Aminotransferase Variants as Probes for the Role of the N-terminal Region of a Mature Protein in Mitochondrial Precursor Import and Processing*

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(Received for publication, September 4, 1997, and in revised form, November 6, 1997)

Of the two homologous isoforms of aspartate aminotransferase that are nearly identical in their folded structures, only the mitochondrial form (mAAT) is synthesized as a precursor (pmAAT). After its in vitro synthesis in rabbit reticulocyte lysate, it can also be efficiently imported into isolated rat liver mitochondria, where it is processed to its native form by removal of the N-terminal presequence. The homologous cytosolic isoenzyme (cAAT) is not imported into mitochondria, even after fusion of the mitochondrial presequence from pmAAT to its N-terminal end. Substitution of the 30-residue N-terminal peptide of the mature portion of pmAAT with the corresponding sequence from the homologous, import-incompetent cytosolic isoenzyme (pcmAAT) does not prevent import but reduces substantially its processing in the matrix. A detectable amount of the pcmAAT chimera is found associated with the inner mitochondrial membrane. Single and double substitution mutants of Trp-5 and Trp-6 at the N-terminal end of the mature protein are imported into mitochondria with efficiency similar to that of wild type. However, replacement of Trp-5 with proline, or of both tryptophans with either alanine (W5A/W6A mutant) or valine and alanine (W5V/W6A mutant), allows import but interferes with the correct processing of the imported protein despite the presence of an intact cleavage site for the processing peptidase. Similar cleavage results were obtained using newly synthesized proteins and mitochondrial matrix extracts. These results indicate that translocation and processing for a precursor are independent events and that sequences C-terminal to the cleavage site are indeed important for the correct maturation of pmAAT in the matrix, probably because of their contribution to the conformation and flexibility of the peptide region surrounding the cleavage site required for efficient processing. The same region from the mature component of the protein may play a role in the commitment of the passenger protein to complete its translocation into the matrix.

Over 90% of mitochondrial proteins are encoded by the nuclear DNA, synthesized in the cytoplasm as precursors with an N-terminal targeting sequence, and imported into the or-

ganelle. For proteins destined to the mitochondrial matrix such as mitochondrial aspartate aminotransferase (mAAT),1 the translocation process involves the initial binding of the precursor form of the protein to the surface of mitochondria, its transport across both mitochondrial membranes mediated by the translocation apparatus, and the final processing and folding of the protein in the matrix (1, 2). In addition to energy in the form of ATP hydrolysis in the matrix and a membrane potential across the inner membrane (Δψ) (1, 2), a partially unfolded, loose conformation of the imported protein appears to be also necessary for efficient translocation (3, 4). The ATP requirement seems to be due primarily to the ATP-dependent action of mitochondrial hsp70 (mt-hsp70) (5). It has been proposed that hsp70 binds the N terminus of the incoming polypeptide as it emerges into the matrix (6, 7). This binding would secure the irreversibility of the process by acting as a molecular ratchet in collaboration with components of the protein import machinery in the inner membrane (8, 9) and additional mitochondrial chaperones (10). Other molecular chaperones in the matrix, including the cpn60/cpn10 chaperonins (5, 11) and mitochondrial cyclophilins (12, 13), mediate the final folding of at least some proteins into their native, active conformations.

At some point during the translocation process, the presequence of most matrix precursors is proteolytically removed to render the mature, naturally found protein. Many mitochondrial precursors are processed in a single step catalyzed by the mitochondrial processing peptidase (14–16), whereas others require an additional cleavage by mitochondrial intermediate peptidase (17). Several bivalent cations stimulate the activity of the mitochondrial processing peptidase (15). Inhibition of processing activity has been observed in the presence of several metal chelators such as EDTA and O-phenanthroline (15, 18, 19). Processing peptidases are highly specific for the precursor forms of mitochondrial proteins (14), the presequences of which are very heterogeneous in sequence although they share some general properties such as the presence of hydroxylated and positively charged amino acids as well as the ability to form amphiphilic α-helices in hydrophobic environments (20). While these properties are known to be important for the targeting function of the presequences (21), the structural features responsible for the specific cleavage of the presequence within

1 The abbreviations used are: mAAT, mitochondrial aspartate aminotransferase; cAAT, cytosolic aspartate aminotransferase; pmAAT, precursor to aspartate aminotransferase; pcmAAT, chimeric precursor; Δψ, membrane potential; mt-hsp70, mitochondrial hsp70; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RRL, rabbit reticulocyte lysate; TPCK, L-1-tosylamido-2-phenylethyl chloromethylketone; HEPES, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.
mitochondria at the exact junction with the N terminus of the mature protein are much less clear. Several cleavage motifs have been suggested, based on mutagenesis analyses and comparison of large numbers of sequences from extension peptides (22, 23).

The contribution of sequences C-terminal to the cleavage site (i.e. N-terminal end of mature protein) to the import and correct processing of mitochondrial precursors is still under debate. Whereas manipulations of sequences 5 residues downstream of the normal processing site of mitochondrial pre-ornithine carbamyltransferase were reported to affect neither import nor correct processing (24), deletion of mature sequences 17 residues down from the maturation site was found to mainly prevent processing of the F$_1$-ATPase $\beta$-subunit within mitochondria (25). More recent studies using either synthetic peptides (26) or chimeric proteins (27, 28) indicate that the amino acid in position +1 C-terminal to the processing site may have a role in determining the fidelity of processing. The lack of apparent consensus among the sequences at the processing sites of mitochondrial precursors has led to the proposal that the conformation of the presequence peptide and the chain flexibility in the region surrounding the cleavage site may be important for the recognition and maturation of precursors in the matrix (29–32). In addition, it is not known whether the overall conformational state of the imported protein or its interaction with molecular chaperones in the matrix might also contribute to the specificity of the cleavage.

Aspartate aminotransferase exists in animal cells in two homologous (over 50% sequence similarity) molecular forms, one located exclusively in the cytosol (cAAT) and the other in the mitochondrial matrix (mAAT). Both are encoded in the nuclear genome and synthesized in the cytosol, yet only the mitochondrial form is competent for translocation into mitochondria. mAAT is synthesized as a precursor with a cleavable extension at its N-terminal end (pmAAT). This N-terminal segment also contains one of the regions of greater dissimilarities between the two AAT isozymes. The presequence of pmAAT from rat liver is 29 residues long and contains two arginine residues, one of them located in position –2 from the maturation site (33). pmAAT therefore belongs to the R-2 class of mitochondrial precursors containing the RX$_1^+$XX$_2$ cleavage motif (22, 32), where X$_1$ is Ala and X$_2$ is Ser for pmAAT. As we show here, processing of imported pmAAT appears to occur in a single step and is dependent on a metalloprotease activity. In this work, we focus on the analysis of the effect of alterations introduced at the N-terminal end of the mature portion of AAT on the processing of the imported protein as well as on the full translocation of the protein to the matrix compartment.

**EXPERIMENTAL PROCEDURES**

**Construction of Chimeric Proteins and Site-specific Mutagenesis—** The cDNA for rat liver pmAAT previously cloned in Bluescript KS (pBSKS-4) (4) was used as the template for all site-directed mutations. The coding region of pmAAT was excised from pBSKS-4 by EcoRI/BamHI digestion and subcloned into the pALTER vector (Promega). The DNA was mutagenized according to the protocol provided by the vendor. In native pmAAT, the cryptophan residues in positions 5 and 6 are coded by TGG. Mutations at either or both of these positions were achieved by using oligonucleotide primers (ranging from 27 to 36 nucleotides in size) in which the Trp codons in the center of the oligonucleotide were changed to the appropriate codon: GCC GCC for W5A/W6A; GTG GCC for W5V/W6A; TTG TTT for W5F/W6F; TCC GCC for W5P/W6P; AAG TTC for W5I/W6I; GCC TTT for W5S/W6S; and TGG for W5V/W6V. mRNAs obtained were translated in rabbit reticulocyte lysate (RRL) (Promega) for 20 min at 30 °C using 25 µg/ml of mRNA and [35]S methionine as the radiolabeled amino acid as described previously (4, 34). Translation reactions were stopped by chilling on ice and adding cycloheximide to a final concentration of 50 µM.

**In Vitro Import of Presequence Proteins into Isolated Mitochondria—** Mitochondria were isolated from male Wistar rat liver by a simple differential centrifugation (36) in a slightly modified buffer (MESH buffer: 220 mM mannitol, 0.1 mM EDTA, 70 mM sucrose, 20 mM HEPPS, pH 7.4). The import reaction was performed essentially as described previously (4, 37). Briefly, 20 µl of [35]S-labeled precursor protein freshly translated in RRL were incubated with 20 µl of isolated mitochondria (5 mg/ml) in MESH buffer for 30 min at 30 °C. After chilling on ice, mitochondria were resolated by centrifugation at 16,000 × g for 4 min at 4 °C. The supernatant was removed and mixed with an equal volume of 2 × SDS-PAGE sample buffer. This fraction represents the protein that does not bind to mitochondria. The pelleted mitochondria were then resuspended in 200 µl of MESH, recovered by centrifugation, and finally resuspended in an appropriate volume of MESH (65 µl or higher). This mitochondrial fraction was either mixed directly with 2 × SDS-PAGE sample buffer (total protein bound to mitochondria), digested with 20 µg/ml 1,1-tosylamide-2-phenylthyl chloromethyl ketone (TPCK)-treated trypsin for 30 min on ice (protein that has been imported), or digested under the same conditions after disrupting mitochondria with 0.1% Triton X-100 (imported protein that is properly folded). Subsequently, samples were analyzed by SDS-PAGE and, at least, an overnight exposure to a Molecular Dynamics PhosphorImager™ screen. The intensity of the radiolabeled mature size in the pelleted mitochondria relative to that of the precursor plus mature bands observed in the complete import reaction was used to calculate the percentage of precursor protein imported.

**Processing of the imported protein was inhibited by incubating mitochondria with 2 mM O-phenanthroline for 10 min at 4 °C before starting import of the pmAAT translation product as described above.** Due to the requirement of extramitochondrial Mg-ATP for pmAAT import and therefore the interference of metal chelators with translocation, the import reaction was supplemented with 5 mM magnesium acetate and 3 mM ATP. Under these conditions, about 30% of the protease-resistant mAAT associated with mitochondria remained as unprocessed precursor (data not shown).

**Subfractionation of Mitochondria—** After isolation by centrifugation, mitochondrial pellets were treated with 100 µg/ml TPCK-trypsin for 30 min on ice to remove the protein associated with mitochondria but not imported. Trypsin digestion was stopped by adding 240 µg/ml soybean trypsin inhibitor when indicated, a mixture of protease inhibitors (leupeptin (5 µg/ml), pepstatin, aprotilin, antipain, elastatin (1 S µg/ml), and phenylmethylsulfonyl fluoride (PMSF) (2 mM)) was added to mitochondria before disruption of the outer membrane by osmotic swelling. Mitochondria were subjected to osmotic swelling by dilution with 10 volumes of ice-cold hypotonic buffer (10 mM potassium phosphate, pH 7.5). After incubation on ice for 30 min, mitoplasts were resolated by centrifugation (16,000 × g for 4 min at 4 °C) and reisolated in the same hypotonic buffer. When indicated, the protease inhibitors (leupeptin (5 µg/ml), and aprotilin and pepstatin (1.5 µg/ml)) were added to the mitoplast preparation. To determine the amount of protein that has been completely translocated into the matrix, mitoplasts were digested for 15 or 30 min on ice with different concentrations of protease K (5, 10, and 20 µg/ml), followed by inhibition of proteinase K with 4 mM PMSF. The integrity of the mitoplasts was examined by monitoring the leakage of the matrix enzyme malate dehydrogenase into the solution using a standard activity assay for this enzyme (38).

To fractionate mitoplasts into their soluble (matrix) and membrane components, samples were frozen in liquid nitrogen and thawed in a sonicator water bath (sonoicing). This freezing/thawing/sonication cycle was repeated three times, and the extract was centrifuged in a Beckman Airfuge for 10 min at 100,000 × g to separate the soluble matrix (supernatant) from the membrane fraction (pellet).

**In Vitro Processing Peptidase Activity—** Mitochondrial matrix extracts were prepared from rat liver mitochondria (5 mg/ml) by osmotic swelling in hypotonic buffer, followed by disruption of the recovered
mitoplasts by cycles of freezing-thawing and sonication as described above in the presence of protease inhibitors leupeptin and pepstatin (2 μg/ml) and PMSF (0.1 mM). Serine protease inhibitors and microbial protease inhibitors do not affect the processing peptidase activity. Processing reactions contained (in a final volume of 10 μl): 2 μl of RRL translation reaction containing 35S-labeled pmAAT, 2 μl of mitochondrial matrix extract, 1 μl of 5 mM MnCl2, 43 units/ml of apyrase, and 5 μl of 10 mM phosphate buffer, pH 7.5. Processing reactions were incubated at 27 °C for 5, 15, or 30 min. Reactions were terminated by the addition of an equal volume of 2× SDS-PAGE loading buffer, and samples were analyzed by SDS-PAGE and autoradiography. The amount of processing was estimated from the intensity of the radiolabeled bands was determined using a Molecular Dynamics PhosphorImager as described previously (39) in 12% polyacrylamide separating gels approximately 5 cm in length. After fixing and drying the gels, the intensity of the radiolabeled bands was determined using a Molecular Dynamics PhosphorImager as described previously (35, 37). All oligonucleotides were synthesized at the University of Missouri Kansas City and purified on NENSORB PREP columns (Du Pont). Restriction enzymes and T4 ligase were obtained from New England Biolabs, and Promega. Proteinase K was from IBI.

RESULTS

Sequence comparisons between the N-terminal end of mature mAAT isolated from rat liver (40) and the complete cDNA of rat precursor pmAAT (35) indicate that processing of pmAAT in vitro occurs by cleavage between Ala-29 and Ser-30 of the precursor polypeptide (Fig. 1). The sequence of the cleavage site and its immediate flanking region is identical in pmAATs from other animal sources (41, 42) but differs from those found in other mitochondrial matrix proteins (23). To analyze the contribution of sequences downstream from the cleavage site to both efficient import and correct processing of rat liver pmAAT, we prepared various fusion protein constructs (Fig. 1) and substitution mutants (Table I). One of the fusion constructs, pcAAT, encodes the presequence of pmAAT fused in frame to the entire cDNA of the homologous cytosolic AAT isozyme, which differs substantially from mAAT in its N-terminal segment (34). The other, pcmAAT, codes both the mitochondrial presequence and 33 amino acids from the N terminus of the cytosolic cAAT fused to the cDNA for residues 34–401 of the mitochondrial mAAT (35). In addition, a set of single and double substitution mutants was prepared targeting the initial part of the N-terminal segment of the mature form of the protein, which is known to be critical for proper protein folding and stability of AAT. Thus, either or both of the tryptophan residues found in positions 5 and 6 of the mAAT N-terminal peptide (third and fourth residue downstream from the cleavage site) were replaced with a variety of hydrophobic, hydroxylated, or even charged residues (Table I). Nevertheless, these mutants retained the wild type Ala-Ser cleavage site.

Table I

<table>
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<td>Mature/100</td>
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<td>55–60</td>
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<td>15–20</td>
<td>Intermediate/100</td>
</tr>
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</table>

a Fraction of mature protein associated with trypsin-treated mitochonria relative to the amount of precursor plus mature protein recovered in the complete import reaction.

b Fraction of imported protein that is resistant to trypsin in the presence of triton.

c Fraction of imported protein having mature or intermediate size.

Figure 1. N-terminal amino acid sequences of the proteins used in this study. The sequences are numbered to maximize sequence homology between the mitochondrial (mAAT; Ref. 33) and cytosolic (cAAT; Ref. 46) isozymes. The first amino acid of the mature sequences (residue 3 in pmAAT and residue 1 in the constructs having the N-terminal peptide from cAAT) is indicated by an arrow. Negative numbering is used for the presequence peptide starting with the C terminus as residue −1. The methionine residue (in parentheses) at the junction between the presequence and cAAT sequence in the chimeric proteins was introduced as a result of the cloning strategy used. The amino acids of pmAAT are shown in bold type, and those of cAAT are displayed in bold type. The pair of tryptophans that were changed by site-directed mutagenesis are underlined.
radiolabeled mature size protein in the pelleted mitochondria treated with trypsin relative to that of the precursor plus mature bands observed in the complete import reaction was used to calculate the percentage of precursor protein imported (ranging from 60% to 70%). Treatment with trypsin in the presence of 0.1% Triton X-100, which disrupts the mitochondrial membranes and thereby allows access of the protease to the internalized protein, showed that about 60–65% of the imported protein was properly folded (Fig. 2, lane 5). This test for folding relies on the well known extreme resistance displayed by folded (native) mAAT toward proteolysis (44). The precursor form pmAAT is also able to fold in to a conformation extremely similar to the known structure of the mature protein (43); only the presequence peptide is susceptible to hydrolysis by low concentrations of trypsin (4), which cleaves after Arg at positions −22 and −2 in the presequence peptide (45). On the other hand, a hybrid protein (pAAAT) consisting of the pmAAT presequence fused to the N terminus of the cytosolic isoform, which can fold rapidly even in RRL (34), not only was not imported into isolated mitochondria, it did not even bind to the organelle (data not shown).

Different behavior was observed for pcmAAT, a chimera in which only the distinctive N-terminal segment of cAAT has been introduced into the N-terminal sequence of pmAAT (Fig. 1). Upon incubation with isolated mitochondria, a substantial amount of precursor-sized protein was recovered bound to mitochondria (Fig. 2B, lane 3). A fraction of this material (40–50%) was resistant to externally added trypsin (Fig. 2B, lane 4), which contrasts with the complete digestion of the precursor-size wild type protein associated with mitochondria. This indicates that pcmAAT has been translocated to a protease-inaccessible location, but most of it remains unprocessed. The small amount of mature-like species associated with mitochondria varied between experiments but usually represented about 20–30% of the protease-resistant imported protein (mature band in Fig. 2B, lane 4). Treatment of mitochondria with trypsin in the presence of detergent indicates that only about 20% of the imported pcmAAT was properly folded, significantly less than for the wild type (Fig. 2, compare lanes 5 in A and B).

Since in the folded precursor only the presequence is susceptible to trypsin (4), the small amount of trypsin-resistant chimera detected under these conditions appears as a mature size band due to the loss of the presequence to trypsin hydrolysis.

**Translocation of pmAAT Trp-5 and Trp-6 Mutants into Isolated Mitochondria**—The results presented above indicate that alterations introduced C-terminal to the peptide bond cleaved by the mitochondrial processing peptidase in pmAAT, i.e. in the N-terminal region of the mature protein, might be important for precise processing. However, the sequence alterations introduced in pcmAAT extend from residue +33 to the amino acid in the +1 position (Met for Ser, Fig. 1). Although methionine residues have been found at both the −1 and +1 positions of maturation sites of mitochondrial precursors (23), it is not clear which of the modifications in the sequence downstream from the processing site in pcmAAT is responsible for the inhibition of processing. To begin to address this question more specifically, we prepared a family of mutants containing the wild type cleavage site sequence, but with single or double substitutions in positions 5 and 6 of the N-terminal peptide of mature mAAT that correspond to residues +3 and +4 C-terminal from the processing site in pmAAT (in the numbering of AAT, the N terminus of mature mAAT is numbered as residue number 3 to maximize sequence alignments with the cytosolic isoform of various species, which contains two additional residues at the N-terminal end; Ref. 46). These two tryptophan residues are particularly intriguing because they are conserved in all mitochondrial mAATs for which sequences are available, whereas the equivalent positions in the cytosolic forms contain either phenylalanine or other hydrophobic residue but not tryptophan (33, 46, 47). They are also known to contribute to the stability of the dimeric structure of the native protein.

The identity of each mutant (listed in Table I) was confirmed by sequencing the cDNA in the region of the mutation. The SDS-PAGE electrophoretic mobilities of the mutant proteins prepared by in vitro transcription/translation of the corresponding cDNAs were identical to that of the wild type protein. The amount of protein synthesized was also essentially comparable to the wild type (data not shown). Several additional faint bands migrating as mature or smaller size species were often observed in the translation reactions of these mutants (Fig. 3, TP lanes). They are also present in the translation reaction of wild type pmAAT (Fig. 2, lane 1), and they probably originate from initiation of translation at internal methionine residues. When these mutant proteins were incubated in RRL with isolated rat liver mitochondria immediately after completing translation, all of them were imported with approximately the same efficiency as judged from the amount of trypsin-resistant protein that appears associated with the mitochondrial pellet.

**Fig. 2. Import of pmAAT and pcmAAT into isolated mitochondria.** 35S-Labeled proteins were synthesized in RRL for 20 min at 30 °C. Import of the translation products was performed by incubation with isolated rat liver mitochondria for 30 min at 30 °C as described under “Experimental Procedures.” Mitochondria were reisolated by centrifugation and left untreated (lane 3), or treated with TPCK-trypsin (20 μg/ml) either in the absence (lane 4) or in the presence (lane 5) of 0.1% Triton X-100. Samples were analyzed by SDS-PAGE and the radioactive protein bands visualized in a PhosphorImager. The sequences of the processing regions are shown below their respective panels, including the expected cleavage sites, which are indicated by an arrow. TP, translation product, 10% of the amount used in the import reaction; IR, unfractionated import reaction; p, precursor; m, mature processed form.
(Table I). However, not all of them were properly processed upon import into mitochondria. Particularly, introduction of proline in position 5 (W5P-pmAAT) results in the appearance of a measurable amount of unprocessed precursor associated with mitochondria and resistant to digestion by trypsin (Fig. 3A). However, the amount of mature-size protein was higher than for pcmAAT and oscillated between 35% and 45% of the protease-resistant (imported) protein associated with mitochondria. The difference in electrophoretic mobilities between the precursor and processed material is identical to that found with the precursor and mature forms of the wild type protein suggesting that the inefficient processing of the W5P mutant probably occurs at the Ala-Ser cleavage site.

Two of the double mutants, W5V/W6A-pmAAT and W5A/W6A-pmAAT, although they can be equally internalized into mitochondria, show a strikingly distinct processing behavior. As shown in Fig. 3C for the W5V/W6A mutant, the translation product associated with trypsin-treated mitochondria is present exclusively as species intermediate in size between unprocessed precursor and mature mAAT. Identical results were obtained for the W5A/W6A mutant (data not shown). It appears that these mutant precursors may be processed at an alternative site within the presequence. Although the precise location of the cleavage has not been established, the molecular weight of the resulting species coincides with that of an intermediate that we have previously obtained and characterized by incubating purified wild type pmAAT with very small concentrations of trypsin. Sequence analysis showed (45) that this shortened precursor was produced by trypsin hydrolysis after Arg at position -22 (Fig. 1). Therefore, the anomalous processing of the W5V/W6A and W5A/W6A mutants probably occurs at/or in the vicinity of this arginine residue. It is important to point out that the translocated mutants appear to be in an incompletely folded state since over 80% of the intermediate-size band disappears upon treatment of mitochondria with trypsin in the presence of detergent (Table I).

By contrast to the above behavior, all of the other pmAAT mutants studied containing either hydrophobic (Ala, Val, Phe, Ile) or hydrophilic (Ser, Thr, Arg) residues in positions 5 or 6 were both imported into isolated mitochondria and processed to a molecular species with electrophoretic mobility similar to that of wild type processed protein (results for W5F/W6A-pmAAT are shown in Fig. 3B to illustrate the behavior of this group of mutants). Furthermore, the efficiency of the processing reaction determined from the fraction of mature-size protein appearing associated with trypsin-treated mitochondria was close to that of wild type precursor (Table I). Many of these mutants fold properly after translocation into the matrix, whereas a few of them (double mutants W5F/W6V, W5F/W6I, and W5F/W6A) fold less efficiently (Table I).

**Location of Imported Proteins within Mitochondria**—The results presented in Figs. 2 and 3 indicate that a substantial fraction of protease-resistant pcmAAT and W5P-pmAAT accumulates within mitochondria as unprocessed species after import in vitro. Between 60% and 70% of the protease-resistant material remained associated with mitoplasts prepared by disruption of the outer membrane by swelling mitochondria under hypotonic conditions (Fig. 4, B and C). This treatment leaves the inner membrane intact (38) but releases the contents of the intermembrane space. The distribution between precursor and processed material remained unchanged after preparation of mitoplasts. Similar treatment of mitochondria that had import either wild type (Fig. 4A) or a pmAAT mutant that is processed normally (Fig. 4D) shows that also about 60% of the translocated proteins is recovered with the mitoplast fraction.

The above results indicate that the unprocessed mutant proteins have been translocated past the outer membrane barrier and are tightly associated with mitoplasts. Further fractionation of the mitoplast preparation into its membrane (pellet) and soluble matrix (supernatant) components by successive cycles of freezing and thawing, sonication, and centrifugation showed that less than 5% of imported wild type or W5F/W6A mutant is recovered in the membrane fraction (Fig. 4, A and D). By contrast, about 45% of the pcmAAT recovered fractionates with the membrane and 55% appears in the matrix fraction (Fig. 4B). In the case of W5P-pmAAT, 25% appears in the membrane pellet and 75% in the soluble fraction (Fig. 4C). The material found in the membrane fraction included both precursor and mature-size species and, in the case of pcmAAT, a small amount of an additional lower molecular weight band (Fig. 4B, lane 5), which most likely is produced by degradation of the radiolabeled protein by endogenous proteases. This conclusion is strengthened by the observation that addition of a mixture of protease inhibitors during the initial swelling of mitochondria and the subsequent disruption of mitoplasts improved the total recovery of material and also decreased the intensity of the
lower molecular weight band. This may also be the cause of the change in distribution of precursor and processed species observed after fractionation of mitoplasts. Nevertheless, the results show clearly the presence of a substantial amount of imported pcmAAT in the membrane fraction. The protein recovered with the matrix fraction displayed a similar distribution between unprocessed and mature-like forms (Fig. 4, lanes 4 in B and C). Therefore, a measurable fraction of these pmAAT mutants, particularly pcmAAT, remains associated with the inner membrane after translocation into mitochondria. However, this alone does not explain the low efficiency of processing since over half of the unprocessed precursor appears in the matrix fraction where it had unrestricted access to the processing peptidase.

Both the wild type protein and a pmAAT mutant that appears to be processed normally (Fig. 4, A and D, respectively) were found mainly in the matrix fraction. The two double mutants (W5A/W6A and W5V/W6A) that are processed to intermediate-size species appeared exclusively in the matrix fraction (data not shown). The overall recovery of radioactive material was over 65% of that initially present in the mitoplasts for the wild type and about 45% for the mutants. These mutants, which appear to remain incompletely folded after translocation (Table I), are found exclusively in the matrix. Thus, the partially folded conformation of at least some of the imported pcmAAT (see lane 5 in Fig. 2B) cannot be the sole reason for its binding to the inner membrane.

Since both mature-sized and unprocessed pcmAAT are found associated with the membrane fraction, the retention of a fraction of mutant pcmAAT in the inner membrane is not merely the result of the presence of the presequence, which is known to have affinity for binding to membranes (48, 49). This conclusion was corroborated by the observation that unprocessed wild type pmAAT, which accumulates in mitochondria treated with the processing peptidase inhibitor O-phenanthroline, was found exclusively in the matrix (data not shown). These results indicate that full translocation and processing of pmAAT are independent steps. Therefore, the retention of a fraction of pcmAAT and, to a lower extent, W5P-pmAAT in the inner membrane is not the consequence of inefficient processing.

In Vitro Processing of pmAAT Variants by Mitochondrial Matrix Extracts—Processing of wild type and mutant pmAAT was also assessed by monitoring the conversion of precursor to mature form upon incubation of aliquots of in vitro translation reactions with a freshly prepared matrix extract as a source of processing peptidase (18, 32). Incubation of wild type pmAAT
freshly translated in RRL with a matrix extract for 15 min at 27 °C results in about 25% conversion of precursor to mature size translation product (Fig. 5, lane 2). This extent of conversion is comparable to the yield of processing observed using other translation products and purified mitochondrial processing peptidase (18, 30, 32). We should point out that the freshly synthesized pmAAT translation product we use as substrate for processing is completely digested by a small concentration of trypsin to fragments too small to be detected in the SDS-PAGE gel (data not shown). Therefore, it is unlikely that the observed hydrolytic conversion of a fraction of precursor into mature species results from the action of unspecific matrix endoproteases. Hydrolysis of an incompletely folded translation product by those proteases would be expected to be at least as extensive as that observed with trypsin. Nonetheless, the processing reaction was supplemented with various protease inhibitors (leupeptin, pepstatin, and PMSF) to minimize the hydrolysis by matrix proteases. In addition, the processing of pmAAT was completely inhibited in the presence of EDTA and O-phenanthroline (Fig. 5, lane 3), which indicates that the reaction is mediated by a metalloprotease present in the matrix crude extract, most likely a mitochondrial processing peptidase whose activity is influenced by metal chelators (15, 18, 19). Interestingly, no processing was observed (Fig. 5, lane 14) when the translation product was preincubated to allow its folding into a trypsin-resistant conformation (4). Apparently, the processing site is not accessible to the processing peptidase once the translation product has acquired a native-like conformation. Furthermore, this lack of processing is not due to the overall inaccessibility of the precursor since trypsin rapidly removes the presequence from the folded translation product by cleaving after Arg at position −2 just one peptide bond upstream from the processing site (4).

Using identical reaction conditions, insignificant processing was observed for the chimera pcmAAT (Fig. 5, lane 5) containing the N-terminal segment from the cytosolic isozyme or for W5P mutant (Fig. 5, lane 8). However, the double substitution mutant W5V/W6A, which is fully processed to an intermediate size species in intact mitochondria, was also converted to species of similar intermediate size between precursor and mature by the matrix extract (Fig. 5, lane 11). This conversion was also completely inhibited in the presence of EDTA and O-phenanthroline (Fig. 5, lane 12), suggesting that it is mediated by the same peptidase that processes the wild type protein. A band corresponding in size to the mature form of pmAAT detected in the reaction containing the W5V/W6A mutant (Fig. 5, lane 11) is present in the original translation reaction (lane 10) and therefore is not generated by incubation with the matrix extract. This band is likely to result from translation initiation at an internal methionine in the mature portion of the protein. The presence of this band in the translation reaction of wild type pmAAT (lane 1) and W5P mutant (lane 7) but not in that of pcmAAT (lane 4) supports this interpretation (the N-terminal sequence of pcmAAT contains two methionines in positions 11 and 33, whereas no methionines exist in the same region of the cAAT sequence as shown in Fig. 1).

Thus, the results obtained for the processing in vitro of these pmAAT variants by matrix extracts, essentially parallel those observed with intact mitochondria, although the efficiency of processing is considerably lower with the matrix extracts. This might explain the almost undetectable processing levels of W5P mutant, which, although inefficiently, appears to be processed in intact mitochondria.

**DISCUSSION**

One of the final steps in the translocation of most mitochondrial precursors into the matrix is the proteolytic removal of the N-terminal presequence peptide that has initially guided the protein to the organelle. Since no intermediate size species are detected during import of wild type rat liver pmAAT into isolated mitochondria (Fig. 2), this precursor is probably processed in a single cleavage step, most likely mediated by the chaperon-sensitive general mitochondrial processing peptidase.

One of the unusual features of the action of processing peptidases appears to be their ability to cleave with great fidelity at the presequence-mature junctions of many different precursor proteins. The almost complete lack of consensus among the sequences at these cleavage sites (22, 23) has led to the proposal that higher levels of structure might be responsible for the specificity of the proteolytic cleavage (24, 30). However, the exact nature of the above mentioned structural requirements is still obscure. This question has been addressed primarily by analyzing the structure of small synthetic peptides comprising the presequence itself or the presequence plus a few residues of the N-terminal mature sequence (31, 50) in membrane-mimetic environments. However, the conformational properties of the cleavage region or its accessibility to the protease may be determined not just by the sequences surrounding the nicked peptide but by the overall conformation of the protein substrate at the time of processing, which in turn might be influenced by its interaction with mitochondrial chaperones. Indeed, our results indicate that the folding state of the precursor substrate affects dramatically the efficiency of the reaction. Analysis of the processing of pmAAT translated in vitro in RRL by a matrix extract clearly shows that the folded, trypsin-resistant polypeptide is no longer processed to the mature form. By contrast, a significant fraction of the newly synthesized unfolded pmAAT can be processed to a mature-size form by this matrix extract (Fig. 5). These results suggest that processing of pmAAT in intact mitochondria probably precedes at least the latest stages of folding of the imported protein. Similar behavior has also been reported for mitochondrial rhodanese (32).

Thus, matrix processing peptidases resemble other endoproteases in their general requirements for cleavage of peptide bonds in non-denatured proteins. Proteolysis of native structures is limited not just by the primary specificity of the endo-
protease or the accessibility of the peptide bond but also by the flexibility of the region proximate to the cleavage site and its ability to adopt a cleavable conformation at the protease active site (51). Hence, local unfolding of the potential cleavage region can be the key factor in determining limited proteolysis. Analysis of the structure of the N-terminal region of mAAm in the folded structure provides a plausible explanation for the inability of matrix extracts to process the folded precursor. In the native folded dimer, the N-terminal arm is part of the subunit interface with residues 3–14 of the mature sequence forming an extended strand that interacts with the other subunit (52). Residues Trp-5 and Trp-6 bind in hydrophobic pockets on the surface of the other subunit, whereas residues Ser-3 and Ser-4 are more exposed to the solvent. Even though the processing site Ala-(−1)-Ser-3 might be still relatively accessible on the surface of the protein, restrictions in the flexibility or conformational adaptability of the polypeptide chain downstream from the cleavage site as a result of quaternary interactions might interfere with the processing peptidase. It is interesting to note that the peptide bond after Arg at position −2 (one peptide unit upstream from the pmAAm maturation site) is readily cleaved by small concentrations of another protease, trypsin (4), confirming that in the folded protein the N-terminal end is exposed on the surface of the folded precursor.

In addition to a partially unfolded conformation of the precursor protein, a number of structural features of the region surrounding the cleavage site have been identified as essential for efficient processing. Within the presequence peptide itself, the presence of positively charged residues both near the cleavage site and at the N-terminal region of the presequence seems to be important for processing (29, 53). A certain degree of flexibility of the presequence peptide also appears to be necessary (31, 32). In this report, we show that the region C-terminal to the processing site is also important for recognition and processing by the liver matrix peptidase. Several alterations affecting the amino acid sequence of the N terminus of the mature subunit of pmAAm interfere with processing. First, the chimeric precursor pmAAm remains essentially unprocessed after import into mitochondria or incubation with a matrix extract. In this precursor, the residues at positions +1 to +4 relative to the cleavage site and beyond have been replaced by the corresponding sequence form the import-incompetent cytosolic isozyme. Despite the fact that methionine residues are found at +1 positions of maturation sites in other precursors (23), in the context of the pmAAm processing region this substitution per se might be responsible for the inhibition of processing. In fact, it has been reported that amino acid substitutions at position +1 affect processing but not import of a chimeric protein containing the presequence of cytochrome b2 fused to dihydrofolate reductase (28).

Second, our results on the processing of a set of pmAAm mutants with substitutions exclusively in the +3 and +4 positions indicate that efficient processing is also directly or indirectly dependent on amino acid determinants present at these locations. From the summary presented in Table I, we can see that the processing peptidase can tolerate almost any residue in either of these positions as long as the other residue remains aromatic (either wild type tryptophan or phenylalanine). The main exception to this trend is the incorporation of Pro in the +3 position (the mutant containing proline in the +4 position is not available). The processing of this mutant (W5P-pmAAT) by intact mitochondria is very inefficient, although better than for pmAAm. It is interesting to note that the pmAAm chimera contains two consecutive proline residues at the +3 and +4 positions. A possible interpretation of these findings is that the substrate recognition site of the peptidase extends to at least the +3 position in the substrate and the enzyme is unable to accommodate a Pro residue in this location, perhaps because of the configuration of the Ser-Pro peptide bond at position +2/+3. Many proteolytic enzymes, including trypsin, show a strong preference for cleaving peptide substrates when the cleaved bond or other bonds close to the cleavage site are in the trans configuration (54). A similar restriction may apply to the matrix processing peptidase substrates if the X-Pro bond in the pmAAm and W5P-pmAAT precursors exists in the cis form or as a mixture of the cis and trans forms at equilibrium. Alternatively, the presence of Pro in this position may have a long range effect on the conformation of the cleavage region that may affect its specific recognition by the peptidase or the proper orientation of the Ala-Ser scissile bond toward the catalytic residues within the peptidase’s active site. Interestingly, even trypsin has difficulties cleaving after Arg at position −2 in the folded W5P-pmAAT.

The reasons for the incorrect processing of the mutants containing non-aromatic residues at the +3 and +4 positions are unclear. Only two such mutants are available (W5A/W6A and W5V/W6A), but in both cases the precursors are converted not to the mature species but to intermediate-size species by intact mitochondria, indicating that cleavage at the normal processing site is prevented. Comparison of the electrophoretic mobility of these intermediate species to that of a shortened precursor produced by trypsin hydrolysis of purified folded pmAAm after Arg at −22 (45) indicates that a cleavage in a region near this Arg at −22 is occurring instead. This alternative processing most likely results from the action of the processing peptidase and not other matrix proteases since the sequence of the unprocessed pmAAm and W5P-pmAAT species that accumulate in the matrix remains intact, and a similar, chelator-sensitive conversion is observed with matrix extracts. It is possible that incorporation in these positions of a residue with a relatively high helical tendency such as Ala might alter the conformation of the processing site. Secondary structure predictions indicate the potential for formation of α-helices at the N-terminal, middle, and C-terminal portions of the sequence separated by flexible linkers (residues −19 to −22 and −12 to −10) (45). The introduction of alanine residues in the region immediately following the junction with the mature sequence might favor the extension of the helical structure through the maturation site. This conformational alteration of the initial residues of the mature protein might preclude recognition by the processing peptidase as proposed for the linker-deleted presequence of another mammalian protein, the aldehyde dehydrogenase precursor (31). This may also lead to the positioning of the wrong peptide region in the active site of the peptidase, which could explain the incorrect processing observed. Analysis of the sequence of the region where the alternative cleavage appears to occur reveals the presence of a sequence motif −22RVLS−19 that resembles the normal maturation site in pmAAm (−22RASS−2) in that it contains the essential Arg in position −2.

Our results raise the issue of how a region of the presequence about 20 residues upstream from the cleavage site can end up being positioned in the catalytic site of the peptidase. A possible explanation is that this region of the presequence represents a normal substrate recognition motif during processing of the wild type protein by the processing peptidase and therefore has a specific binding site in the peptidase. Alterations in the conformation of the processing site may lead to changes in the positioning of the N-terminal region of the presequence in the

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catalytic site that result in the cleavage of the wrong peptide bond. This interpretation is supported by previous studies indicating the importance of basic residues, particularly arginine, at positions distant from the cleavage site (position −22 in pmAAT) for recognition by the matrix processing peptidase (29, 30). Based on these findings, it was proposed that both the proximal and distal arginine residues found in presequences interact with the peptidase active site (29). The presence of a flexible linker region containing glycine and/or proline residues in the middle of the presequence (positions −11 and −10 in pmAAT) would facilitate the positioning of these two distant regions of the presequence in the active site. Indeed, removal of the RPG linker found in aldehyde dehydrogenase has been shown to render the protein unprocessable (31), suggesting that presequence flexibility might be necessary, although probably not sufficient, for correct processing.

Several of the pmAAT mutants that show altered processing appear to have difficulties folding properly after import into the matrix (Table I). Therefore, it may be argued that misfolding or aggregation of the partially folded substrate is the reason for the anomalous processing observed. However, no such correlation exists between the folding and the processing properties of other mutants. Whereas the W5F/W6A mutant, which remains partially unfolded after translocation, is processed correctly and as efficiently as the wild type, only a fraction of imported W5F-pmAAT, which folds properly, is converted to mature.

Even though all of the pmAAT forms analyzed in this work are translocated to a protease-inaccessible location inside mitochondria with yields similar to the wild type, a fraction (about 50%) of internalized pmAAT is recovered in the inner membrane fraction. Apparently, alteration of the N-terminal section of pmAAT interferes with some of the last stages of its translocation into the matrix. According to current models for protein import into mitochondria, irreversible translocation across the inner membrane requires the ATP-dependent interaction of precursors with mt-hsp70 (13, 55, 56), which recognizes both the presequence peptide and incoming mature segments of precursor proteins (7). The presence of the N-terminal sequence from cAAT at the N terminus of pmAAT might hinder the initial interaction of mt-hsp70 with emerging segments of the mature protein immediately following the presequence. As a consequence, some of the protein may slide back into the membrane. This interpretation is consistent with our previous findings, indicating that the N-terminal segment of the mature protein sequence represents a recognition site in pmAAT for cytosolic hsp70 (35, 57) and that cAAT does not bind to cytosolic hsp70 (35, 58). Thus, the N-terminal region of mature mAAT contains information for both correct processing and full translocation of the mAAT precursor into the matrix. It is possible that both processes involve the action of molecular chaperones in the matrix. We propose that intermolecular interactions with molecular chaperones involved in the translocation and folding of the imported protein may contribute to the correct conformation and orientation of the processing region to allow recognition and correct processing by the processing peptidase. According to this hypothesis, mutations C-terminal from the maturation site may affect its interaction with other matrix proteins and thereby its accessibility and general suitability as substrate for the processing peptidase.

In summary, this study provides valuable insight into several aspects of the translocation and maturation of precursors in mitochondria. First, the hybrid precursor pcAAT is not translocated into mitochondria in vitro, which indicates that the presequence peptide is necessary but not sufficient for import. Additional information must reside within the sequence of the passenger protein. Second, translocation and processing are independent events. However, certain alterations of the N-terminal region of the mature protein affect the efficiency of both. Third, the requirements for substrate recognition and specific hydolysis by processing peptidases might not be that different from those of other endoproteases for hydrolysis of peptide bonds in native proteins. In addition to information contained in the presequence peptide, efficient and correct processing are dependent on amino acid determinants located C-terminal to the cleavage site. The permissive primary specificity characteristic of the mitochondrial processing peptidases probably arises from a catalytic site that can accommodate a great variety of different amino acid residues at the −1 and +1 positions. However, to achieve the correct orientation of the active bond toward the catalytic groups, the enzymes may display “conformational specificity” for regions both upstream and downstream from the cleavage site. Any amino acid substitution perturbing this as yet unknown structure would affect the processing reaction. The desired conformation can be directed by the amino acid sequence of the region or imposed by intermolecular interactions with components present in the mitochondrial matrix, particularly molecular chaperones involved in the translocation and folding of many imported proteins.

Acknowledgment—We thank Dr. Joseph Mattingly for preparing the pcmAAT chimera used in this study.

REFERENCES

N-terminal Role in Mitochondrial Precursor Processing
