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Research article

Redox conditions specify the proteins synthesised by isolated chloroplasts and mitochondria

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SUMMARY. **In chloroplasts and mitochondria 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 (in chloroplasts) or with respiratory substrates (in mitochondria). Redox state of specific electron carriers may therefore regulate expression of specific genes in chloroplasts and mitochondria. The results are consistent with the hypothesis that chloroplast and mitochondrial genomes encode proteins whose synthesis must be regulated by electron transport in photosynthesis and respiration.**

Abbreviations

DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU: 3-(3,4 dichlorophenyl)-1,1-dimethylurea; DTT: dithiothreitol; PQ: plastoquinone; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SHAM: salicylhydroxamic acid; UQ: ubiquinone; TCA: tricarboxylic acid.

INTRODUCTION

Chloroplasts and mitochondria are eukaryotic subcellular organelles that contain extra-nuclear genetic systems, yet whose primary functions are energy transduction in photosynthesis and respiration. Their genomes encode a limited but relatively constant subset of proteins involved in energy transduction, together with components of the genetic systems themselves.[1,2] Although chloroplasts and mitochondria almost certainly evolved, via intracellular symbiosis, from free-living bacteria,[3,4] most chloroplast and mitochondrial proteins are now encoded in the cell nucleus and synthesised in the cytosol, in precursor form, for subsequent transport into each organelle. There is currently no agreed explanation for the retention of the limited but apparently essential genomes of chloroplasts and mitochondria, nor for the relative constancy of the subset of their proteins encoded and synthesised in situ. A recent suggestion is that chloroplast and mitochondrial genetic systems are required in order to permit direct redox regulatory control of gene expression in response to changes in photosynthetic and respiratory electron flow.[5,6] Here we show that isolated chloroplasts and mitochondria indeed synthesise specific subsets of proteins when incubated in the presence of different redox reagents, electron donors, and electron transport inhibitors. Changes in redox state of electron carriers may therefore determine the products of organelle gene expression in vivo. Such regulatory control may provide for adaptive responses, at the genetic level, that serve to maintain redox poise, optimize energy transduction, and minimize inappropriate electron transfers or 'redox stress'.

MATERIALS AND METHODS

Chloroplasts were isolated from shoots of 8 day old pea (*Pisum sativum* L.) plants, grown at 20°C with a 12-hour day. Protein synthesis assays were based on the method of Mullet et al.[7] The isolated chloroplasts were preincu-

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bated for 15 min at room temperature with HEPES/KOH buffer, pH 8.0, 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. Labelling of synthesised proteins was initiated by addition of 30 μCi of ^{35}S-methionine (>1000 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.

Mitochondria were isolated[8,9] from green leaves from 11–12 day-old peas and equal volumes (5 pl) of a concentrated mitochondrial suspension were preincubated for 15 min, on a shaker, at 25°C in a reaction buffer (0.4 M mannitol, 60 mM KCl, 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KH_2PO_4, 4 mM ADP, 4 mM ATP, 2 mM Na_2GTP, 0.1% BSA, 25 μM amino acids [all protein amino acids except methionone], pH 7.0), together with different redox reagents or electron transport inhibitors. Labelling of synthesised proteins[9,10] was initiated by addition of 30 μCi of ^{35}S-methionine (>1000 Ci/mmol). Synthesis was performed either in the presence or absence of added respiratory substrates (1 mM Na pyruvate and 10 mM malic acid). Erythromycin (400 μg/ml), a specific inhibitor of chloroplast protein synthesis, was included in the reaction mixture to exclude contamination by chloroplast proteins. After labelling for 75 min, excess resuspension buffer containing unlabelled methionine was added. Samples were then centrifuged and resuspended in electrophoresis sample buffer. An equal amount of protein was added to each lane of the gel.

RESULTS

Figure 1 shows an autoradiograph of ^{35}S-methionine-labelled proteins separated by SDS-PAGE after their synthesis de novo by chloroplasts isolated from pea. Lanes 1 and 8–9 show the water-soluble products of chloroplast protein synthesis in the light. Lanes 11 and 18–19 show the corresponding thylakoid membrane-derived products of protein synthesis in the light. It is seen that the electron transport inhibitor DCMU (which blocks the Q_B site of the photosystem II reaction centre, causing the plastoquinone (PQ) pool to become oxidized) completely inhibits synthesis of both sets of pro-

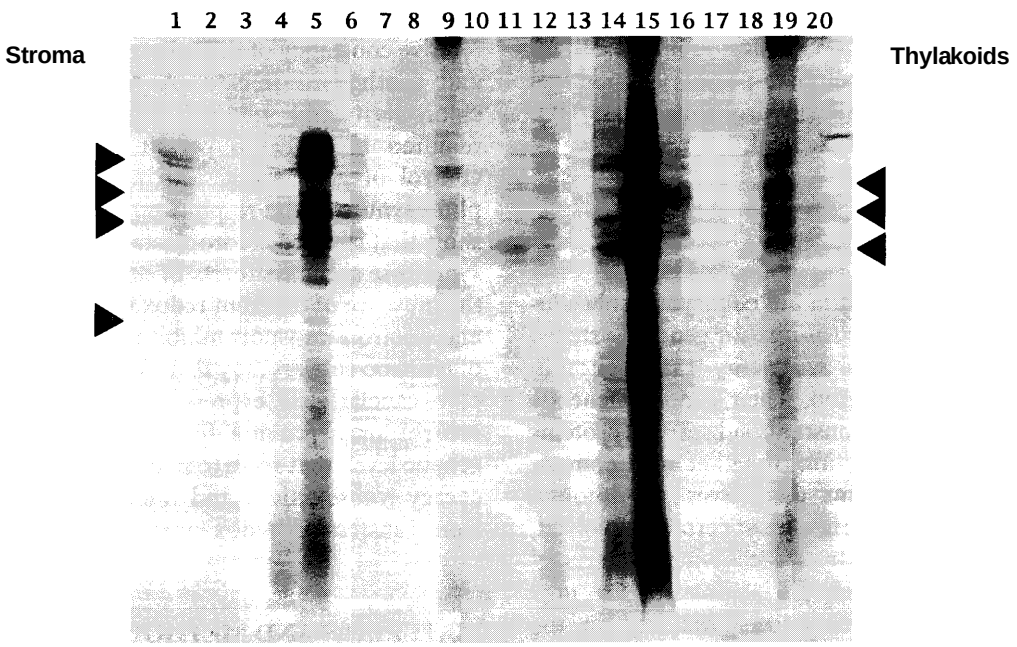

Fig. 1 — Autoradiograph of ^{35}S-labelled SDS-PAGE gel (15%) showing proteins synthesised in isolated pea chloroplasts in the presence of added redox reagents and electron transport inhibitors. Lanes 1–10 show synthesis of stromal proteins and lanes 11–20 show thylakoid proteins. Lanes 1 and 11, controls (light); lanes 2 and 12, controls (dark); lanes 3 and 13, potassium ferricyanide (20 mM); lanes 4 and 14, sodium ascorbate (20 mM); lanes 5 and 15, dithiothreithol (20 mM); lanes 6 and 16, sodium dithionite (20 mM); lanes 7 and 17, anaerobic; lanes 8 and 18, DCMU (10 μM); lanes 9 and 19, DBMIB (2 μM); lanes 10 and 20, duroquinol (1 mM). The positions of specific protein bands with changed intensity compared to the controls (lanes 1–2 and 11–12) are indicated (closed arrow heads).

teins (lanes 8 and 18), while the inhibitor DBMIB (which, at this concentration, blocks plastoquinol oxidation, keeping the PQ pool reduced) permits synthesis of some of each group of proteins while inducing synthesis of others (lanes 9 and 19). This result indicates that electron carriers located between the Q_B and Q_0 sites of the photosynthetic chain may exert redox regulatory control over synthesis of certain chloroplast proteins of both the thylakoid and the stroma.

In darkness, synthesis of both stromal (lane 3) and thylakoid (lane 13) proteins is abolished by the presence in the medium of the strong oxidising agent potassium ferricyanide. This result is consistent with the effect of DCMU on light-dependent protein synthesis, since light absorbed by photosystem I then oxidises electron carriers located after the acceptor side of photosystem II. Also in darkness, presence of the mild reducing agent sodium ascorbate results in synthesis of a subset of the proteins made in the light, a subset (especially at low molecular weight) of the proteins made in the light in the presence of DBMIB, and induces synthesis of a further subset of both stromal (lane 4) and thylakoid proteins (lane 14). A corresponding result is obtained when dithiothreitol (DTT) replaces ascorbate in dark-incubated chloroplasts (lanes 5 and 15). With DTT, certain bands are found in common with each of the other treatments, while a further subset in each chloroplast fraction is seen to be synthesised only in the presence of DTT. DTT is a slightly stronger reducing agent than ascorbate, and is routinely included in chloroplast and mitochondrial protein synthesis assays as a specific reagent for protection of sulphydryl groups. The strong reductant sodium dithionite again produces its own specific class of stromal (lane 6) and thylakoid (lane 16) products of protein synthesis in isolated chloroplasts in darkness. Anaerobic conditions in darkness (lanes 7 and 17) appear to abolish protein synthesis, as seen also with ferricyanide (3, 13) and DCMU in the light (8, 18). The reducing agent duroquinol specifically replaces plastoquinol as an electron donor to the cytochrome b_6/f complex. In chloroplast protein synthesis in darkness, duroquinol supports synthesis of a subset of the proteins synthesised in the light in the presence of DBMIB, in both the stromal (lane 10) and thylakoid fraction (lane 20). In sharp contrast with the autoradiograph, no specific differences in Coomassie-blue staining were seen between any of the lanes of the gel (results not shown).

Figure 2 shows the results of the corresponding experiment carried out on mitochondria isolated from pea leaves. Lanes 1 and 8–9 show the products of mitochondrial protein synthesis in the presence of added respiratory substrates. Lanes 2–7 show the products of

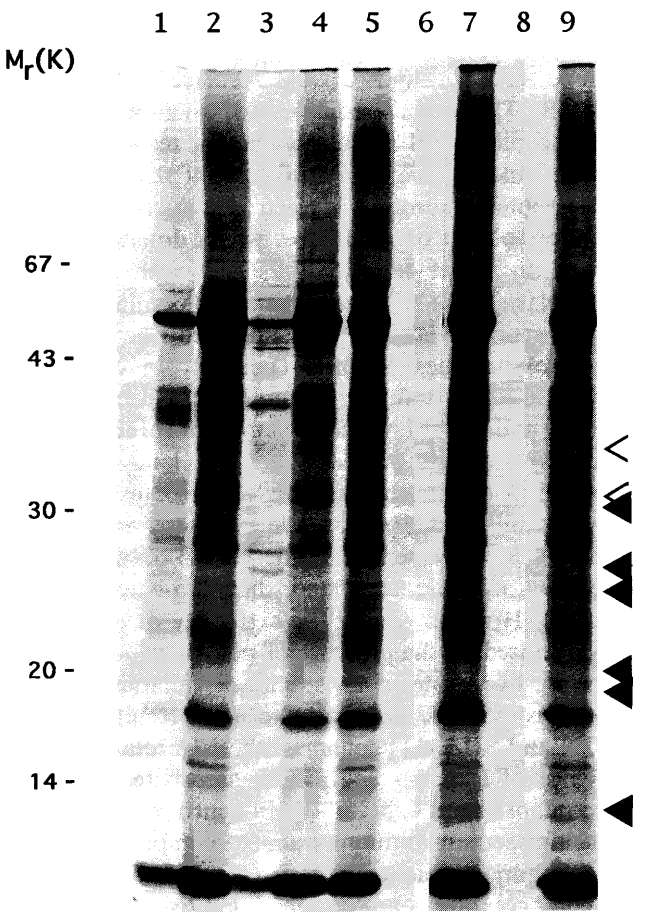

Fig. 2 — Autoradiograph of ^{35}S-labelled SDS-PAGE gel (12–20%) showing proteins synthesised in isolated pea mitochondria in the presence of redox reagents and electron transport inhibitors, as follows. Lane 1, control (respiratory substrates added); lane 2, control (no respiratory substrates added); lane 3, potassium ferricyanide (20 mM); lane 4, sodium ascorbate (20 mM); lane 5, dithiothreithol (20 mM); lane 6, sodium dithionite (10 mM); lane 7, duroquinol (1 mM); lane 8, rotenone (50 μM), malonic acid (5 mM) and dicumarol (20 μM). Rotenone is an inhibitor of complex I (which oxidises NADH produced by the TCA cycle), while malonic acid inhibits complex II (which oxidases succinate to fumarate, as part of the TCA cycle). The additional NAD(P)H-dehydrogenases found in the inner mitochondrial membrane of plants are both inhibited by dicumarol. Lane 9, KCN (1 mM) and SHAM (1 mM). KCN and SHAM inhibits the terminal oxidases cytochrome c oxidase and the alternative oxidase respectively. Specific protein bands which increase (filled arrow heads) or decrease (open arrow heads) compared to the control (lane 2) are indicated.

mitochondrial protein synthesis in the absence of added respiratory substrates. The same general conclusion as that from Figure 1 holds for the results of the experiment in Figure 2: the products of mitochondrial protein synthesis are determined by redox potential, and a specific subset of proteins is therefore synthesised in the presence of each combination of inhibitors and in the presence of different redox reagents. In Figure 2 the respiratory substrates sodium pyruvate and malic acid replace

illumination, and the electron transport inhibitors rotenone, malonic acid and dicumarol or potassium cyanide (KCN) and SHAM replace DCMU and DBMIB. The simultaneous use of rotenone, malonic acid and dicumarol in the presence of respiratory substrates (lane 8) results in oxidation of the ubiquinone (UQ) pool and subsequent electron carriers, since these inhibitors all act on complexes which donate electrons to UQ. In agreement with the result with DCMU in chloroplasts (Fig. 1, lanes 8 and 18), mitochondrial protein synthesis is completely inhibited when the quinone pool becomes oxidized (lane 8). In contrast, a reduced electron transport chain (obtained by addition of KCN and SHAM, which are inhibitors of the terminal oxidases) supports protein synthesis (lane 9). In the presence of the strong oxidising agent potassium ferricyanide, mitochondrial protein synthesis is inhibited (lane 3), in agreement with the results obtained when rotenone, malonic acid and dicumarol were added (lane 8). However, under the extremely oxidizing condition caused by the presence of potassium ferricyanide (lane 3), two proteins are specifically retained and one protein is induced. Consistent with the result in lane 9, (KCN and SHAM) protein synthesis is retained in the presence of the reducing agents ascorbate, DTT and duroquinol (lanes 4, 5 and 7). In contrast, the stronger reductant sodium dithionite almost completely inhibits mitochondrial protein synthesis (lane 6). The majority of redox conditions are accompanied by a specific pattern of mitochondrial protein synthesis, although no differences in protein synthesis could be detected between the control (lane 2) and the lanes with the milder reducing agents ascorbate and DTT (lanes 4 and 5). As with chloroplasts, no specific differences in Coomassie blue-staining could be seen between any of the lanes of the gel (results not shown).

DISCUSSION

The hydrophilic redox reagents used in these experiments (ferricyanide, ascorbate, DTT, dithionite) are unlikely to have had any direct, general chemical effects on chloroplast or mitochondrial transcription or translation, since each reagent has both positive and negative effects on ^{35}S-incorporation into different bands. Apart from this specificity, chloroplast envelopes and mitochondrial outer membranes are impermeable to hydrophilic reagents. Duroquinol is the only redox reagent used here that is likely to penetrate the intact organelles. The hydrophilic redox reagents at substrate concentrations in the suspending medium are assumed to affect the redox state of electron carriers in the intact organelles. Such an effect would require transfer of reducing equivalents across the chloroplast envelope and mitochondrial outer membrane, an assumption to be tested. We also think it unlikely that any of the electron transport inhibitors influenced gene expression directly, and quite improbable that they all did so. The inhibitors were mostly present at micromolar concentrations, and have well-defined effects at specific sites in photosynthetic and respiratory electron transport.

The correspondence between effects of external redox reagents and electron transfer partial reactions selected by donors and inhibitors suggests that redox states of specific electron carriers determine the pattern of protein synthesis in isolated chloroplasts (Fig. 1) and mitochondria (Fig. 2). Specificity in redox control of protein synthesis is suggested by the qualitative differences between lanes in the autoradiographs (Figs 1 and 2), and is not easily reconciled with purely chemical effects on total gene expression or protein turnover.

Free radical-induced protein degradation[11] and inhibition of transcription[12] must be considered as factors that may contribute to at least some of the differences between lanes seen in Figures 1 and 2. However, it would be difficult to explain purely by these means the remarkable specificity of the observed redox effects in increasing labelling of some bands while decreasing labelling of others. In addition, no differences were observed in the relative intensity of Coomassie blue staining between lanes, therefore any proteolysis contributing to differences in labelling would have to be specific not only to particular polypeptides, but also to newly-synthesised polypeptides that have incorporated ^{35}S-methionine during the incubation period. In our view, the simplest interpretation of these results (Figs 1 and 2) is that they arise primarily from redox control of protein synthesis.

We have not yet identified the products of protein synthesis in these experiments, though a number of them can be inferred from relative mobility on SDS-PAGE in conjunction with previous experiments[7–10] and with the complete nucleotide sequences of chloroplast[13,14] and mitochondrial[15,16] genomes. The fractionation of the ^{35}S-labelled chloroplasts (Fig. 1) may also assist identification. For example, the heaviest major band of the chloroplast soluble fraction (most heavily labelled in lane 5) is predicted to be the 55 kDa Rubisco large subunit, rbcL.

The results presented here suggest that chloroplast and mitochondrial gene expression is tightly and specifically controlled by the redox state of electron carriers within each organelle. We propose that such redox control occurs also in vivo. This conclusion is consistent with the idea that the primary function of chloroplast

and mitochondrial genetic systems is to maintain redox homeostasis by permitting adjustment of the stoichiometry and composition of electron transport complexes to changing environmental conditions.[6,17]

In view of the prokaryotic ancestry of chloroplasts and mitochondria[3,4] and of the existence of redox regulatory control of transcription in bacteria,[17] we suggest that the specific redox regulatory effects described here arise from the action on organelle transcription of a number of specific redox regulatory components, though redox effects on translation and RNA processing may also be involved. Furthermore, as each chloroplast (Fig. 1) and mitochondrial (Fig. 2) gene product seen here responds to redox potential in a specific way, we further propose that expression of each protein-coding gene of chloroplasts and mitochondria is under redox control. Redox sensors, response regulators and activators[17] may place expression of each gene under the control of redox state of one or more specific sites in photosynthetic and respiratory electron transport.

Redox control of organellar gene expression may serve to adjust the stoichiometry of electron carriers in response to altered environmental and metabolic conditions.[18] This adjustment may maintain efficiency of energy transduction while minimizing inappropriate electrochemistry such as reduction of oxygen to superoxide by semiquinones and direct production of singlet oxygen by chlorophyll triplet states.

We consider that our results are consistent with the hypothesis[5,6] that the primary function of organelle genomes is the encoding in situ of proteins whose synthesis is thereby able to respond rapidly to changes in redox potential. Organelle genes encoding components of their own genetic systems may serve a secondary function in permitting the maintenance and replication of the extra-nuclear genetic systems whose primary function is maintenance of correct redox balance in eukaryotic cells. Chloroplast and mitochondrial genomes may thus be an essential requirement for the protection of eukaryotic cells from the indiscriminate reactivity of free radicals, an unavoidable consequence of the bioenergetic requirement for electron transfer in photosynthesis and respiration.

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