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Protein synthesis by isolated pea mitochondria is dependent on the activity of respiratory complex II

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Abstract In isolated pea (*Pisum sativum* L.) mitochondria incorporation of ^{35}S-methionine into newly synthesised proteins was influenced by the presence of site-specific inhibitors of the respiratory electron-transport chain. These effects were not produced by changes in the rate of respiratory electron transport itself nor by changes in ATP concentration. Protein synthesis was inhibited by inhibitors of ubiquinone reduction but not by inhibitors of ubiquinol oxidation. By the use of additional inhibitors at specific sites of the respiratory chain, different oxidation-reduction states were obtained for the different complexes in the electron-transport chain. It was found that electron transport through succinate:ubiquinone oxidoreductase (respiratory complex II) was specifically required for protein synthesis, even when all the other conditions for protein synthesis were satisfied. We suggest that a subunit of complex II, or a component closely associated with complex II, is involved in a regulatory system that couples electron transport to protein synthesis.

Key words Mitochondria · Protein synthesis · Succinate dehydrogenase · Regulation of gene expression

Introduction

Control of gene expression in response to changes in redox potential has been shown to occur in bacteria as well as in nuclear gene expression in eukaryotic systems (Iuchi and Lin 1993; Meyer et al. 1994; Mosley et al. 1994). This control allows cells optimally to exploit changing environmental conditions. In bacteria, two-component systems control gene expression by means of "redox sensors", defined as electron carriers that initiate control of gene expression in response to changes in redox potential, and "redox response regulators", DNA-binding proteins that affect gene expression under the action of one or more redox sensors (Allen 1993a). In eukaryotic cells, environmental changes causing alterations in the redox potential of components of electron-transport chains will be rapidly detected in chloroplasts and mitochondria, organelles with their own genetic system. Taking into account the prokaryotic origin of these organelles (Palmer 1992), it is reasonable to expect that redox regulatory systems are also present in chloroplasts and mitochondria (Allen 1993b, c).

Results of recent investigations are consistent with redox regulation of organelle gene expression. By the use of specific electron-transport inhibitors Pearson et al. (1993) showed that RNA synthesis in isolated chloroplasts is favoured by oxidation of the cytochrome b_6f complex. Using the same approach in isolated potato mitochondria Wilson et al. (1996) showed that radiolabelled UTP incorporation into RNA was regulated by the redox poise of the Rieske iron-sulphur protein. Danon and Mayfield (1994) showed redox regulation of the translation of chloroplast *psbA*-messenger RNAs in *Chlamydomonas reinhardtii* in vitro. Translation of these RNAs required binding of nuclear-encoded translational activators. Both translation and binding were found to be inhibited under oxidising conditions (Danon and Mayfield 1994). Furthermore, changes in protein synthesis in response to changes in the redox potential of the reaction medium have been demonstrated in isolated chloroplasts and mitochondria (Allen et al. 1995).

Besides regulating mitochondrial gene expression, the redox state of mitochondria is important for the expression of nuclear genes encoding mitochondrial components. In *Nicotiana tabacum* L., inhibition of electron-transport at the site of complex III by the addition of antimycin A has been shown to result in an increase of the expression of the nuclear gene *Aox1*, which encodes the alternative oxidase of plant mitochondria (Vanlerberghe and McIntosh 1994).

In the present report different respiratory chain inhibitors and respiratory substrates were used in order to alter

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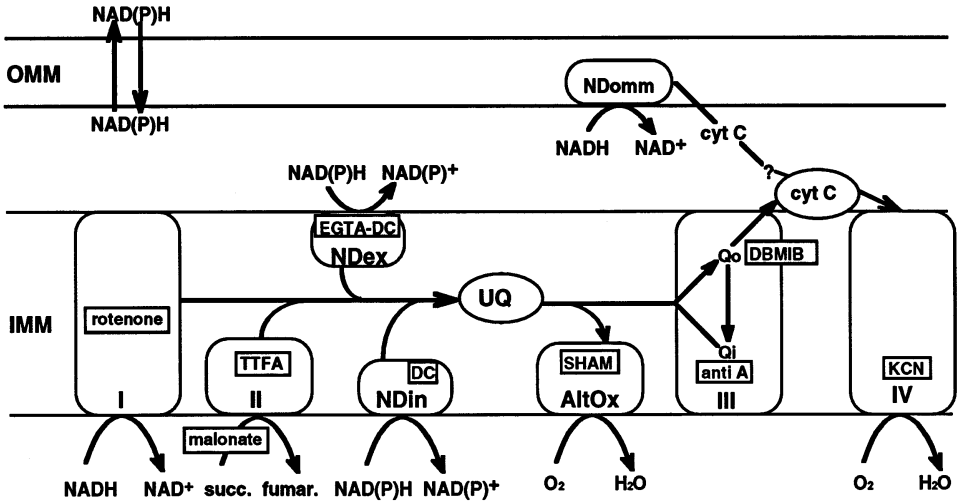


Fig. 1 Schematic presentation of the inner membrane of plant mitochondria and respiratory chain components. Electron transport from NAD(P)H to O_2 involves three of the four multiprotein complexes (*I, III* and *IV*), which are located in the inner membrane. Complex *II* oxidises succinate (*succ.*) to fumarate (*fumar.*) transferring electrons to the UQ-pool and subsequent electron carriers. Electron flow within complex III involves the two centres of cytochrome b (Q_i and Q_o). ATP synthesis by oxidative phosphorylation occurs by means of coupling of the electron flow through complexes I, III and IV to proton transport across the inner membrane. The alternative oxidase (*AltOx*) and NAD(P)H dehydrogenases [*NDin* (internal) and *NDex* (external)] are specific to plants. Respiratory inhibitors and their action sites are indicated. *Anti A* Antimycin A; *DC* dicumarol; *NDom* outer membrane NADH dehydrogenase; *TTFA* thenoyltrifluoroaceton. Adapted from Rasmusson (1994)

the redox potential of specific complexes in the electron-transport chain and analyse corresponding changes in protein-synthesis patterns. Figure 1 shows a schematic presentation of the mitochondrial membrane and components of the respiratory chain, indicating the site of action of the inhibitors used in these experiments.

Materials and methods

Mitochondrial isolation. Mitochondria were isolated from young pea (*Pisum sativum* L.) leaves (11–12-days-old, grown at 20 °C with a 12-h day) according to Boutry et al. (1984), with modifications according to Håkansson et al. (1988).

Redox-regulated translation. Protein synthesis in isolated mitochondria was performed mainly according to Allen et al. (1995). In 10-ml sterile tubes 80–100 µg of protein (≤5 µl of mitochondrial suspension) were added to 100 µl of synthesis medium (5 mM KH_2PO_4, 2 mM GTPdiNa, 0.4 M mannitol, 60 mM HEPES, 10 mM $MgCl_2$, 25 µM of each of the amino acids excluding methionine, 4 mM ADP(K) and 4 mM ATP, 1% bovine serum albumin, pH 7.0). Different respiratory substrates and electron-transport inhibitors were included according to Table 1. After 15 min of incubation at 25 °C on a shaker, 20–30 µCi of ^{35}S-methionine was added to each tube, and the tubes were incubated again for 75 min. To stop the translation, 350 µl of suspension medium (plus 10 mM methionine) was added and the samples transferred to microfuge tubes. After centrifugation for 4 minutes at 16 000 g in a microcentrifuge, the supernatants were removed and the pellets dissolved in 30 µl of electrophoresis sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris/HCl pH 6.8, bromophenol blue and 1% DTT). The solvents DMSO and ethanol (Table 1) had no effect on ^{35}S-methionine incorporation at the final concentrations (not higher than 1% v/v and 1.5% v/v respectively) obtained in experiments with water-insoluble inhibitors (data not shown) . Half of each sample was separated in 12–20% SDS-polyacrylamide gradient gels.

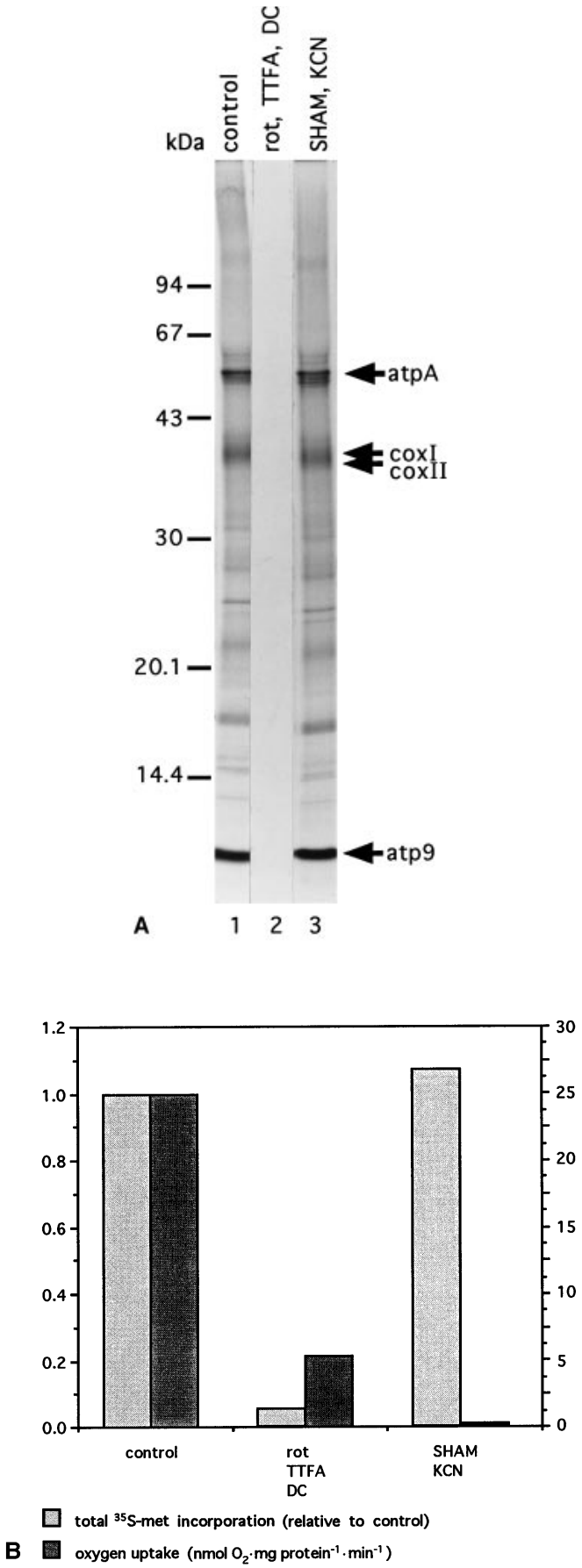
Table 1 Concentrations of respiratory substrates and respiratory chain inhibitors used, unless otherwise stated in figure texts

Common name	Final concentration	Solvent
Antimycin A	0.4 µM	H_2O
$CaCl_2$	1 mM	H_2O
DBMIB	2 µM	Ethanol
Dicumarol	20 µM	DMSO
Duroquinol	0.1, 0.5 or 1 mM	Ethanol
KCN	1 mM	H_2O
Malic acid	10 mM	H_2O
Malonate	5 mM	H_2O
Myxothiazol	15 µM	Ethanol
NADH	1 mM	H_2O
Rotenone	50 µM	DMSO
SHAM	1 mM	DMSO
Na-pyruvate	1 mM	H_2O
Succinate	5 mM	H_2O
TTFA	0.1 mM	Ethanol

Estimation of ^{35}S-incorporation into mitochondrial proteins. Incorporation of ^{35}S-methionine was estimated by the densitometry of autoradiographs using a Personal Densitometer, Molecular Dynamics, and ImageQuant (v 1.11) as a quantification computer program. All the values are expressed relative to the control value except for Fig. 7 where the absolute values of the detected signal were employed.

Measurement of oxygen consumption. Oxygen uptake of the isolated mitochondria was measured with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) at 25 °C in a total volume of 0.4 ml of the *in organello* synthesis medium. The effect of the different additions on oxygen uptake was calculated from the traces and plotted in panel B, Figs. 2, 3 and 4.

Estimation of ^{35}S-methionine uptake. Mitochondria were incubated, according to the procedure for translation, with 200 µM of chloramphenicol to prevent mitochondrial translation. After incubation, mitochondria were centrifuged, washed twice with washing medium (0.4 M mannitol and 10 mM KH_2PO_4) and re-suspended in 30 µl of



the same medium. Half of the volume was mixed with sample buffer at double solute concentration and analysed by gel electrophoresis and autoradiography. The other half was mixed with ReadySafe scintillation liquid (Beckman) and, following the manufacturer's instructions, estimation of the isotope was performed in a LS 6000IC Beckman scintillation counter. ^{35}S-methionine was obtained from Amersham. Chemicals for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. Other chemicals used were from Sigma or Boehringer-Mannheim.

Results

Figures 2–6, panel A, show autoradiographs of ^{35}S-methionine-labelled proteins separated in SDS-polyacrylamide gels after their synthesis de novo in intact mitochondria isolated from pea (*P. sativum* L.). Panels B show rates of oxygen consumption and/or total ^{35}S-methionine incorporation corresponding to the different additions shown in panels A.

Effects of reducing and oxidising conditions on mitochondrial protein synthesis

Figure 2 summarises the effect on ^{35}S-methionine incorporation into mitochondrial proteins obtained by inhibiting electron-transport at the beginning or the end of the respiratory chain (see Fig. 1). The polypeptide pattern observed in the control (Fig. 2A, lane 1) corresponds to mitochondrial translation products reported before, where the 55-kDa band is most likely the α subunit of the F_1-ATPase, the 39–40-kDa bands are the cytochrome *c* oxidase subunits I and II, and the 12-kDa band is subunit 9 of the ATPase (Lonsdale 1989). Under oxidising conditions created by the presence of the inhibitors rotenone, thenoyltrifluoroacetone (TTFA) and dicumarol, which cause inhibition of complexes donating electrons to the ubiquinone (UQ) pool (see Fig. 1), protein synthesis is almost completely inhibited (Fig. 2A, lane 2). On the other hand, salicylhydroxamic acid (SHAM) and potassium cyanide (KCN) inhibit electron transfer from the respiratory chain to O_2, causing all the complexes of the respiratory chain to become reduced in the presence of oxidisable substrates (see Fig. 1). Under such reducing conditions mitochondrial protein synthesis is retained (Fig. 2A, lane 3). The observed differences in protein synthesis (lane 2 vs lane 3) are not correlated with respiratory activity, as measured by oxygen uptake, since both sets of inhibitors cause a dras-

Fig. 2A, B Effects of respiratory chain inhibitors on ^{35}S-methionine-labelling of proteins synthesised by isolated pea mitochondria. Malic acid and Na-pyruvate were added to all the reactions as respiratory substrates. Respiratory chain inhibitors were added as in Table 1. **A** autoradiograph of proteins separated by SDS-PAGE. **B** effect of inhibitors on total amounts of ^{35}S-methionine-incorporation into mitochondrial proteins and on oxygen consumption. The incorporation of ^{35}S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample

tic reduction of oxygen consumption (Fig. 2B). In agreement with these results, and as previously described (Allen et al. 1995), inhibition of protein synthesis was also obtained in the presence of the oxidising agent potassium ferricyanide, while no inhibition occurred in the presence of the reducing agent DTT.

Complexes III and IV

Figure 3 shows the oxygen uptake and polypeptide labelling obtained in the presence of different inhibitors that act specifically on complex III (Esposti et al. 1994). SHAM, an inhibitor of the alternative oxidase, was used together with the complex-III inhibitors in order to rule out the possibility of electron flow through the alternative oxidase. Antimycin A (Fig. 3A, lanes 2, 3, 4, and 6) interacts with the Q_i-site of complex III (Fig. 1) and causes the reduction of cytochrome b. The inhibitors 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) (Fig. 3A, lanes 2, 5 and 6) and myxothiazol (Fig. 3A, lane 3), on the other hand, act specifically on the Q_o-site of complex III (Fig. 1). Together with antimycin A, myxothiazol and DBMIB cause the oxidation of cytochrome *b*. No major differences were seen in the pattern of ^{35}S-methionine incorporation between samples incubated in the presence of Q_i- or Q_o-site inhibitors. Therefore, it is unlikely that there is a regulatory component for the redox control of protein translation within complex III.

Inhibition of complex III (Fig. 3A) causes the oxidation of complex IV while addition of SHAM and KCN (Fig. 2A, lane 3) cause its reduction; no difference in protein patterns, as compared to the control, was observed in these two cases. It is unlikely therefore that the observed effects on protein synthesis seen in Fig. 2 (lane 2 compared with lane 3) results from the action of any redox sensor in complex IV.

This conclusion is strengthened by further experiments. As stated earlier, oxidation of the ubiquinone pool and subsequent electron carriers, caused by the presence of rotenone, TTFA and dicumarol, resulted in the inhibition of protein synthesis. This effect could not be reversed by the addition of up to 1 mM of duroquinol (data not shown), which is able to feed electrons to the respiratory chain at the ubiquinone pool level, thus reducing complexes III and IV.

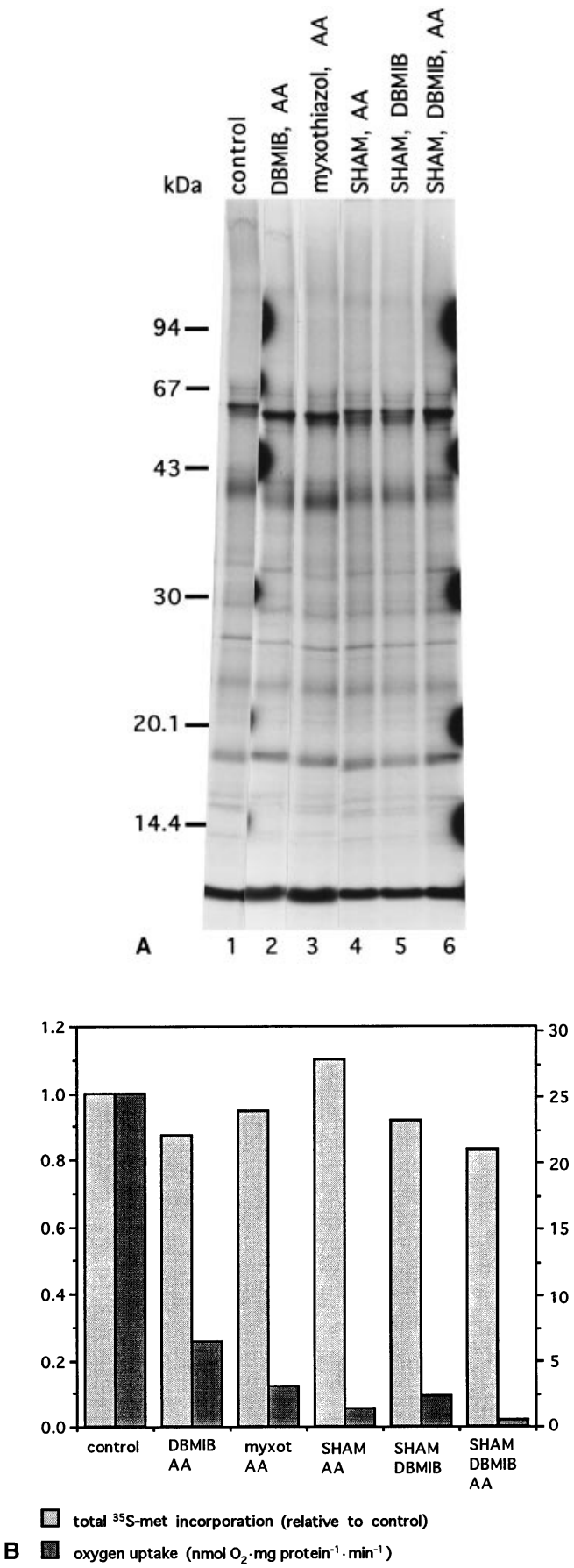
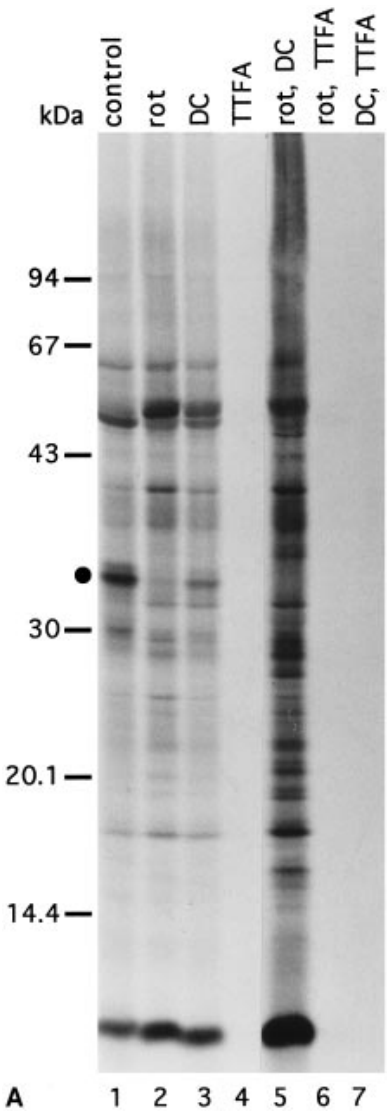
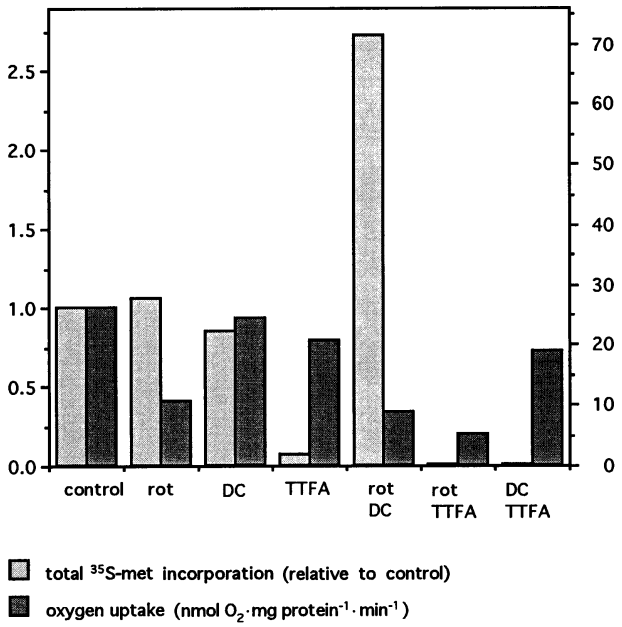


Fig. 3A, B Effects of complex-III inhibitors on ^{35}S-methionine-labelling of proteins synthesised by isolated pea mitochondria. Malic acid and Na-pyruvate were added to all the reactions as respiratory substrates. The alternative oxidase inhibitor SHAM was included in some of the reactions. Respiratory chain inhibitors were added as in Table 1. **A** autoradiograph of proteins separated by SDS-PAGE. **B** effect of inhibitors on total amounts of ^{35}S-methionine-incorporation into mitochondrial proteins and on oxygen consumption. The incorporation of ^{35}S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample





Complexes donating electrons to the ubiquinone pool

To distinguish between the respiratory chain components located prior to the UQ-pool, different combinations of inhibitors for these complexes were employed (Fig. 4). The presence of TTFA in these experiments showed that the activity of complex II is necessary to support the incorporation of ^{35}S-methionine into mitochondrial proteins (Fig. 4A, lanes 4, 6 and 7).

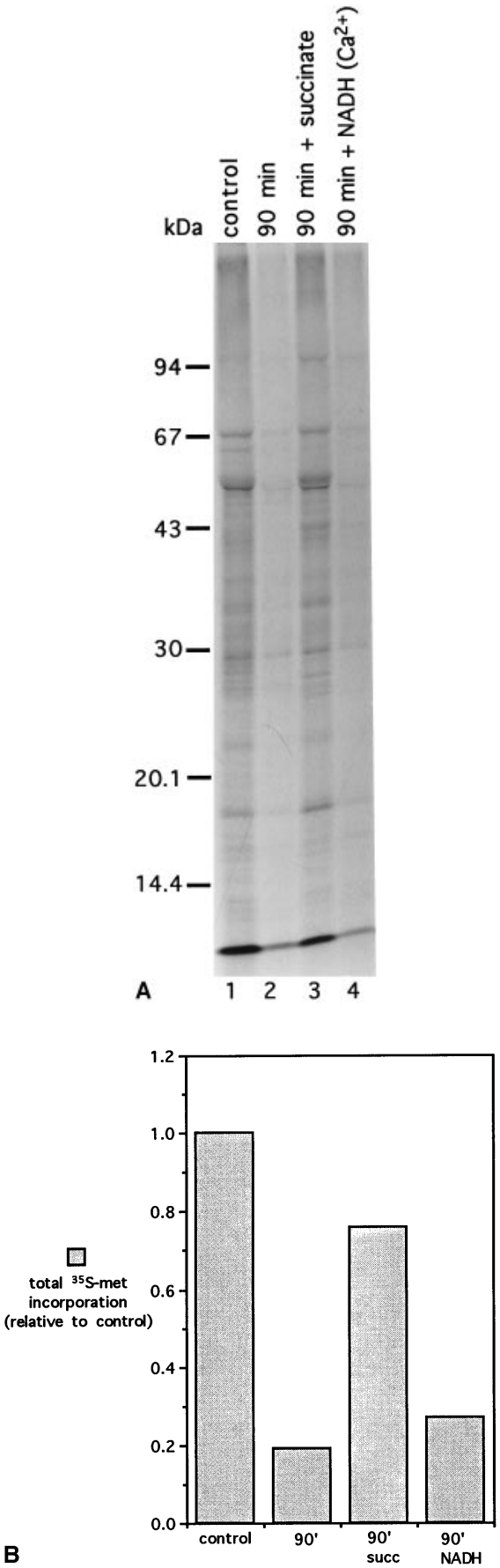
In contrast with this observation, inhibition of complex I (addition of rotenone, lane 2) or the internal and external NADH dehydrogenases (addition of dicumarol, lane 3) does not affect protein synthesis. However, inhibition of all NADH dehydrogenases (rotenone plus dicumarol, lane 5) has a stimulatory effect on mitochondrial protein synthesis. Our results suggest that the activity of complex II is required for protein synthesis, since the addition of TTFA always causes an inhibition of protein synthesis (Fig. 4, lanes 4, 6 and 7; Fig. 2, lane 2). Inhibition of all the NADH-dehydrogenases, on the other hand, results in higher amounts of ^{35}S-methionine incorporation, but only when complex II is still active (Fig. 4A, lane 5 vs Fig. 2, lane 2). Besides these general effects on protein synthesis, alterations in the labelling intensity of specific protein bands were sometimes found after the addition of a respiratory inhibitor (Fig. 4A). These alterations in protein patterns were, however, not consistent.

As described earlier (Fig. 2B), no correlation between oxygen uptake and the incorporation of ^{35}S-methionine into mitochondrial proteins was observed (Fig. 4B). Oxygen consumption is still high in the presence of TTFA (since the activity of complex I maintains respiratory activity) while protein synthesis is inhibited. By contrast, the addition of dicumarol together with rotenone reduces oxygen consumption to half of the control level, while protein synthesis is strongly induced.

Inactivation of the TCA cycle

It was important to determine whether the effect of TTFA on mitochondrial protein synthesis was a result of a direct inhibition of electron-transport within the complex or of an indirect inhibition of the TCA cycle.

Fig. 4A, B Effects of respiratory chain inhibitors on ^{35}S-methionine-labelling of proteins synthesised by isolated pea mitochondria. These inhibitors prevent electron donation to the UQ-pool. Malic acid and Na-pyruvate were added to all the reactions as respiratory substrates. Respiratory chain inhibitors were added as in Table 1. **A** autoradiograph of proteins separated by SDS-PAGE. The *dot* represents a protein band with alterations in intensity between experiments. **B** effect of inhibitors on total amounts of ^{35}S-methionine-incorporation into mitochondrial proteins and on oxygen consumption. The incorporation of ^{35}S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample



Incubation at 25 °C

In further experiments, isolated mitochondria were allowed to consume endogenous substrates by pre-incubation in synthesis medium, at 25 °C, on a shaker for 90 min. Labelled translation was initiated by the addition of ^{35}S-methionine. The polypeptide pattern obtained after pre-incubation (Fig. 5A, lane 2) showed a substantial decrease in labelling compared to that of the control (lane 1). Partial restoration of the protein pattern was attained by the addition of succinate before the labelled translation was initiated (lane 3).

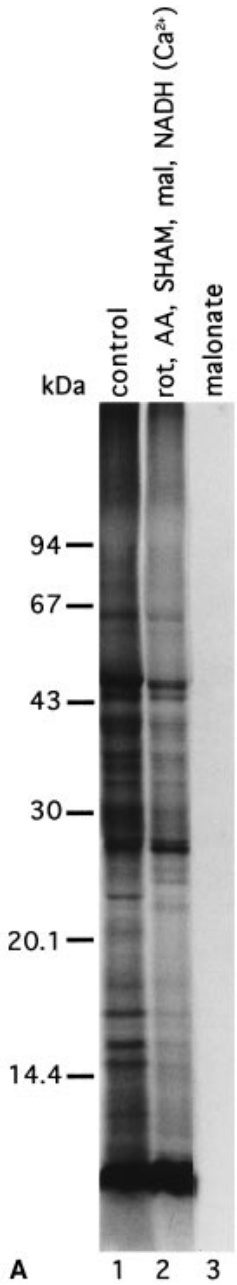
Figure 5 also shows the results of the addition of NADH with Ca^{2+} (addition of Ca^{2+} is necessary for activation of the external NADH dehydrogenase) instead of succinate (Fig. 5A, lane 4). In contrast to succinate, this respiratory substrate was unable to restore the original translation pattern to any degree. Oxidation of succinate (25.7 nmol $O_2 \cdot$ mg of protein$^{-1} \cdot$ min^{-1}) and NADH (18.7 nmol $O_2 \cdot$ mg of protein$^{-1} \cdot$ min^{-1}) was measured in the oxygen electrode with the mitochondria incubated as described above, in order to show that the mitochondria were still able to oxidise these substrates after the pre-incubation period.

These results further point to a specific role of complex II in the regulation of mitochondrial protein synthesis.

Addition of malonate

Malonate, another inhibitor of complex II, has the same effect on protein synthesis as TTFA (Fig. 6A, lane 3), although it affects electron flow through the complex in a different way, namely by blocking the active site of the large subunit of succinate dehydrogenase (Wilson et al. 1996). TTFA, on the other hand, interacts with the quinone-binding site of the complex (Ramsay et al. 1981; Chauveau and Roussaux 1996). The presence of malonate causes the oxidation of the complex, and at the same time inhibits the TCA cycle. Reduction of complex II, in the presence of malonate, was achieved by the reverse-flow of electrons from the ubiquinone pool. Electrons were fed through the external NADH dehydrogenase by the addition of NADH and Ca^{2+}. If at the same time electron-transport through complex I and III (addition of rotenone, antimycin A) and the alternative oxidase (presence of SHAM) is inhibited, a flow of electrons from the reduced ubiqui-

Fig. 5A, B Restoration, by addition of succinate, of ^{35}S-methionine-incorporation into mitochondrial proteins after depletion of endogenous substrates by pre-incubation for 90 min. Synthesis medium was used as a pre-incubation medium. **A** autoradiograph of proteins separated by SDS-PAGE. *Lane 1* control; *lane 2* incubation for 90 min; *lane 3* addition of succinate after incubation for 90 min; *lane 4* addition of NADH and Ca^{2+} after incubation for 90 min. Final concentrations of additions as in Table 1. **B** effects on total amounts of ^{35}S-methionine-incorporation into mitochondrial proteins. The incorporation of ^{35}S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample



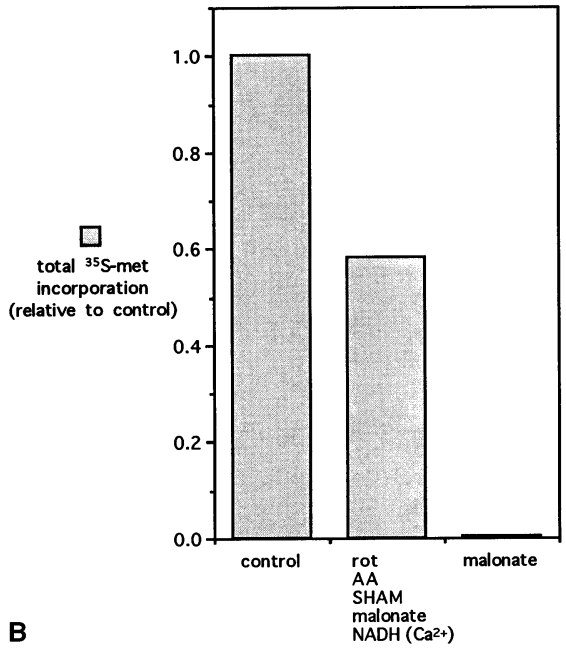


Fig. 6A, B Restoration, by re-reduction of complex II, of the ^{35}S-labelled mitochondrial protein pattern obtained in the presence of malonate. Final concentrations of additions as in Table 1. **A** autoradiograph of proteins separated by SDS-PAGE. **B** effects on total amounts of ^{35}S-methionine incorporation into mitochondrial proteins. The incorporation of ^{35}S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample

none pool to complex II occurs. With this procedure a partial recovery of the initial rate of labelling was observed (Fig. 6A, lane 2).

Influence of ATP concentration

In addition to the investigation of the effect of electron-transport inhibitors, the effect of ATP concentration was studied, in order to rule out the possibility that the results obtained (Fig. 4) merely reflect changes in the rate of ATP production produced by the different inhibitors. ATP titrations were carried out in the absence and presence of 4 mM of ADP (Fig. 7A and B).

In both cases, absence and presence of ADP, the addition of TTFA caused an inhibition of mitochondrial protein synthesis at all the ATP concentrations employed. The higher values of incorporation at low ATP concentrations, in the presence of ADP (Fig. 7B), were mainly a result of the higher incorporation of label into a single protein, the proposed *atp*9 gene product (data not shown; for position of *atp*9 see Fig. 2A).

In contrast to TTFA, addition of the NADH-dehydrogenase inhibitors, rotenone and dicumarol, did not change ^{35}S-methionine incorporation as compared to the control. At the highest ATP concentrations (4 mM) the addition of dicumarol and rotenone increase protein synthesis relative to that of the control, possibly because of high concentrations of ATP in the absence of respiratory inhibitors. Previous work (Lind et al. 1991) has shown that the incorporation of ^{35}S-methionine was inhibited by the external addition of ATP.

On the basis of these observations (Fig. 7), we conclude that the observed effect of TTFA on mitochondrial protein synthesis is not a result of changes in ATP concentration. While the highest values of incorporation for the control were seen at around 3 mM of ATP (Fig. 7A), not even 4 mM of ATP was enough to support protein synthesis in the presence of TTFA.

As mentioned before, respiratory activity and the incorporation of label showed no correlation, strengthening the conclusion that the observed effects are not the result of

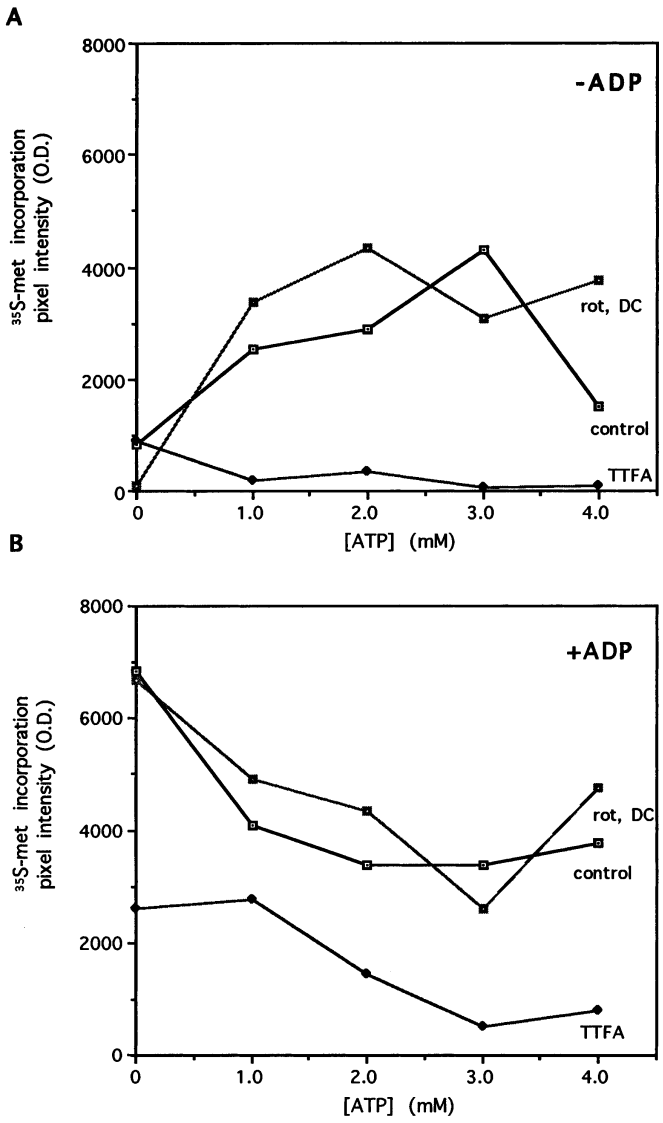


Fig. 7A, B Effect of different concentrations of ATP on the incorporation of labelled methionine into mitochondrial proteins. Malic acid and Na-pyruvate were added to all the reactions as respiratory substrates. **A** in the absence of ADP. **B** in the presence of 4 mM of external ADP

changes in ATP concentration caused by the presence of the different respiratory inhibitors.

Effect of TTFA on amino-acid uptake

It could be argued that the effect of TTFA on mitochondrial protein synthesis is a result of changes in the uptake of ^{35}S-methionine caused by the presence of this respiratory inhibitor. In order to investigate this possibility, mitochondria were incubated in medium containing chloramphenicol, an inhibitor of mitochondrial protein synthesis, in the presence and absence of TTFA. The uptake of labelled methionine was estimated by scintillation counting of the washed and re-suspended mitochondrial pellet. No significant differences ($P = 0.8773$, 95% confidence level) in the uptake of labelled methionine were observed between samples with (155 440 ± 12 538 cpm) and without TTFA (151 213 ± 22 422 cpm).

Discussion

Oxidising conditions of the mitochondrial electron-transport chain created by the inhibition of the electron-donating complexes (Fig. 2) inhibit mitochondrial protein synthesis. In previous work (Allen et al. 1995) addition of the oxidising agent potassium ferrycyanide was shown to have a similar effect, suggesting that mitochondrial protein synthesis is controlled by oxidation-reduction reactions. These results favour the hypothesis that mitochondrial translation is inhibited when some component of the respiratory chain, which therefore acts as a redox sensor (Allen 1993a), becomes oxidised. In agreement with this, previous work (Danon et al. 1994) has shown that reducing conditions promote translation in isolated chloroplasts. Pearson et al. (1993) and Wilson et al. (1996), on the other hand, showed that UTP incorporation into chloroplast and mitochondrial RNA, in isolated plant organelles, was inhibited when specific components of the electron-transport chain were reduced. These examples of the redox regulation of organelle gene expression in plants might indicate differential regulation depending on the level of gene expression.

It was found that the activity of complex II is necessary for mitochondrial protein synthesis (Fig. 4). Inhibition of complex I and at the same time of alternative NADH dehydrogenases, on the other hand, promotes protein synthesis. The increase in the rate of incorporation of labelled methionine into mitochondrial proteins upon inhibition of the NADH dehydrogenases could result from the production of inhibitory concentrations of ATP when these complexes are active (Fig. 7). However, since rotenone and dicumarol also increase protein synthesis at low ATP concentrations, these results could indicate the presence of at least two regulatory points. Low activity of the NADH dehydrogenases together with an active complex II promotes protein synthesis. It could be argued that this effect reflects a physiological state where the production of reducing power by the TCA cycle exceeds the limits of its consumption by the respiratory chain. Under such conditions there will be a demand of new components of the electron-transport chain, and thus protein synthesis needs to be up-regulated. Under these same conditions succinate dehydrogenase activity will be high. Alterations in the redox state of the succinate:ubiquinone oxidoreductase might therefore be a key regulatory point for protein synthesis in mitochondria. Care has to be taken, though, in interpreting these results since all experiments were carried out with isolated mitochondria, separated from the rest of the cell.

While succinate dehydrogenase creates a direct connection between the TCA cycle and the respiratory chain, all other TCA-cycle enzymes are located in the matrix. Succinate dehydrogenase activity depends on succinate pro-

duction and therefore TCA-cycle activity. The activity of the TCA cycle depends on the oxidation of NADH and $FADH_2$ by the respiratory chain. Succinate dehydrogenase is also one of the most active enzymes in isolated mitochondria, showing very complex kinetics, suggesting a possible regulatory activity. However, no evidence for a regulatory role of succinate dehydrogenase in the TCA cycle has been found (Wiskich and Dry 1985).

The absence of incorporation of ^{35}S-methionine into newly synthesised polypeptides after TTFA treatment could be caused either by inhibition of the TCA cycle or by the blocking of electron-transport through complex II. In cases where mitochondria were starved of endogenous respiratory substrates before labelled translation was initiated (Fig. 5) the TCA cycle becomes inhibited, since it is depleted of substrates (NAD^+). In these cases, partial recovery of translation with the addition of succinate prior to the initiation of labelled translation indicates that the activity of the TCA cycle is not correlated with the regulation of mitochondrial protein synthesis. This conclusion is strengthened by experiments where, in the presence of malonate, protein synthesis was supported by an excess of electrons in the ubiquinone pool, having complex II as the only acceptor (Fig. 6, lane 2). This result suggests that the electron flow within the complex is sufficient to support mitochondrial protein synthesis, at least partially, in spite of the inhibition of the TCA cycle. The lack of a full recovery of protein synthesis could reflect the fact that is difficult to obtain full reduction of complex II through flow-back from the UQ-pool. We suggest that the effect of TTFA on protein synthesis is most likely the result of a change in the redox state of complex II or of some component closely associated with this complex.

Oxidation of succinate to fumarate causes reduction of the FAD bound near to the active site of the large subunit (Fp) of complex II (Cramer and Knaff 1991; Igamberdiev and Falaleeva 1994). Electron transfer from FAD to the ubiquinone pool apparently involves two of the three iron-sulphur clusters (S-1 and S-3). The role of the other centre (S-2) is not clear, but a complex interaction with S-1 has been found. Although the different inhibitors (malonate and TTFA) for complex II used in our investigation have different sites of action, they both inhibit mitochondrial protein synthesis. Malonate competes with succinate for the active site on Fp, causing oxidation of the whole complex. TTFA, on the other hand, blocks the electron flow from S-3 to the ubiquinone pool, perhaps at the binding site of the quinone pair (Ramsay et al. 1981), which would lead to reduction of the complex. Recent work (Chauveau and Roussaux 1996) has shown two possible sites of inhibition by TTFA and corroborated its direct interaction with S-3, which would imply a partial reduction of complex II in the presence of TTFA. However, since electron flow within the complex has not been well established, we cannot determine how the redox state of the different components is affected by TTFA, and thus we cannot attribute the function of a redox sensor to any specific component of the complex. Since the mid-point redox potentials (E_{m7}) for the different components are known (Cramer and Knaff 1991), it should be possible to identify the redox sensor within the complex by redox titration (Allen and Holmes 1986).

We conclude that our results are consistent with the hypothesis that the mitochondrial genetic system permits direct redox regulatory control of gene expression by respiratory electron-transport (Allen 1993b). The specific requirement for the activity of complex II suggests that a redox sensor involved in such a regulation is contained within, or close to, complex II. Further work is required in order to establish the mechanisms which couple electron-transport to protein synthesis in mitochondria, and to determine whether this is the only level of gene expression at is which such control is exerted.

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