The critical residues involved in targeting and processing of the soybean alternative oxidase to plant and animal mitochondria was investigated. Import of various site-directed mutants into soybean mitochondria indicated that positive residues throughout the length of the presequence were important for import, not just those in the predicted region of amphiphilicity. The position of the positive residues in the C-terminal end of the presequence was also important for import. Processing assays of the various constructs with purified spinach mitochondrial processing peptidase showed that all the −2-position mutants had a drastic effect on processing. In contrast to the import assay, the position of the positive residue could be changed for processing. Deletion mutants confirmed the site-directed mutagenesis data in that an amphiphilic α-helix was not the only determinant of mitochondrial import in this homologous plant system. Import of these constructs into rat liver mitochondria indicated that the degree of inhibition differed and that the predicted region of amphipilic α-helix was more important with rat liver mitochondria. Processing with a rat liver matrix fraction showed little inhibition. These results are discussed with respect to targeting specificity in plant cells and highlight the need to carry out homologous studies and define the targeting requirements to plant mitochondria.

The role of the presequence in targeting proteins to mitochondria has been well established for a variety of species using chimeric constructs containing passenger proteins and deletion studies where removing the presequence abolished mitochondrial import (1, 2). This process requires the presence of cytosolic factors (3–5) and recognition event(s) at the surface of the mitochondria (6). Inside the mitochondria precursors proteins are processed to a mature form by specific peptidase(s), which requires information located within the presequence for correct recognition of the cleavage site (1, 7). Despite the fact that the sequence of a large number of presequences are known, no consensus sequence for mitochondrial targeting and subsequent processing are defined. This is because of large variations in the length of presequences, which display no primary amino acid sequence homology (7). However mitochondrial targeting presequences share some common features such as enrichment of positively charged amino acids and a lack of negatively charged amino acids and display an enrichment for alanine, leucine, and serine (7). Furthermore they are normally predicted to have the ability to form an amphiphilic α-helix (8).

Mitochondrial presequences can be roughly divided into two domains, an N- and a C-terminal domain (8, 9). The N-terminal has the potential to form the α-helix and is proposed to function in targeting of the precursor (10). The C-terminal domain contains the information necessary for proper cleavage of the presequence (10) but does not exhibit any preference to a secondary structure (11). The functions of both domains are independent of each other, but they may overlap (9).

Positive charges and the position of the positive charge appear to be important for mitochondrial targeting. Studies using site-directed mutants of rat ornithine transcarbamylase precursor (pOTC), pig aspartate aminotransferase, and rat aldehyde dehydrogenase indicate the critical role of arginine residues located in the N-terminal part of the presequence for import (12–14). Human pOTC appears to be different; the mutation of some individual arginines (arginine 23) had a drastic inhibitory effect on import, whereas changing arginine 26 to glycine had no effect on import (15). In contrast, mutagenesis of arginine 23 to glycine of rat pOTC affected processing but not import (12). To our knowledge this discrepancy has not been resolved. Experiments whereby negatively charged residues are incorporated into the presequence also affected import (16). This suggests that mitochondrial protein import depends on a net positive charge on the presequence that may be required at different stages of translocation (1), possibly through interaction with the acid bristle of the translocation pore, as suggested by Haucke and Lithgow (17).

As there is no known primary structure homology at the cleavage site between different precursor proteins, it is intriguing how these sites are recognized and cleaved by the general mitochondrial processing peptidase (MPP). An arginine residue located two amino acids upstream of the start of the mature protein has been proposed to act as the processing signal (7). The mutagenesis studies outlined above indicated that the arginine residues involved in processing were not required for import. However certain arginine residues may overlap in function, e.g. human pOTC, where changing an arginine residue resulted in loss of import and processing (15).

Only a few studies have looked at the requirements for precursor targeting to plant mitochondria. Studies with the F1β presequence from Nicotiana plumbaginifolia showed that the first half of the 54 amino acid presequence could support mitochondrial targeting although at a much lower efficiency (18). Likewise, the superoxide dismutase from maize showed that only small deletions in the C-terminal region of the presequence could be tolerated (19). Few studies have addressed...
Targeting of a Plant Mitochondrial Protein

The requirement for processing of precursor proteins in plants. The –2 arginine rule does hold for the soybean alternative oxidase precursor (20). Surprisingly, processing of the N. plum-baginifolia Fββ was inhibited by synthetic peptides, which had a predicted ability to form a helical structure, suggesting a structural requirement for processing in this case (21).

Therefore to define the requirements of a mitochondrial pre-sequence for targeting and processing in plants, we have carried out studies with the soybean alternative oxidase precursor, an inner membrane protein responsible for cyanide-insensitive respiration (22). We have carried out site-directed mutagenesis with the authentic protein to avert any potential problems with the nature of the passenger protein and linker sequences (23). This is the first study to investigate the role of specific residues required for plant mitochondrial import. Also it is the first investigation of the processing requirements of plant MPP by site-directed mutagenesis.

MATERIALS AND METHODS

Synthesis of the Soybean Alternative Oxidase Precursor and Derivatives—The alternative oxidase (AOX) from soybean contains a presequence of 41 amino acids. Mutagenesis was performed using altered site-directed mutagenesis (Promega, Madison, WI) or quick-change site-directed mutagenesis (Stratagene) kits as per the manufacturer’s instructions, and mutants were confirmed by sequencing.35S-Labeled precursor was synthesized as described by Whelan et al. (24). The mutations introduced were designated by the position relative to the start site of the mature protein, followed by the three-letter abbreviation for the amino acid introduced, i.e., −2Gly means the amino acid glycine two residues upstream of the start of the mature protein. A list of the mutations is shown in Fig. 1C.

Soybean and Rat Liver Mitochondrial Isolation and in Vitro Imports—Mitochondria were isolated from 7-day-old soybean (Glycine max L. Merr. c.v. Stevens) cotyledons as described by Day et al. (25). Import of the soybean alternative oxidase mutants was performed as by Whelan et al. (20). Rat liver mitochondria were isolated from approximately 30 g of tissues (26). In vitro mitochondrial imports were performed similar to the soybean mitochondrial imports, except that dithiothreitol was omitted, as import was found to be lower in the presence of dithiothreitol with rat liver mitochondria, and the 20-min incubation of the reaction was performed at 30 °C. The results were analyzed using a MacBAS 1000 according to manufacturer’s instructions (Fuji). Import studies with all mutant precursor proteins were also accompanied by a valinomycin control (see Fig. 2) to ensure that all PK-protected protein generated was dependent on a membrane potential (data not shown).

Processing Studies and Western Blotting—Isolated rat liver mitochondria were resuspended in fractionation buffer (1 mM methionine, 30 mM Tris-Cl, pH 8.0). The suspension was cooled on ice for 5 min before sonication. Sonication was performed 3 times on a sonicator (Branson) setting of 4 for 10 s with a 2-min cooling on ice inbetween. The fractions were separated by centrifugation at 15,000 × g for 30 min at 4 °C. The supernatant was collected and diluted 2-fold for use in processing assays. Processing mix contained the following: 20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 20 mM sodium ascorbate, 20 mM N-ethylmaleimide, and 20% glycerol, and the pH was adjusted to 7.4. Import and processing studies of the soybean alternative oxidase but not the MPP (27, 28). The reaction was incubated at room temperature for 15 min followed by the addition of dithiothreitol to 10 mM to inactivate N-ethylmaleimide. The mix was then incubated with 2 μl of radiolabeled precursors. The samples were analyzed similar to the import assay.

Processing of the soybean alternative oxidase and the mutant precursors was performed using purified spinach MPP (29). Western analysis was carried out with 40 μg of purified mitochondria using antibodies to the alternative oxidase (30).

Densitometric Analysis—Quantiﬁcation of imports was performed as described by Dessi and Whelan (31). Quantitations of imports were determined by densitometric analysis on the raw image scans using the MacBas v2.0 software (Fuji). The efficiency of import and processing was calculated based on density of precursor and mature bands. The percentage of import was calculated as follows (−PK and +PK denotes bands in the non-PK-treated and PK-treated samples, respectively.

\[
\frac{\text{mature + precursor}(+PK)}{\text{mature + precursor}(-PK)} \quad \text{(Eq. 1)}
\]

The percentage of wild type processing was set to 100%, and the percentage of processing of the mutants was calculated relative to the wild type. All data taken fell within the linear range of the MacBAS 1000, and all assays were carried out at least three times.

RESULTS

The Presequence of the Alternative Oxidase—The AOX presequence was analyzed using MITOPROT (32) and Robson-Garnier analysis (33) to determine the amphiphility and secondary structure, respectively. The AOX presequence has the potential to form an amphiphilic α-helix (32)(Fig. 1A) between residues 9 and 25 followed by no predictable structure after residue 26 (Fig. 1B), in agreement with the two-domain structure of mitochondrial targeting presequences (8, 9). To demonstrate the requirement for the presequence in mitochondrial targeting of the AOX, the mature AOX form (AOX ΔP) was constructed. This was not imported into isolated soybean and rat liver mitochondria (Fig. 2, A and B, lanes 6 to 8) under conditions that clearly supported the import of the wild type AOX (Fig. 2, A and B, lanes 1 to 3). (The identity of the additional bands P and M will be discussed with the deletion mutants below.) From this experiment it was concluded that the presequence was responsible for targeting the AOX to the mitochondrion in a membrane potential-dependent manner (Fig. 2, A and B, lanes 4 and 5), and site-directed mutagenesis was carried out to determine critical residues for import and processing in plants and animals (Fig. 1C).

Role of the –2 to +2 Region in Targeting and Processing—The –2 arginine was the major focus as it is common to other precursor proteins (34). Import using isolated soybean mitochondria showed that the greatest effect was seen with the 2Gly and –2Thr, where at least 30% inhibition was consistently observed (Fig. 3A, Table I (note that the import efficiencies are normalized as indicated under “Materials and Methods” because of different precursor translation efficiencies). The mature form generated upon import with these two mutants had a slower mobility (higher \(M_r\)) compared with the wild type AOX, indicating that processing upstream of the correct site had taken place. When the processing site was deleted (Δ−1+1 mutant), import proceeded normally, except this time the mature form generated had a greater mobility (lower \(M_r\)) compared with the control. An overall affect of the various –2 mutations was the accumulation of imported precursor forms, up to 40% in the case of the –2Gly, –2Thr, and Δ−1+1 mutants.

Imports with the –2 mutant precursor proteins were also carried out with isolated rat liver mitochondria (Fig. 3B, Table I). In agreement with the soybean mitochondria experiments, no complete inhibition of import was observed, and import was inhibited by 20% with most mutants. However, in the case of rat liver mitochondria, the Δ−1+1 mutant had a faster mobility (Fig. 3B). Similar to soybean, for some mutants, significant amounts of imported precursor form was observed (Fig. 3B).

Processing studies of the –2 mutants were also conducted using isolated spinach MPP (35) and a rat matrix fraction (36). The wild type AOX was efficiently processed by the isolated spinach MPP but except for the +2Gly and Δ−1+1 mutants, most of the –2 mutants inhibited processing drastically (Fig.

\[ \text{[mature + precursor] + PK} \]
FIG. 1. Structural analysis of the soybean alternative oxidase presequence. A, hydrophobic moment plot analysis of the presequence calculated at a \( \delta = 95\). B, secondary structure prediction (see text). C, mutant forms of the soybean alternative oxidase presequence. Arrowhead represents the start site of the mature protein of soybean alternative oxidase (45). The number along the bottom refers to the position of the amino acid relative to the start of the mature protein (see “Materials and Methods”). Dashes indicate amino acids removed. Preseq, presequence.

FIG. 2. Import of the soybean alternative oxidase precursor protein into soybean cotyledon mitochondria (Mit) (A) and rat liver mitochondria (B). Lanes 1 and 6, precursor protein. Lanes 2 and 7, precursor protein incubated with mitochondria. Lanes 3 and 8, as lanes 2 and 7, with PK added. Lanes 4 and 9, as lanes 2 and 7, with valinomycin (Val) added. Lanes 5 and 10, as lanes 4 and 9, with PK added. P and M denote precursor and mature forms of AOX. Additional products observed are labeled P’ and M’ (see text).

4A, Table I). In contrast, processing using the rat matrix fraction was not affected except for the -2Phe mutant (Fig. 4B, Table I).

Roles of Residues in the -10 to -2 Region—The AOX presequence contains three glycine residues at positions -4, -6, and a -10 arginine (a putative processing signal) (37). A -4Arg could not complement the inhibition of import seen with the -2Gly (Fig. 5A), and even though the efficiency of processing (in the import assay) was not increased with the -2Gly, -4Arg mutant compared with the -2Gly mutant, the fidelity of processing was restored (Fig. 3A and 5A). The -10Gly mutant decreased import by 35%, similar to the inhibition seen with the -2Gly (Fig. 5A). The double mutant -2, -10Gly inhibited import by ~70%, and this could not be restored by the presence of an arginine as shown in the -2, -10Gly, -4Arg mutant (Fig. 5A, Table I). Therefore the position of the -2 arginine residue is important as well as the positive charge; if charge alone was the only parameter, it would be expected that the -2, -10Gly, -4Arg would be imported with a similar efficiency to the single mutants -2Gly or -10Gly.

The AOX presequence contains a -10 arginine, a putative processing signal for two-step processing by MPP and mitochondrial intermediate peptidase, respectively (37). A -7 serine residue is considered important for two-step processing (38). Therefore we changed this -7 residue to a serine to see if the -10 arginine could act as a processing signal. It was found that the -7Ser, -2Gly mutant had the effect that the majority of the product was processed upon import into soybean mitochondria (Fig. 5A). However it had a slower mobility than the wild type AOX, indicating processing upstream of the correct site, possibly using the -10 arginine as a processing signal. In the case of rat liver mitochondria, a similar effect was seen with the -2, -10Gly mutants that import was inhibited; however, only by 40% compared with 70% with soybean cotyledon mitochondria (Fig. 5A, Table I). Again, the presence of a -4Arg could not restore processing of the -2Gly in the import assay (Fig. 3B), but the mature form generated with the -2Gly, -4Arg mutant had the effect that the majority of the product was processed upon import into rat liver mitochondria, the -7Ser could restore the processing efficiency in the import assay with the -2Gly (-7Ser, -2Gly mutant) and to a lesser degree with the -2Thr mutant (Fig. 3B and 5B).

In the rat liver import assays, precursors containing the -2Gly mutation, except the -2Gly, -4Arg mutant, the mature form generated had a slower mobility compared with the wild type. This is in contrast to the soybean import assays where only the -2Gly and -2Thr mutants had this effect. However, in rat mitochondrial import, the presence of the -10Gly mutation with the -2Gly affected the formation of the mature product, whereby a slower mobility mature form was generated (denoted by a double minus sign). This suggests that in the
FIG. 3. Import of the mutant soybean alternative oxidase precursor proteins with mutations in the -2 to the +2 region into soybean cotyledon mitochondria (A) and into rat liver mitochondria (B). Each panel represents a different precursor protein, which is designated on top, containing three lanes corresponding to the first three lanes of import as indicated in Fig. 2. Import efficiency is indicated and refers to import of precursor and mature bands in the last lane in each panel as indicated under “Materials and Methods.” A circled minus sign or plus sign above certain panels denotes mobility of the imported mature form, which is lower or higher compared with the wild type AOX, respectively. A circled double minus sign denotes a much lower mobility.

TABLE 1

| Precursor | Plant Import | Plant Processing | Animal Import | Animal Processing |
|-----------|--------------|------------------|--------------|------------------|
| Wild-type | 100          | 100              | 100          | 100              |
| -2Gly     | 65           | 16               | 80           | 100              |
| -2Leu     | 80           | 20               | 80           | 85               |
| -2Lys     | 80           | 10               | 100          | 80               |
| -2Ala     | 90           | 11               | 100          | 80               |
| -2Thr     | 70           | 0                | 80           | 80               |
| -2Phe     | 80           | 0                | 90           | 65               |
| -2Gln     | 80           | 0                | 90           | 85               |
| -2His     | 80           | 13               | 80           | 75               |
| +2Gly     | 65           | 23               | 90           | 70               |
| Δ-1+1     | 100          | 100              | 70           | 85               |
| -4Arg     | 100          | 60               | 80           | 80               |
| -2Gly,-4Arg | 70     | 56               | 80           | 85               |
| -10Gly    | 65           | 70               | 80           | 50               |
| -2,-10Gly | 35           | 9                | 60=           | 45=              |
| -2,-10Gly,-4Arg | 35 | 57 | 40= | 50= |
| -7Ser,-2Gly | 75       | 65               | 80           | 75               |
| -7Ser,-2Thr | 50       | 0                | 90           | 65               |
| -20Gly    | 70           | 0                | 70           | 75               |
| -30Gly    | 75           | 0                | 90           | 65               |
| -35Gly    | 75           | 16               | 90           | 100              |
| -20,-30Gly | 20     | 60               | 0            | 50               |
| -20,-35Gly | 40     | 30               | 20           | 70               |
| -30,-35Gly | 25     | 0                | 10           | 65               |
| -20,-30,-35Gly | 0 | 100 | 0 | 75 |
| AOX 9-36  | 30           | 0                | 75           | 0                |
| AOX Δ1-17 | 15           | 100              | 45           | 100              |

The import efficiencies refer to the combined amount of the imported mature and precursor product. The mutants are outlined in Fig. 1C, and the symbols used are as outlined in Figs. 3 and 4. For Plant, import was with soybean cotyledon mitochondria and processing was with purified spinach MPP. For Animal, import was with rat liver mitochondria and processing was with rat liver mitochondrial matrix fraction.

The role of residues in the -2 to -10 region was also analyzed in processing reactions (Fig. 4). In contrast to the import studies, the -4Arg mutant restored processing with spinach MPP, processing of the -2Gly mutant (16%) was increased by greater than 3-fold to approximately 50% by the -4Arg mutant, and the processed product generated had the same mobility as wild type AOX. The processing of the -2,-10Gly mutant was increased from 9 to 57% in the -2,-10Gly,-4Arg mutant. The ability of the -4Arg to restore significant processing to both these mutants but have no effect on import indicates that these arginine residues play a critical role in import distinct from a role in processing. Similar to what was seen with the import assays into soybean, the -7Ser mutant could restore processing of the -2Gly mutant but not the -2Thr mutant.

In contrast to purified spinach MPP, processing by the rat matrix of the -10Gly mutant was inhibited by 50%, and the -2,-10Gly mutant was also inhibited by greater than 50%, generating a lower mobility product (Fig. 4B). The other notable difference compared with spinach MPP was that the -4Arg did not restore the processing efficiency of the -2,-10Gly (but did change the product to that similar to wild type AOX), and the -7Ser did not restore the processed product of the -2Gly to the same mobility as wild type AOX. This may be because of the fact that the -10 arginine can act as a processing signal with rat matrix but not with spinach MPP.

Role of Positive Residues in the Presequence on Import and Processing—From import experiments using both soybean and rat liver mitochondria, it was evident that changing any single positive residue at the -20, -30, or -35 position had some (≥25%) inhibitory effect (Fig. 6) similar to the level seen with changing the -2 or -10 arginines individually (Figs. 3 or 4). Changing two positive residues inhibited import into both soybean and rat liver mitochondria, overall more so in the latter (Fig. 6, Table 1). For rat liver mitochondrial import, complete inhibition was seen with the -20,-30Gly mutant, and inhibition was almost complete with the -30,-35Gly mutant. This is much greater than the expected additive effect seen with the individual mutants. The double mutants did inhibit import into soybean mitochondria, but it was notable that import efficiency was twice as high compared with rat liver mitochondrial import (Fig. 6, Table 1). The triple mutant completely abolished import with both sets of mitochondria.

Processing by purified spinach MPP was significantly inhibited by each of the changes of the N-terminal positive residues. It would appear that the double mutant, which included the -20 position (i.e., -20,-30Gly, or -20,-35Gly), relieved this inhibition of processing to some extent compared with the single mutant (Fig. 4). The -30Gly mutant was not processed

presence of the -2Gly mutant, the wild type -10 arginine acted as a processing signal. The presence of a -7Ser with the -2Gly (-7Ser,-2Gly mutant) appears to have increased the efficiency at which the rat mitochondria utilized the -10 arginine residue as a processing signal in the import reaction. A serine in the -7 position would be predicted to change the residues to that of the consensus required for -10 arginine processing in animals (7). The -10Gly mutant alone had little effect on the mobility of the product obtained (Fig. 5B).

The role of residues in the -2 to -10 region was also analyzed in processing reactions (Fig. 4). In contrast to the import studies, the -4Arg mutant restored processing with spinach MPP, processing of the -2Gly mutant (16%) was increased by greater than 3-fold to approximately 50% by the -4Arg mutant, and the processed product generated had the same mobility as wild type AOX. The processing of the -2,-10Gly mutant was increased from 9 to 57% in the -2,-10Gly,-4Arg mutant. The ability of the -4Arg to restore significant processing to both these mutants but have no effect on import indicates that these arginine residues play a critical role in import distinct from a role in processing. Similar to what was seen with the import assays into soybean, the -7Ser mutant could restore processing of the -2Gly mutant but not the -2Thr mutant.

In contrast to purified spinach MPP, processing by the rat matrix of the -10Gly mutant was inhibited by 50%, and the -2,-10Gly mutant was also inhibited by greater than 50%, generating a lower mobility product (Fig. 4B). The other notable difference compared with spinach MPP was that the -4Arg did not restore the processing efficiency of the -2,-10Gly (but did change the product to that similar to wild type AOX), and the -7Ser did not restore the processed product of the -2Gly to the same mobility as wild type AOX. This may be because of the fact that the -10 arginine can act as a processing signal with rat matrix but not with spinach MPP.
at all by spinach MPP, compared with 60% efficiency with the −20,−30Gly mutant. In contrast the −20,−30Gly mutant was processed at the least efficiency (along with the −10Gly) with rat liver matrix of all the mutants tested (Fig. 4, Table I). The triple mutant −20,−30,−35Gly was processed by rat liver matrix and spinach MPP (Fig. 4, Table I), so the inhibition of spinach MPP seen with the single and double mutants was relieved by the triple mutant. This indicates that the inhibition of processing seen with spinach MPP by changing positive residues in this region of the presequence may have been a result of changes in the overall structure of the presequence rather than any of the positive residues playing a specific role in binding or catalytic process of MPP.

Is the PK Protected Precursor Form Truly Imported?—One effect of many of the single mutants in soybean was that the imported form was largely precursor as opposed to mature form (Fig. 3A). It is possible that the hydrophobic nature of the AOX protein may allow it to be embedded into the outer membrane where it may display some PK resistance (22). To investigate if the precursor form was truly inside the mitochondria, we tested if the imported precursor form had the same PK insensitivity as the imported mature form of wild type AOX. When the PK concentration was increased to 100 μg/ml, it was evident that the imported precursor form of the −2Gly mutant displayed a similar degradation profile as the imported mature form of wild type AOX (Fig. 7, A and B). Furthermore we analyzed the state of the endogenous AOX protein in soybean mitochondria under these conditions (Fig. 7C). The endogenous AOX proteins are
designated as AOX2 and AOX3, as determined by direct N-terminal sequencing in a previous study (39). It was evident that under these conditions, the imported AOX (both precursor and mature forms from wild type and −2Gly mutant) displayed similar percentage degradation with increasing PK, as did the endogenous AOX in the mitochondria. Therefore it can be concluded from these experiments that the PK-protected precursor was truly imported into mitochondria.

**Structure Requirements Sufficient for the Import of AOX**—As mentioned above, in *vitro* translation of the AOX precursor (and mutants) was also accompanied with a lower molecular weight product (*P*) below the authentic precursor protein (Fig. 8A). Furthermore, import into soybean mitochondria always resulted in a product with a higher mobility than that of the authentic mature (M′) form, which is never present in rat liver mitochondria imports (Fig. 8A versus 8B). However, this band is not PK-protected and was generated in the absence of membrane potential (Fig. 2A, lane 4). This indicated that it was not necessary for the *P* to cross the inner mitochondrial membrane in a ΔΨ-dependent manner for the generation of M′.

The *P* protein is thought to be because of the internal methionine initiating the start of translation (i.e. methionine at position 18) and not because of an early termination product. It is possible that this form can be imported into the mitochondria and can be processed to the authentic mature form. To investigate this we created the Δ1−17 construct (Fig. 1C). As expected in the *in vitro* translation, only one band was seen with the same mobility comparable with the *P* seen with the wild type AOX translation. When import into soybean and rat liver mitochondria were performed, this construct was imported and processed to the correct mature form with different efficiencies of 15 and 45%, respectively (Fig. 8, A and B, lanes 7−9). This indicated that the C-terminal 24 amino acids could support import into rat liver mitochondria 3 times more efficiently than in soybean.

We then created a mutant in which the presequence encompasses only the predicted α-helical region (AOX 9−36) (Fig. 1C), and imports were performed to test if this structural feature alone was capable of directing the AOX precursor to the mitochondria. Translation of this construct resulted in two bands with the lower mass band because of an internal methionine (as discussed above). The import figure shows the efficiency of import for each product; these values cannot be added to give an overall efficiency, as they represent the efficiency of import from each band (Fig. 8). The efficiency of import presented in Table I is for the upper, authentic band. Import into soybean and rat liver mitochondria showed 20 and 40% formation of PK-protected mature forms, respectively (Fig. 8, A and B, lanes 4 to 6). This indicated that the helical structure supported import twice as much in rat liver as in soybean. The mutagenesis studies outlined above showed the requirements of positively charged residues throughout the presequence for import into soybean mitochondria.

The processing efficiency of the Δ1−17 mutant was largely unaffected, presumably as the authentic processing site was still intact. As expected, the AOX 9−36 construct was not processed because of deletion of the authentic processing site.

**DISCUSSION**

The transit sequence of the triose-phosphate translocator, which has the predicted ability to form an amphiphilic α-helix, has been shown to contain mitochondrial-targeting ability (40, 41). We have carried out this study with the authentic “passenger” protein in an attempt to avoid any differential import with passenger proteins (23, 40−42). We have also carried out experiments with rat liver mitochondria to compare import into another higher eukaryote.

**Import of the Site-directed Mutants**—Import of the various site-directed mutants into soybean mitochondria indicated that positive residues throughout the presequence were important for import. In fact, changing any single positive residue resulted in a decrease in import by 20 to 35%. With soybean mitochondria, changing the −2 arginine or −10 arginine had a slightly greater effect than changing the positive residues upstream (Table I). Rat liver mitochondria displayed a similar trend with the single mutants. Surprisingly for both soybean and rat liver mitochondria, substitution of an arginine residue at the −4 position for a glycine could not restore the import ability of the −2Gly mutant, indicating that the position of this arginine residue was important for import in this presequence. In the import experiments with soybean mitochondria, the −2Gly mutant gave a mature product with a slower mobility (apparent higher *M*′) and a significant amount of imported precursor form, but the −2Gly, −4Arg mutant produced a mature form upon import that appeared similar to wild type AOX. This double mutant (−2Gly, −4Arg) did not restore the import or processing efficiency in the import experiments, just the size of the mature product obtained. In contrast, with purified spinach MPP, the −2Gly, −4Arg mutant was processed 4 times as efficiently as the −2Gly mutant alone and restored the processed product to the same size as the wild type AOX.

The double −2 and −10 arginine mutations had an additive effect in both soybean and rat liver. However, the effect was greater with soybean mitochondria where import of −2, −10Gly mutant was inhibited by 65%, compared with 40% seen with rat liver mitochondria. Mutating two or more positive residues at the −20, −30, and −35 positions, however, did have a greater inhibition on import than that predicted from the individual mutants, and this effect was much greater with rat liver mitochondria than that with soybean. The −20Gly, −30Gly, and the −35Gly individual mutants gave 30, 10, and 10% inhibition of import alone in rat liver, whereas any two inhibited import by 80% or greater (Table I). In soybean, a similar effect was seen, but the inhibition of import with the double mutants was not as drastic; all double mutants imported into soybean mitochondria with twice the efficiency as they did into rat liver mitochondria (Table I). The triple mutant (−20, −30, −35Gly) of these residues completely inhibited import into both sets of mitochondria.
mitochondria.

The conclusion from the site-directed mutagenesis study on the effect of import into soybean mitochondria with the AOX precursor are (i) positive residues in the C-terminal region, at the −2 and −10 position relative to the mature protein, play an important role in import; (ii) changing the position of the positive residue at the −2 position to −4 does not restore import.

Processing of the Site-directed Mutants—Surprisingly, the majority of mutants had a drastic effect on processing with purified spinach MPP. All mutants of the −2 arginine inhibited processing by 80% or greater. Additionally the processed product in the −2Gly and −2Leu mutants had a slower mobility, strongly suggesting that processing did not take place at the correct site. Even the −4Arg mutant alone decreased processing by 40%, although this mutant had no inhibitory effect on import. However the −4Arg mutant could restore processing in the −2Gly mutant, the processing of the −2Gly, −4Arg mutant being almost the same as the −4Arg mutant alone, which was almost 4-fold higher than the −2Gly mutant. Additionally the mature size product obtained had a similar mobility to wild type AOX. This suggested that the −2 arginine residue in the wild type AOX plays two different roles, a role in import, where it is essential in the −2 position, and a role in processing, where the position can be changed to some degree. The other mutation that could restore processing was a −7Ser mutant; 80% of the imported product in the −7Ser, −2Gly mutant was mature form compared with only approximately 35% in the presence of the −2Gly mutant. In the processing assay, the −7Ser mutant restored processing of the −2Gly mutant from 16 to 65%.

We also carried out processing studies with rat liver matrix. In contrast to spinach MPP, processing was efficient with the majority of the mutants. We believe that the activity detected in the rat matrix represented true processing as (i) no significant proteolysis was evident; (ii) the size products generated on processing with the rat matrix fraction corresponded closely to that generated upon import with rat liver mitochondria (the same trend in mobility shift of the mature form with various mutants was seen with the processing experiments as in the import experiments); (iii) although the majority of mutants had no great effect on processing the double −2, −10Gly mutant inhibited processing by 50%, −2 and/or −10 arginine residues have previously been characterized as processing signals with rat liver MPP (7, 12, 15).

In conclusion the effect of the various site-directed mutants on processing with purified spinach MPP were (i) that all −2 mutants had a drastic effect on processing; (ii) that double mutants that could not restore import could restore processing; (iii) that even positive residues at the −20, −30, and −35 positions of the wild type AOX seem to play some role in processing; and (iv) that the −10 arginine residues does not act as a cryptic processing signal with spinach MPP, even though an −4Arg mutant apparently can.

Does an Amphiphilic α-Helix Play a Crucial Role in Plant Mitochondrial Targeting?—The deletion mutant Δ1–17 does not contain the positive residues at −35 and −30 positions. The second construct was a 9 to 36 mutant encompassing the predicted amphiphilic α-helix region. Import into isolated soybean mitochondria indicated that the Δ1–17 mutant and the 9 to 36 mutant were imported poorly. This indicated that an amphiphilic α-helix alone was not sufficient to support efficient import. In contrast both these deletion mutants displayed significant import into rat liver mitochondria, with at least 45% efficiency of the wild type AOX. Although the Δ1–17 mutant had the first portion of the potential amphiphilic α-helix deleted, it was still predicted to contain some amphiphilic helical character. Therefore an α-helical amphiphilic element alone cannot support efficient import with soybean mitochondria, but it can with rat liver mitochondria.

Determinants of Import and Processing in Different Species—This study indicates that positives residues throughout the presequence were required for import. This is further supported by the fact that the amphiphilic α-helix alone could not support efficient import. Although a similar trend was seen with rat liver mitochondria, it was evident that the N-terminal residues play a more prominent role in import than they did with soybean mitochondria. This can be seen with the deletion constructs where the predicted amphiphilic α-helix region supported import into rat liver mitochondria. Purified spinach MPP had a stringent requirement for processing, with all −2 position mutations having a drastic effect. The −10 arginine could not act as a cryptic processing signal, although a −4Arg mutant and a −7Ser mutant could restore processing. A direct comparison with rat MPP was not possible, as we used matrix extract; however it was apparent that the processing by this extract was not affected to the same degree by the mutations in AOX. It was apparent from the import assays that the processing specificity differed between the two organisms, as the products obtained upon import into rat liver mitochondria differed in size to those obtained upon import into soybean mitochondria (Table I). The difference in efficiency in processing the various mutants and the size products obtained in the import assays confirms that organism differences exist in processing that cannot be attributed to the difference between a purified MPP and a matrix extract.

With the plant processing studies there was a strong discrepancy between the inhibition of cleavage obtained with the import assays and that obtained with purified MPP. In the import assays with soybean mitochondria, greater processing of the various mutant precursor proteins was seen compared with the processing studies. Processing could be restored without the restoration of import ability, indicating that the effect on both processes may be different. However as more efficient processing was seen in the import assays, it suggests that (i) either additional isoforms of MPP or other processing peptidases are
present in plant mitochondria or (ii) that additional components help MPP to process precursor proteins upon import into intact mitochondria. In the first instance, we have reported a soluble specific peptidase in both spinach and soybean that can process several mitochondrial precursor proteins (43). It is possible that the requirement for processing with the membrane-bound MPP is more strict, as it is incorporated into a large multi-subunit complex, and that another less complex, soluble peptidase does not have as strict requirements. The results from processing with the rat liver matrix are in agreement with this. The hypothesis that the membrane-bound MPP represents the primitive situation and was moved to the matrix to increase efficiency or range (44) is also in agreement with the results obtained here. The second possibility is that additional factors such as chaperones may bind to the presequence and present it in a proper conformation for processing to MPP. Again, processing with the rat liver matrix, which may contain these factors, supports this hypothesis. However, as purified MPP processes the wild type AOX precursor efficiently in the absence of any added factors, it is difficult to imagine that the single amino acid change affects binding of a chaperone in the translation lysate to the extent that processing is completely inhibited, but import, which has been shown to require several chaperones, is much less affected (4).

The other important conclusion from this study is that an amphipathic α-helix alone is not the only requirement for plant mitochondrial import. Although a predicted helical region can support import with low efficiency, it is not an absolute requirement, nor it is sufficient for efficient import. Therefore, it cannot be presumed that precursor protein from other organelles, such as chloroplasts, which have the ability to form an amphipathic helix, will necessarily have to be specifically excluded from importing into plant mitochondria. The question of how import specificity is maintained in plants has been raised many times. It is important to note that specificity may be the result of many steps. Here we have shown that the requirement for import does not solely depend on an amphipathic α-helix in plants. Other steps such as binding to specific chaperone factors may also be involved in specificity, so that overall a number of discriminating steps maintain specificity (3). No single step alone may dictate 100% specificity but may combine so that the overall specificity of import is very high.

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