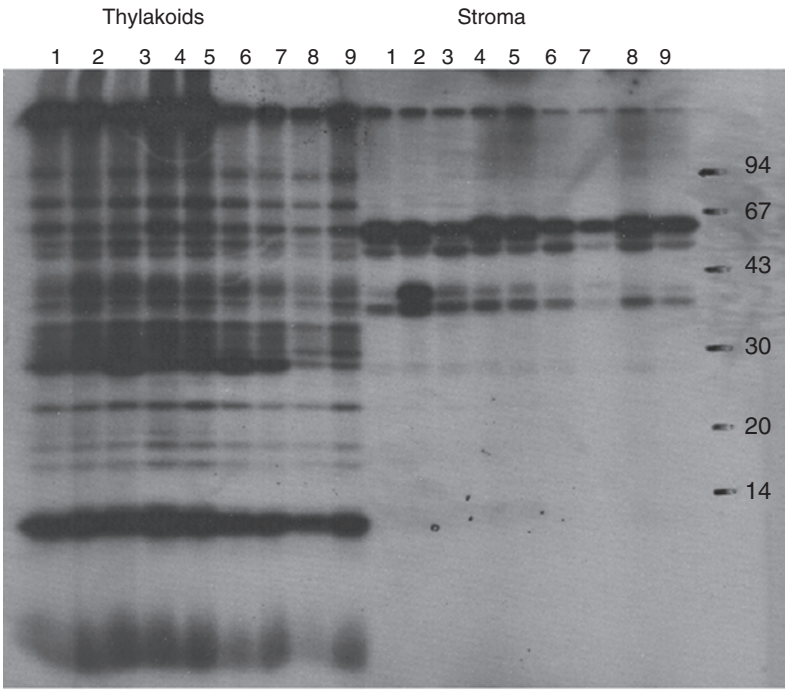

Fig. 2 Autoradiograph of SDS-PAGE gel (15%) showing ^{32}P incorporation from [^{32}P]-Pi into phosphoproteins of isolated, intact pea chloroplasts separated subsequently into soluble (Stroma) and membrane (Thylakoid) fractions. Treatments (1) Dark + ATP, (2) Dark + ATP + Ferricyanide (20 mM), (3) Dark + ATP + Ferrocyanide (20 mM), (4) Dark + ATP + Dehydroascorbate (20 mM), (5) Dark + ATP + Ascorbate (20 mM), (6) Dark + ATP + Dithiothreitol (20 mM), (7) Dark + ATP + Dithionite (10 mM), (8) Dark + ATP + DCMU (10 μM), (9) Dark + ATP + Methyl viologen (50 μM) + DCMU (10 μM). The positions of molecular weight markers are indicated

and the proteins which are phosphorylated. Without this precise regulation, the electron transport chains will generate reactive oxygen species which will not only damage the organelle it is in, but will cause generalised protein damage and mutation in the nuclear DNA. Keeping DNA in chloroplasts and mitochondria is dangerous, but not as dangerous as having electron transport chains operating without permission to regulate themselves.

Our results are consistent with gene expression within chloroplasts being governed by the redox state of electron carriers involved in energy transduction in photosynthesis (Allen 1993a, 2003). The conclusion that chloroplast and mitochondrial genomes serve to permit redox regulation of gene expression has very wide implications for cell evolution and for the structure, function, and properties of eukaryotic cells (Allen et al. 2005, 2007).

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