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Studies on the import and processing of the alternative oxidase precursor by isolated soybean mitochondria

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Abstract

Import of the synthetic precursor of the alternative oxidase from soybean was shown to be dependent on a membrane potential and ATP. The membrane potential in soybean mitochondria may be formed either by respiration through the cytochrome pathway, or through the alternative oxidase pathway with NAD^+-linked substrates. Import of the alternative oxidase precursor in the presence of succinate as respiratory substrate was inhibited by KCN. Import in the presence of malate was insensitive to KCN and SHAM added separately, but was inhibited by KCN and SHAM added together (inhibitors of the cytochrome and alternative oxidases respectively). Import of the alternative oxidase was accompanied by processing of the precursor to a single 32 kDa product in both cotyledon and root mitochondria. This product had a different mobility than the two alternative oxidase bands detected by immunological means (34 and 36 kDa), suggesting that the enzyme had been modified *in situ*. When the cDNA clone of the alternative oxidase was modified by a single mutation (−2 Arg changed to −2 Gly), the processing of the precursor was inhibited.

Introduction

Plant mitochondria contain a branched mitochondrial electron transport chain. In one branch, electrons reduce oxygen in a reaction catalysed by cytochrome oxidase, which is sensitive to KCN. The second pathway is catalysed by the alternative oxidase, which is KCN-insensitive but inhibited by SHAM and *n*-propyl gallate [18]. The alternative oxidase is found in other organisms such as fungi but is best characterised in plants [3, 6, 7, 14]. It is a quinol oxidase on the inner mitochondrial membrane which is not linked to proton translocation across the inner membrane and by-passes the cytochrome bc_1 complex and cytochrome oxidase [18].

A role for the alternative oxidase has been clearly defined in thermogenic plants. In these plants a large increase in alternative oxidase activity coincides with pollen maturation and heat produced by the activity of the alternative oxidase is used to volatilise compounds to attract insects which aid in pollination [21]. Three proteins which increase dramatically with thermogenesis

have been shown to be responsible for alternative oxidase activity in the thermogenic spadix of *Sauromatum guttatum* and monoclonal antibodies have been raised to these proteins [7, 8]. All three proteins cross-react with these antibodies indicating that they are similar. These antibodies have been used to identify alternative oxidase in other plants as well as fungi [10, 11, 14]. In non-thermogenic plants the role of the oxidase is unclear, although all plants examined to date have some activity which may vary between tissues [6, 11, 18].

The identification of the protein(s) involved in alternative oxidase activity has allowed the cloning of the genes for these proteins. Alternative oxidase cDNAs have been cloned from *S. guttatum* [24], *Arabidopsis thalinia* [12], *Glycine max* [32] and the yeast *Hansenula anomala* [25]. This has facilitated the study of the expression of the alternative oxidase and the factors that control it. The alternative oxidase is induced under conditions that inhibit the function or assembly of the cytochrome pathway. This has been shown both for plant cell cultures and yeast where antimycin A, which inhibits electron transport through cytochrome bc_1, induces the alternative oxidase [25, 29]. Inhibition of mitochondrial protein synthesis in *Neurospora crassa* also induces the alternative oxidase [14], as does wounding in potato tubers [10] and cold treatment of tobacco and wheat [16, 27].

To date, two nuclear genes have been reported for most nuclear-encoded mitochondrial proteins, such as the adenine nucleotide translocator in maize [33] and the F1 β subunit of ATP syntase in *Nicotiana plumbaginifolia* [1]. A single nuclear gene for the alternative oxidase (*Aox1*) has been identified and cloned from *S. guttatum* [23] and a single chromosomal localisation has been reported for *Arabidopsis* [12]. However, three different protein bands in *S. guttatum*, with apparent molecular masses of 37, 36 and 35 kDa, are seen on western blots. In soybean, shoot mitochondria have two bands which cross-react with alternative oxidase antibodies whereas in roots only a single band is seen [8, 11, 22, 26]. The intensity of the two bands in the shoots of soybean varies with tissue, developmental age and with light conditions [20]. To date there has been no detailed account of alternative oxidase uptake and processing by plant mitochondria, and the nature of the multiple protein bands on immunoblots has not been identified.

In the present work we report our investigation of the *in vitro* import and processing of an alternative oxidase precursor with soybean mitochondria. Tissue-dependent differences in the appearance of two proteins was investigated using mitochondria isolated from cotyledons and roots.

Materials and methods

Production of precursor protein and in vitro *protein import*

^{35}[S]-methionine-labelled soybean alternative oxidase precursor was produced in a coupled transcription-translation rabbit reticulocyte lysate system (T_NT) from Promega (Madison, WI) using T_7 RNA polymerase. One μg of double-stranded plasmid containing an alternative oxidase cDNA clone from soybean [32] was incubated in a 50 μl reaction for 2 h according to the manufacturer's instructions. Seven-day old, light-grown plants were used for the isolation of cotyledon and root mitochondria as described by Day *et al.* [5]. *In vitro* import experiments were carried out with the post-ribosomal supernatant from the translation lysate [31] except that 5 mM succinate was used as respiratory substrate unless otherwise indicated. When malate (5 mM) was used as a respiratory substrate for import, pyruvate, TPP and NAD^+ were added to a final concentration of 5 mM, 2 mM and 1 mM, respectively. Import in the presence of malate was carried out at pH 6.5 for efficient oxidation of malate [15]. Import was allowed to proceed for 20 min at room temperature (22–25 °C) after which reactions were transferred to ice and some reactions treated for 30 min with Proteinase K as indicated in figure legends. Finally, 2 mM PMSF was added, mitochondria were pelleted, lysed in SDS-PAGE sample buffer and analysed on 12% gels, followed by autorad-

iography. Valinomycin and oligomycin were added to 1 μM each, where indicated. Respiratory competence and energy coupling of the isolated mitochondria were investigated using a Rank oxygen electrode (Cambridge, UK) [11].

Processing studies

Processing of the synthetic precursor was carried out in a reaction volume of 20 μl containing 15 mM Tris pH 8.0, 2 mM $MnCl_2$ and 0.5% Triton X-100. Mitochondria were added to 50% (v/v) to double-strength reaction buffer containing precursor protein, typically 0.5 μl containing 5000 cpm. The reaction was allowed to proceed for 30 min at room temperature. The reaction was terminated by the addition of an equal volume of double-strength gel sample buffer and immediately heated to 95 °C for 5 min. Processed products were examined by 12% SDS-PAGE followed by autoradiography. Two-dimensional gel electrophoresis was carried out by isoelectric focusing with ampholytes ranging from pH 3–10, followed by SDS-PAGE in a BioRad Protean II gel apparatus [13, 19]. The pH range of the 2-D gels was determined by incubating 1 cm slices in 1 ml of distilled H_2O, shaking for 10 min and measuring the pH using a pH meter from Radiometer (Copenhagen).

Site-directed mutagenesis

The mutated alternative oxidase precursor was produced by *in vitro*, site-directed mutagenesis using the Altered sites *in vitro* mutagenesis system from Promega (Madison, WI) according to the manufacturer's instructions. An oligonucleotide with a single-base change (shown below) was used to change amino acid 39 from arginine to glycine:

Normal: 5′-GGT GGT GTG AGG AGT GAG AGC-3′
5′-Gly Gly Val *Arg* Ser Glu Ser-3′

Mutated: 5′-GGT GGT GTG GGG AGT GAG AGC-3′
5′-Gly Gly Val *Gly* Ser Glu Ser-3′

Immunological studies

Western analysis was carried out using a Millipore dry blot apparatus and Hybond C membrane (Amersham, Sydney), according to the manufacturer's instructions. Monoclonal antibodies raised against the alternative oxidase from *S. guttatum* [7, 8], were used to probe the membranes. Cross-reacting bands were visualised using horse radish peroxidase-linked secondary antibody with ECL (Amersham, Sydney).

Results

In vitro *import studies*

The production of ^{35}[S]-methionine-labelled alternative oxidase precursor from the soybean cDNA clone led to the production of one main product with an apparent molecular mass of 36 kDa (P in Figs. 1A and 1B, lane 1). This is very close to the predicted molecular mass of 36.5 kDa derived from analysis of the cDNA sequence [32]. The production of the alternative oxidase precursor was very efficient compared to other clones (data not shown) and this may be due to the enhancement of translation efficiency caused by the five methionines at the start of the clone. An increase in translation efficiency has been reported previously for duplicated start codons in an *in vitro* translation system [30].

When the precursor protein was incubated with mitochondria isolated from soybean cotyledons and roots, a smaller form with an apparent molecular mass of 32 kDa was produced (Figs. 1A and 1B, lane 2). It was concluded from this that the presequence had been removed upon import and this form of the alternative oxidase is thus termed the mature form (M). The first 41 amino acids of the soybean alternative oxidase precursor contain many features common to mitochondrial targeting sequences, being rich in arginine and serine, and lacking acidic residues. This sequence may form an amphiphilic α-helix [28, Fig. 6A]. The 41 amino acids assumed to be the presequence have a predicted molecular mass of

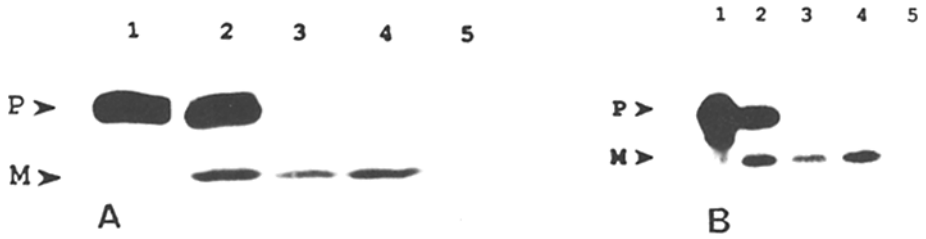

Fig. 1. Import of the alternative oxidase into soybean cotyledon and root mitochondria. A. Cotyledon mitochondria. Lane 1, precursor (P) alone, representing 50% of the precursor added to the import reaction; Lane 2, precursor with isolated mitochondria; an additional mature (M) band is generated; lane 3, as lane 2 with PK added to a concentration of 1 μg/ml; only the mature band is evident; lane 4, as lane 3 with PK added to 2 μg/ml; again, only the mature form is generated; lane 5, as lane 4 with Triton X-100 added prior to PK treatment. B. As A but with root mitochondria.

4 kDa. Thus the apparent size of the mature form is in close agreement to the predicted size of 32.5 kDa [32]. The mature form was insensitive to externally added proteinase K (PK) indicating that it had been imported into the mitochondria (Figs. 1A and B, lanes 3 and 4). Although 1 μg/ml final concentration of PK was sufficient to remove all the precursor remaining outside the mitochondria, 2 μg/ml did not degrade any of the imported precursor and the latter concentration of PK was used subsequently to ensure that all unimported precursor outside the mitochondria was removed (Figs. 1A and 1B, lanes 2, 3 and 4). Lysis of the mitochondria with Triton X-100 allowed the added PK to digest imported protein (Figs. 1A and 1B, lane 5). Examination by SDS-PAGE of the post-mitochondrial supernatants from the import reactions provided further proof that all of the precursor was digested in the presence of PK in contrast to the reactions in the absence of PK (data not shown).

As soybean cotyledon mitochondria display high levels of cyanide-insensitive respiration, we were interested to see if this alternative pathway could support import. To this end, we carried out import in the presence of SHAM, a specific inhibitor to the alternative pathway, and in the presence of KCN, a specific inhibitor of the cytochrome pathway. The results are shown in Figs. 2 and 3. Figure 2 shows *in vitro* import with succinate as a respiratory substrate. The inhibition of import by valinomycin and oligomycin indicates that import was dependent on the presence of a membrane potential (Fig. 2, lanes 2, 3, 4 and 5). Examination of bound but not imported precursor, by washing the post-import reaction with import buffer alone, indicates that binding in the presence of valinomycin and oligomycin was similar to the control. Non-specifically bound proteins have been shown to be removed by such wash procedures [31]. Thus alternative oxidase falls into the Class I category of precursors which bind efficiently in the absence of a membrane potential. The alternative oxidase has two putative hydrophobic, transmembrane helices which may assist binding, as in other class I proteins [9]. The

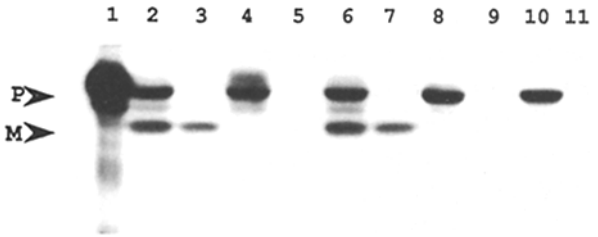

Fig. 2. Import of the alternative oxidase into cotyledon mitochondria with succinate as a respiratory substrate. Lane 1, precursor alone; lane 2, precursor with mitochondria; lane 3, as lane 2 with PK added to a final concentration of 2 μg/ml; lane 4, precursor and mitochondria in the presence of valinomycin and oligomycin; lane 5, as lane 4 with PK added to a final concentration of 2 μg/ml; no imported products are detected; lane 6, precursor and mitochondria with 2 mM SHAM; lane 7, as lane 6 with PK added to a final concentration of 2 μg/ml; imported product is evident; lane 8, precursor and mitochondria in the presence of 2 mM KCN; lane 9, as lane 8 with PK added; lane 10, precursor and mitochondria in the presence of 2 mM KCN and 5 μM oligomycin; lane 11, as lane 10 with PK added, no imported product is evident.

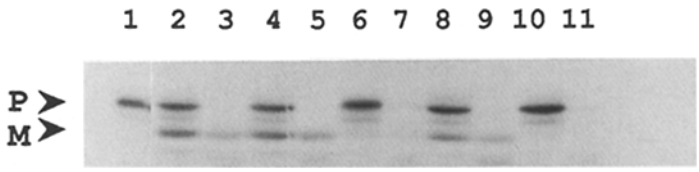

Fig. 3. Import of the alternative oxidase into cotyledon mitochondria with malate as a respiratory substrate. Lane 1, precursor alone; lane 2, precursor and mitochondria; lane 3, as lane 2 with PK added; lane 4, precursor and mitochondria with KCN added to 2 mM; lane 5, as lane 4 with PK added; imported product is evident; lane 6, precursor and mitochondria with KCN (2 mM) and SHAM (2 mM) added; lane 7, as lane 6 with PK added; import is inhibited; lane 8, precursor and mitochondria with SHAM added to 2 mM; lane 9, as lane 8 with PK added; lane 10, precursor and mitochondria in the presence of KCN, SHAM and oligomycin; lane 11, as 10 with PK added.

addition of SHAM alone had no effect on import (Fig. 2, lanes 6 and 7), while addition of KCN alone inhibited import almost completely (Fig. 2, lanes 8 and 9).

Figure 3 shows the results of import experiments using malate as a respiratory substrate. Import was carried out at pH 6.5 so that malate could be oxidised efficiently by malate dehydrogenase [15]. Import in the presence of KCN with malate as a substrate proceeded to the same extent as the control (Fig. 3, lanes 2 and 3 vs. lane 4 and 5), whereas the addition of KCN and SHAM together inhibited import (Fig. 3, lanes 6 and 7). SHAM alone had no effect on import (Fig. 3, lanes 8 and 9). Residual import in the presence of KCN and SHAM was inhibited by the addition of oligomycin (Fig. 3, lanes 10 and 11) indicating that it was supported by an ATPase-generated membrane potential.

These results show that import of the alternative oxidase into soybean mitochondria can be supported by either the alternative or cytochrome pathway, providing that at least one proton-translocating site (at complex I) is operative to energise the inner membrane. Thus it was necessary to use NAD-linked substrates in the presence of KCN. It should be noted that, in general, import with succinate as a respiratory substrate was more efficient than with malate (compare Figs. 1 and 2 to Fig. 3). This may be due to the higher rate of respiration with succinate as substrate in this tissue [4] or to the difference in the pH of the medium in the two reactions.

Root and shoot processing of the alternative oxidase precursor

We have previously shown that soybean cotyledon mitochondria contain two proteins detectable with alternative oxidase antibodies, whereas in root mitochondria, only a single band is seen [11] (see Figs. 4B and 4C). However, no difference could be detected in precursor import and processing between isolated cotyledon and root mitochondria, in a large number of import experiments (about 20 experiments). In both types of mitochondria, the same size mature protein was

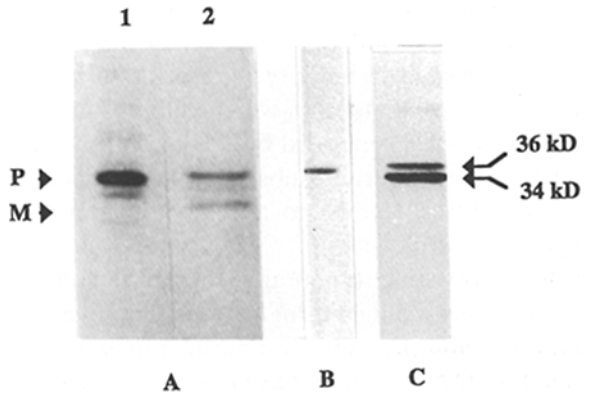

Fig. 4. Comparison of the *in vitro* imported products with the alternative oxidase proteins detected by western analysis. A. Lane 1, precursor alone; lane 2, imported products detected by autoradiography. B. 25 μg of root mitochondria probed with antibodies to the alternative oxidase and positive band visualised by ECL. C. As A except that lane 2 was probed with antibodies to the alternative oxidase and positive bands visualised by ECL.

generated, with approximate equal efficiency (Fig. 1).

To determine the relationship between the synthetic precursor band, its cleaved mature product, and the mitochondrial protein bands seen on western blots, mitochondria samples from an *in vitro* import experiment were subjected to SDS-PAGE and blotted onto nitrocellulose. The blot was then firstly probed with antibodies raised against the alternative oxidase and then exposed to X-ray film (Fig. 4). Since the amount of *in vitro* ^{35}[S]-methionine-labelled precursor was below the levels of detection with these antibodies, the immuno-blot (Figs. 4B and 4C) shows only the *in vivo* alternative oxidase protein bands which react with antibody, while the autoradiograph (Fig. 4A) shows only the precursor and its processed mature form after import. Although the *in vitro* synthesised precursor and the upper band detected by western analysis of cotyledons display the same apparent molecular weight (36 kDa), the lower band detected by western analysis in cotyledons and the mature form generated upon *in vitro* import differ by ca. 2 kDa (Figs. 4A and 4C). The *in vitro* imported mature form was calculated to have an apparent molecular mass of 32 kDa and the lower band seen in western blots of cotyledon mitochondria had an apparent molecular mass of 34 kDa. Mutation experiments (see below) indicate that the correct processing site was being recognised in the *in vitro* import experiments and we therefore conclude that the 32 kDa generated by cleavage of the precursor after import is subsequently modified in some manner, leading to a change in apparent molecular mass.

It is interesting to note that root mitochondria contain only a single form of the alternative oxidase protein with a similar molecular mass to that of the synthetic precursor (36 kDa) (Fig. 4B). Yet mitochondria isolated from roots are quite capable of cleaving the imported synthetic precursor to a smaller mature form of 32 kDa. Thus the precursor must also be modified in root mitochondria after cleavage. An alternative hypothesis is that the bands seen on western blots are the products of different genes.

To investigate the identity of the proteins further, we analysed the alternative oxidase proteins by 2-D gel electrophoresis (Figs. 5A, 5B and 5C).

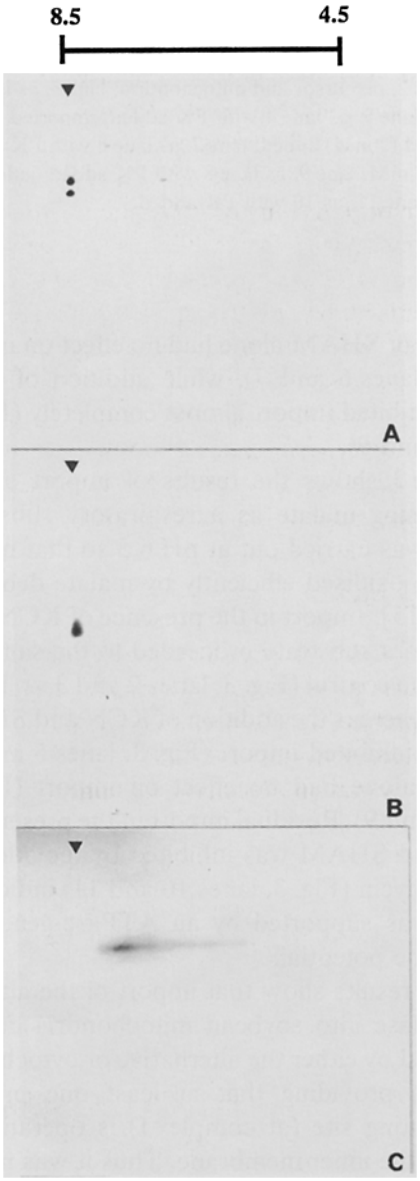

Fig. 5. Two-dimensional gel analysis of the alternative oxidase proteins. A. 40 μg of isolated soybean cotyledon mitochondria separated by 2-D gel analysis and alternative oxidase proteins detected with monoclonal antibodies to the alternative oxidase. B. As A except that isolated root mitochondria were used. C. ^{35}[S]-methionine *in vitro* synthesised precursor analysed by 2-D gel electrophoresis. The precursor was detected by autoradiography. The arrows indicate the origin (top) of the isoelectric dimension.

Isolated mitochondria from cotyledons and roots, together with *in vitro* synthesised ^{35}[S]-labelled precursor, were analysed by 2-D gel electrophoresis. With soybean roots and cotyledons, a single and double spot, respectively, could be detected after the gel was blotted (Figs. 5A and 5B). However these spots remained at the origin of the gel and their mobility on the isoelectric gel was obviously restricted. The synthetic precursor, on the other hand, migrated into the gel (Fig. 5C). The precursor protein thus clearly differs from the *in vivo* mitochondrial forms of the alternative oxidase. The isoelectric point for the synthesised precursor is between 8.0 and 8.5, which compares favourably with the predicted isoelectric point from the protein sequence of 8.3. The predicted isoelectric point of the mature protein is 7.1 and therefore it would be expected to migrate into the 2-D gel. It is possible that modification of the protein prevented this from occurring.

Processing studies

The start of the mature protein has been defined in *S. guttatum* by direct N-terminal sequencing [24]. Alignment of the presequences of the three known plant alternative oxidases shows that although they vary in length, the arginine at position −2 is conserved (Fig. 6A). Arginine at −2 is a common processing signal in mitochondrial targeting presequences [9, 28]. To investigate the role of −2 arginine, we have used site-directed mutagenesis to change it to a −2 glycine and investigated the effect of the mutation on processing. Processing of the normal alternative oxidase precursor by cotyledon mitochondria led to the production of a 32 kDa band, in agreement with the import studies described above (Fig. 6B, lane 3). Addition of 5 mM EDTA reduced the extent of processing (Fig. 6B, lane 4) while 5 mM 1,10-orthophantroline (a characteristic inhibitor of the mitochondrial processing peptidase [9]) inhibited the processing completely (Fig. 6B, lane 5).

Incubation of the −2 mutant form of the alternative oxidase with cotyledon mitochondria did not yield a processed band (Fig. 6B, lane 6). The difference in intensity between the mutant and normal precursor was due to differences in the transcription-translation efficiency for the two clones. In the experiment shown, the amount of lysate added was kept constant so that reactions conditions would be identical. Increasing the amount of mutant precursor did not lead to detection of any processing. The results shown here, and alignment of the predicted soybean alterna-

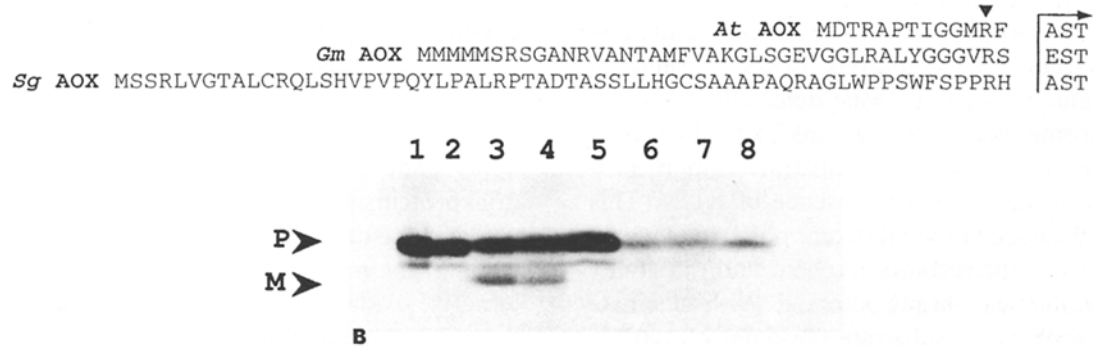

Fig. 6. Defining the start of the soybean alternative oxidase mature protein. A. Alignment of amino acids of the putative presequence for all plant alternative oxidases sequenced to date. The absolutely conserved arginine is indicated by a vertical arrow. The start of the mature protein is indicated by the horizontal arrow as defined by Rhoads and McIntosh [24]. At, *Arabidopsis thaliana*; Gm, *Glycine max*; Sg, *Sauromatum guttatum*. B. Analysis of the processing of the normal and mutated alternative oxidase precursor; lane 1, 0.5 μl of lysate with normal alternative oxidase precursor; lane 2, 2 μl of mutated precursor; lane 3, 0.5 μl of normal precursor with 50 μg of cotyledon mitochondria; a processed product is evident; lane 4, as lane 3 with EDTA added to 5 mM; lane 5, as lane 3 with 1,10-orthophantroline added to 5 mM; lane 6, as lane 3 with mutated precursor used; lane 7, as lane 6 with EDTA added to 5 mM; lane 8, as lane 6 with 1,10-orthophantroline added to 5 mM.

tive oxidase with the known mature N-terminal protein sequence data from *S. guttatum*, suggest that the soybean mature protein begins at residue 41 [24, 31]. Although −2 arginine has been shown to be an important signal for processing in other organisms, this is the first time it has been demonstrated using plant mitochondria with a mutated plant precursor.

Discussion

We have investigated the import and processing of the alternative oxidase in soybean mitochondria. *In vitro* experiments with isolated cotyledon and root mitochondria indicate that the precursor is efficiently taken up by the mitochondria and that the presequence is cleaved efficiently. Both cotyledon and root mitochondria import and process the precursor to a single 32 kDa product, despite the differences between root and cotyledon mitochondria in the number of alternative oxidase proteins detected by immunological analysis [11, 20, 26]. Protein import into isolated soybean mitochondria is similar to that in other organisms, being dependent on a membrane potential and ATP. Addition of valinomycin and oligomycin inhibits import [9].

Either of the alternative or cytochrome pathways can support import of the alternative oxidase precursor, but this is dependent on the respiratory substrate employed. In the presence of succinate, no import was detected when the cytochrome pathway was inhibited by KCN whereas with malate as substrate import proceeded normally in the presence of KCN. This difference is due to the different points of entry of electrons to the respiratory chain and the ability to generate a membrane potential. With succinate as the respiratory substrate the point of entry is via succinate dehydrogenase and electrons travel directly to ubiquinone. In the presence of KCN electrons can travel to the alternative oxidase but no transmembrane potential is generated. However, with malate as the respiratory substrate electrons may enter the respiratory chain via complex I, and even in the presence of KCN a membrane potential can be generated via complex I. Addition of both SHAM and KCN with malate as a respiratory substrate inhibits import because both terminal oxidases are blocked. The inhibition is not complete as some residual import can be detected (5% or less of the substrate driven rate). This residual import was inhibited by oligomycin, an inhibitor of the mitochondrial ATPase, indicating that import in the absence of electron transport can be supported by an ATP-generated membrane potential. Import in the absence of added ATP was undetectable (not shown).

To investigate the identity of the alternative oxidase bands detected *in vivo*, we compared directly the synthesised precursor, the processed (i.e. the cleaved) form generated upon import, and the bands detected by western blot analysis of cotyledon and root mitochondria. Surprisingly, we found that the upper band in cotyledons and the single band in roots have the same apparent molecular mass as the *in vitro* synthesised precursor. This suggested that either the upper form *in vivo* is the unprocessed precursor or that the precursor is processed upon import and subsequently modified to give a similar apparent molecular mass. However, the upper and lower bands in cotyledons have similar isoelectric points and the isolated root mitochondria, while containing only the upper band, are capable of processing the imported precursor to a 32 kDa form. The precursor also has a different mobility on 2-D gels compared to the forms detected with monoclonal antibodies. This strongly suggests that the upper band seen in western blots of mitochondrial proteins is a modified form of the alternative oxidase, distinct from the precursor (but see below). The nature of the modification of the alternative oxidase is not known, but possible candidates include binding of quinone or insertion of a metal centre.

The lower band of the alternative oxidase in cotyledon mitochondria also appears to be different from that of the cleaved precursor after import. This could be due to a different modification of the same protein subsequent to cleavage of the pre-sequence, but could also be due to the

expression of a second alternative oxidase gene (the gene copy number for this protein has not been determined in soybean). In this context, it is also possible that the 32 kDa processed precursor protein is a third alternative oxidase protein which does not react with the monoclonal antibody used in western blots. This is unlikely given that this antibody was used to isolate a cDNA clone from *Sauromatum guttatum*, and that the *Sauromatum* clone was subsequently used to isolate the soybean cDNA used here to synthesise the precursor protein. Definitive evidence awaits direct sequencing of the proteins but initial attempts in soybean have been unsuccessful. Whatever the case, it is clear that the difference between root and cotyledon mitochondria in the number of alternative oxidase proteins seen in immunoblots, is not due to differential cleavage of a precursor protein after import.

As there was an apparent difference between the mature form generated upon *in vitro* import and the lower band detected *in vivo* by western blot analysis, we attempted to define the processing site. Sequence comparison of all published alternative oxidase presequences indicate that they contain a conserved arginine (Fig. 6A). N-terminal sequence data from *S. guttatum* alternative oxidase indicates that this arginine is most likely located in a −2 position relative to the start of the mature protein [28]. −2 Arg has been reported to be a common processing signal in mitochondrial precursors from other organisms and this appears to be the case in a large number of plant mitochondrial precursors [2]. Conversion of the −2 arginine to a glycine residue completely inhibited processing, indicating that residue 41 in fact corresponds to the start of the mature protein. Mutation of the −2 arginine has been shown to inhibit processing in other organisms and has been proposed to be a processing signal in plants [2]. Mitochondrial targeting signals are in general not well conserved at a sequence level, and the alternative oxidase presequence varies in length from 13 amino acids in *Arabidopsis*, to 41 in soybean, and 61 in *S. guttatum* [12, 24, 32]. However, the −2 arginine is well conserved generally in plants and absolutely in the alternative oxidase genes sequenced to date [12, 24, 32]. Thus we conclude that this residue is indeed essential for processing.

Conclusion

We have studied the import of the alternative oxidase into isolated soybean mitochondria. This import can be supported by either the cytochrome or alternative pathway providing that NAD-linked substrates are used. Comparison of the products obtained following import, with those detected by immunological analysis *in vivo*, indicate that the imported alternative oxidase protein may undergo modification, giving rise to proteins with different apparent molecular mass in soybean.

Acknowledgements

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