Precursor Protein Is Readily Degraded in Mitochondrial Matrix Space if the Leader Is Not Processed by Mitochondrial Processing Peptidase

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It is not known why leader peptides are removed by the mitochondrial processing peptidase after import into the matrix space. The leaders of yeast aldehyde dehydrogenase (pALDH) and malate dehydrogenase were mutated so that they would not be processed after import. The recombinant nonprocessed precursor of yeast pALDH possessed a similar specific activity as the corresponding mature form but was much less stable. The nonprocessed pALDH was transformed into a yeast strain missing ALDHs. The transformed yeast grew slowly on ethanol as the sole carbon source showing that the nonprocessed precursor was functional in vivo. Western blot analysis showed that the amount of precursor was 15–20% of that found in cells transformed with the native enzyme. Pulse-chase experiments revealed that the turnover rate for the nonprocessed precursor was greater than that of the mature protein indicating that the nonprocessed precursor could have degraded. By using carbonyl cyanide m-chlorophenylhydrazone, we showed that the nonprocessed precursor was degraded in the matrix space. The nonprocessed precursor forms of precursor yeast malate dehydrogenase and rat liver pALDH also were degraded in the matrix space of HeLa cell mitochondria faster than their corresponding mature forms. In the presence of o-phenanthroline, an inhibitor of mitochondrial processing peptidase, the wild type precursor was readily degraded in the matrix space. Collectively, this study showed that the precursor form is less stable in the matrix space than is the mature form and provides an explanation for why the leader peptide is removed from the precursors.

The vast majority of mitochondrial matrix space proteins are nuclear encoded and synthesized as precursor proteins with an extension of amino acids at their N-terminal end that functions as a mitochondrial import signal sequence (1, 2). After import, the leader sequence is removed by the action of a protease, the mitochondrial processing peptidase (3–6). It is not known why it is necessary for the leader to be removed. It was shown that pre-fumarase was less active than its mature form (7). In contrast, the recombinantly expressed rat liver pALDH had essentially the same specific activity as the mature form of the enzyme, but the precursor was thermally less stable than was the mature form (8). It appears that in some cases removing the leader might produce a more stable mature protein.

Deletion of the mitochondrial processing peptidase was lethal to aerobically growing yeast (9). This implies that in vivo it is necessary to convert some, if not all, of the precursor proteins to their mature counterpart. It is not known what is the fate of a precursor protein should it remained unprocessed. Recently, however, investigators found that the leaders cleaved by MPP are degraded to smaller fragments by two protease enzymes present in plant mitochondria and chloroplast (10).

These enzymes are not reported to be found in yeast or mammalian cells. While studying structure/functional relationships of leader sequences (11–13), we were able to convert some into ones that became nonprocessable and found that they were imported into mitochondria, but the long term fate of the protein was not investigated. It is known that synthetic peptide corresponding to the leader sequence can bind to the mitochondrial membranes (14). It is possible then that the nonprocessed precursor proteins might bind to the matrix side of the inner membrane after they were translocated into the matrix space. Alternatively, the presence of the leader sequence might affect the rate of folding as well as the stability of the protein, thus increasing the rate of degradation.

The three-dimensional structure of the mature form of human liver mitochondrial ALDH, but not the precursor, has been determined (15, 16). The first seven residues were not detected, so it cannot be stated with certainty where the leader might be located on the precursor molecule. It possibly could be located on the surface of the subunit as the first seven residues of mature protein appeared to be located on the surface. Should this be the case then the leader would be exposed to solvent. If for other proteins the N termini are buried in the protein, then it is apparent why it must be removed before the protein folds in the matrix space. A number of diseases have been reported that are a result of missing a nuclear coded matrix space protein

4 The abbreviations used are: pALDH, precursor aldehyde dehydrogenase; ALDH, aldehyde dehydrogenase; mutant-pALDH, the yeast R18Q,R22Q double mutant precursor ALDH, which is nonprocessable; pMDH, precursor yeast malate dehydrogenase; mutant pMDH, K7Q,R8Q double mutant precursor MDH; CCCC, carbonyl cyanide m-chlorophenyl hydrazone; MPP, mitochondrial processing peptidase; RGP, Arg-Gly-Pro.
(17). A mutation occurring in the leader could make it nonprocessable, and then, if the precursor proved to be unstable, an explanation for finding a decreased level of protein can be offered.

We undertook a study to investigate the fate of a nonprocessed precursor protein. By employing a yeast strain that had three of its chromosomal ALDHs disrupted (18) and modifying yeast pALDH so it became nonprocessable, it was possible to study the fate of a nonprocessed precursor ALDH in vivo. We went on to also use rat pALDH and precursor malate dehydrogenase (pMDH) in HeLa cells to test further the stability of the nonprocessed precursor proteins. We report here that the nonprocessed precursor of yeast pALDH was active but rapidly degraded in the mitochondrial matrix space. We show here that rat nonprocessed pALDH and yeast nonprocessed pMDH were also degraded in matrix space faster than their corresponding mature forms.

**MATERIALS AND METHODS**

**Yeast Strains and Growth Conditions—**Saccharomyces *cerevisiae* strain was used to isolate yeast mitochondria for the import reactions. The triple ALDH-disrupted yeast strain HWTHL10, which was previously constructed in this laboratory (18), was used for ALDH expression in yeast, yeast growth complement assays, and pulse-chase experiments. Nontransformed yeast was grown in YPD (1% yeast extract, 2% peptone, and 2% glucose). Transformed yeast was grown in acetate medium (2% glucose, 0.5% acetate, pH 5.7, 0.67% yeast nitrogen base, and 0.074% complete supplement mixture lacking uracil) or in ethanol medium (2% ethanol, 0.5% glucose, 0.67% yeast nitrogen base, and 0.074% complete supplement mixture lacking uracil).

**Cloning of ALDH and MDH Genes—**Yeast and rat mitochondrial pALDHs as well as the mutated form of the rat liver enzyme were previously cloned (11, 18). They were subcloned in the *Escherichia coli* expression vector pT7-7 and the bacterial/yeast shuttle vector pRS316m that carried the yeast pALDH native promoter and terminator. The yeast R18Q,R22Q mutant pALDH was prepared by overlapping-extension PCR and subcloned in the above two vectors. The mature ALDH was prepared by deleting the presequence portion (residues 2–25) and subcloned into pT7-7. pMDH was cloned using yeast genomic DNA as a template, and primers for PCR were selected on the basis of the published sequence. The pMDH was cloned into pET-24a vector. The mutant pMDH was made by overlapping extension PCR and subcloned to the pET-24a vector.

**Mitochondrial Import—**[35S]Methionine-labeled pALDHs were synthesized using a TnT Quick kit (Promega). Isolation of yeast mitochondria was conducted according to a published protocol (19). The import of precursor proteins into isolated yeast mitochondria was carried out as described previously (20). HeLa cells were kindly provided by Prof. Steven Broyles (Purdue University). The cells were suspended in import buffer and homogenized. Mitochondria were isolated by differential centrifugation as performed previously (21). For import of proteins, TnT-synthesized precursor proteins were added to isolated HeLa cell mitochondria and incubated for 30, 60, or 90 min. The import reaction for pMDH and mutant pMDH volume was 100 μL. After incubation for 30 min, 30 μL was removed and proteinase K was added to terminate the import reaction. The remaining 70 μL was kept at room temperature. After another 30 min of incubation, 30 μL was removed and proteinase K was added to this 30 μL to stop the import reactions. The remaining 40 μL was kept at room temperature for another 30 min. 30 μL was then removed, and proteinase K was added. For each time period, proteins imported into mitochondria were determined. To investigate precursor protein degradation in HeLa cell mitochondria, a slightly different protocol was employed. The TnT-synthesized precursor proteins were incubated with isolated HeLa cell mitochondria. The import reaction volume was 100 μL and was incubated for 30 min at 30 °C. After incubation for 30 min, proteinase K was added to the 100-μL import reaction to terminate the import. A 30-μL sample was removed. The mitochondria were centrifuged, and the proteins imported within 30 min were determined. The remaining 70 μL was kept at room temperature. After another 30 min of incubation, 30 μL was removed. The mitochondria were isolated, and the proteins imported were determined. The remaining 40 μL was kept at room temperature for another 30 min. Finally, a 30-μL sample was removed. Mitochondria were isolated, and the amount of imported proteins was determined.

The inhibition of MPP by o-phenanthroline was performed with yeast mitochondria. Yeast mitochondria were isolated as described (4). Purified mitochondria were incubated with 0, 2, 4, 6, or 8 mM o-phenanthroline on ice for 15 min to inhibit MPP. Import of wild type rat pALDH was then performed with mitochondria incubated with each concentration of o-phenanthroline. The import was carried out as outlined for HeLa cell mitochondria. Briefly, import was carried out for 30 min, and then proteinase K was added. A 30-μL sample was removed, and mitochondria were isolated. The amount of protein imported after 30 min was quantified. The remaining 70 μL of the reaction solution was kept at room temperature in the presence of proteinase K to destroy all proteins not imported into mitochondria. After 30 min another 30-μL aliquot was removed; mitochondria were isolated, and the amount of imported protein was quantified.

**Protein Expression and Purification—**Plasmids carrying yeast ALDH genes on the pT7-7 vector were transformed into the *E. coli* strain BL21(DE3)-RIL CodonPlus (Novagen) and then induced overnight at 16 °C with 0.4 mM isopropyl β-D-thiogalactoside in Terrific Broth to express the aldehyde dehydrogenases. Cells were harvested, resuspended in the buffer (20 mM sodium phosphate pH 7.4, 2.0 mM EDTA, 1.0 mM dithiothreitol, and 1.0 mM phenylmethylsulfonyl fluoride), and then lysed by a French cell press at 4 °C. The samples were clarified and then...
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treated with 5 mg/ml protamine sulfate. After dialysis, proteins were loaded on a DEAE-Sephadex column (Sigma) and then eluted with a linear NaCl gradient (0–500 mM). Samples were analyzed for catalytic activity assay with propionaldehyde as substrate and NAD as coenzyme and then subjected to SDS-PAGE and Western blot analysis. Fractions containing the highest activities were collected and dialyzed against an EDTA-free buffer (20 mM sodium phosphate, pH 7.4, and 0.1 mM dithiothreitol). The protein was further separated on a pre-packed 5-ml Econo-Pac CHT-II cartridge (Bio-Rad) by elution with a sodium phosphate gradient (20–250 mM). Enzyme assays or activity staining was carried out essentially as described previously (18).

Yeast Growth Rate Comparison—Transformed HWTHL10 cells were first grown to the same cell density in acetate-containing medium. Cells were washed twice with water, resuspended in the ethanol medium, and then inoculated into the ethanol liquid medium or on agar plates. The initial cell density was manipulated to be the same for the various yeast cells harboring different plasmids. After a period of growth at 30 °C, cell densities of the liquid culture or colony sizes on agar plates were compared.

Protein Extraction from Yeast—Yeast cells were lysed by French cell press in a nondenaturing buffer (100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) followed by centrifugation to prepare nondenatured protein samples that could be used in the activity assays. For the Western blot analysis, proteins were also extracted by treating the yeast cells at 97 °C for 10–15 min in the SDS buffer (200 mM Tris-HCl, pH 6.8, 400 mM dithiothreitol, 4% SDS, 20% glycerol, and 0.2% bromophenol blue) followed by centrifugation to clarify the solutions.

Yeast ALDH Level Comparison—The ALDH levels in yeast were compared by activity staining and Western blot analysis. Transformed HWTHL10 cells were initially grown to same cell density in the acetate medium. Cells were washed twice with water and then re-grown in the ethanol medium. ALDHs were extracted from the yeast cells using the above homogenization method followed by activity staining or extracted by the above SDS-treating method followed by Western blot analysis. The ALDH levels were compared by analyzing the band densities with the Chemilumager (Alpha Innotech).

Submitochondrial Localization of ALDH—Radiolabeling and mitochondrial import reactions with the yeast wild type pALDH and mutant pALDH were performed in two separate Eppendorf tubes. The nonimported pALDHs were destroyed by proteinase K treatment. Mitochondria with imported pALDHs were combined, pelleted, washed with the mitochondrial suspension medium (0.6 M mannitol and 10 mM Tris-HCl, pH 7.4), and then resuspended in the same medium. The mitochondria were then subjected to digitonin treatments (0, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30% (w/v)) (22) for 60 s and then immediately diluted with the mitochondrial suspension medium. Supernatants and pellets were separated by centrifugation at 48,000 × g for 20 min at 4 °C and then subjected to SDS-PAGE analysis followed by quantification with the PhosphorImager.

**Yeast pALDH leader sequence**

| MFSRSTLC | KTSASSIG | RLQLRYF |

**Rat liver pALDH leader sequence**

| MLRAALSTAR | RGP | RLSRLL |

**Yeast pMDH leader sequence**

| MLRSVA | KRAFSSTVANP |

**FIGURE 1. Leader sequences of yeast and rat pALDH and yeast pMDH.** The arginine and lysine residues in shown in boldface were mutated to glutamine to make the leader nonprocessable in the matrix space. The leader sequence of rat pALDH was solved, and it forms helix-linker-helix conformation (11).

**Pulse-Chase and Immunoprecipitation**—Pulse-chase experiments were performed based upon those designed by Fujiki and Verner (23). Transformed yeast was grown in the acetate medium to mid-log phase. Cells were washed twice with water, resuspended at 0.5–1.0 A_{600} in 10 ml of the ethanol medium lacking methionine, and then incubated for 1–2 h at 30 °C. Cells were collected and then resuspended in 1 ml of the methionine-free medium. Pulse was performed at 30 °C for 10–20 min by the addition of 20–50 μCi of [35S]methionine in the presence or absence of 20 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP). Cells were washed once with water after centrifugation and then resuspended in the methionine-free medium. Chase was initiated by the addition of 100 mg/ml unlabeled methionine. Aliquots were taken at different times. Chase was terminated by the addition of 50 mM NaN_3 and then incubated on ice. Proteins were extracted by SDS treatment. Immunoprecipitation was performed according to the protocols provided by Santa Cruz Biotechnology, the manufacturer of protein A-agarose.

**Limited Digestion of Precursor Proteins with Proteinase K**—Wild type rat pALDH and mutant precursor R17Q were incubated with proteinase K at a concentration of 0.28 μg/ml for 0, 30, 60, and 90 min. The samples were run on 10% SDS-PAGE followed by PhosphorImager analysis.

**RESULTS**

**In Vitro and in Vivo Import of Nonprocessed Yeast pALDH**—From the amino acid sequence of yeast pALDH, we predicted that the precursor had a leader peptide consisting of 24 amino acids because the amino acid sequence RXHXR starts at position 18, a consensus site recognized by mitochondrial processing peptidase (Fig. 1) (3). The positively charged residues at positions 18 and 22 were mutated to glutamine to destroy the purported processing site. The wild type and the mutant pALDH were incubated with purified yeast MPP and found that the wild type was processed, whereas the mutant was not (Fig. 2A). To investigate the fate of a nonprocessed precursor in the matrix space, the wild type and the nonprocessed pALDH were employed in an *in vitro* and *in vivo* import.
A processing and import of yeast pALDH. A, processing of yeast pALDH mutants by purified MPP. In vitro processing of native pALDH and mutant pALDH was performed as described under "Materials and Methods," employing a 30-min incubation at 27 °C. Lanes 1 and 2 indicate native pALDH. Lanes 3 and 4 indicate mutant pALDH. Lanes 1 and 3 indicate translated protein. Lanes 2 and 4 indicate translated protein after incubation with purified MPP. p, precursor; m, mature. B, mitochondrial import of pALDH. Radiolabeled pALDHs were incubated at 30 °C with isolated yeast mitochondria for 30 min. The mitochondria were separated into 2 equal aliquots, and 1 aliquot was treated with proteinase K. The mitochondria were re-isolated and treated with SDS-PAGE loading buffer. Proteins were separated on SDS-PAGE followed by Western blot analysis. Lane 1, pALDH, no proteinase K treatment after import; lane 2, pALDH with proteinase K treatment after import; lane 3, mutant pALDH, no proteinase K treatment after import; lane 4, mutant pALDH with proteinase K treatment after import. C, ALDH expression in the HWTHL10 strain carrying pALDH genes. Transformed HWTHL10 was grown at 30 °C for several days in the ethanol medium. Proteins were extracted from the yeast with the SDS treatment method and separated on SDS-PAGE followed by Western blot analysis. Lane 1, pALDH; lane 2, mutant; lane 3, pALDH.

study. TNT-synthesized wild type and the mutant pALDH were incubated with isolated yeast mitochondria. The data showed that the wild type yeast pALDH and the mutant precursor (R18Q, R22Q) proteins both were imported as expected. The wild type was processed whereas the R18Q, R22Q double mutant was not processed (Fig. 2B).

The triple ALDH-disrupted yeast strain HWTHL10, previously constructed in this laboratory (18), was used here to test for the in vivo import and processing of the modified pALDH leader. This yeast strain was transformed with the recombinant plasmids carrying the yeast pALDH genes and also with empty vector pRS316m as control. Western blot was used to determine whether or not pALDH was produced (Fig. 2C). The wild type ALDH gene produced a mature-size ALDH implying that the precursor was imported and processed just as it was in the in vitro study. The R18Q, R22Q double mutant gene produced a precursor size ALDH implying that this form could have been imported but not processed.

Both pALDH and the Mutant pALDH Are Localized in Mitochondrial Matrix Space—To verify that the mutant nonprocessed pALDH was translocated to the matrix space and not stuck inside the inter-membrane space, a fractionation study after import was performed. TNT-synthesized wild type and nonprocessed pALDH were incubated with isolated yeast mitochondria, and after proteinase K digestion, a digitonin fractionation was used to determine where in the yeast mitochondria were the ALDHs localized. Cytochrome b2, an inter membrane space protein, appeared in the supernatant fraction starting from 0.05% digitonin (w/v) (Fig. 3). Aconitase, a matrix protein, started to appear in the supernatant starting from 0.10% digitonin. The mature ALDH and the nonprocessed mutant pALDH had similar distributions and were found essentially in the same fractions with aconitase. These findings led us to suggest that the both proteins were imported into the mitochondrial matrix space.

The Nonprocessed pALDH Is Functional in Vivo—The inability of triple disrupted yeast strain to grow on ethanol allowed us to compare the growth rate on ethanol to determine whether the nonprocessed pALDH was functional. Yeast strain HWTHL10 carrying the empty vector pRS316m did not grow on ethanol, whereas yeast carrying the wild type pALDH did gene grow (Fig. 4). Yeast carrying the mutant pALDH gene also grew but at a slower rate compared with the cells carrying the wild type precursor (Fig. 4). The yeast growth rates in liquid medium showed that the mutant grew at ~10% that of the wild type. These results indicated that the mutant pALDH could...
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partially complement growth of the deleted yeast strain on ethanol medium, showing that the enzyme was functional in vivo.

Less Mutant pALDH Accumulated in the Yeast Compared with the Wild Type ALDH—To determine the amount of imported ALDH in mitochondria, HWTHL10 cells carrying different recombinant plasmids were transformed. Cells grown to the same A_{600} were found to possess the same amount of total protein, as determined by the Bradford method. The extracts from cells with the same amount of total protein were either separated by SDS-PAGE followed by Western blot analysis or separated by isoelectric focusing gel electrophoresis followed by activity staining. The Western blot revealed that the amount of mutant pALDH was only 15–20% of mature ALDH. The activity staining showed that the activity of mutant pALDH was also ~15% of mature ALDH. This indicated that the mutant pALDH was nearly as active as the mature ALDH. The slow yeast growth of nonprocessed pALDH on ethanol as we observed here was a result of having a lower total activity of aldehyde dehydrogenase in the cells.

Yeast Mature ALDH and the Mutant pALDH Had Similar Specific Activity—To verify that the mutant pALDH and mature ALDH had the same specific activity, both proteins were expressed in E. coli. It was possible to purify the mutant pALDH to homogeneity. The specific activity was found 21 units/mg protein. The mature ALDH co-eluted with a non-ALDH protein and was purified to just 50% as judged by SDS-PAGE analysis followed by quantification. The solutions of enzymes with same amount of aldehyde dehydrogenase activity, determined by fluorometric assays, were subjected to SDS-PAGE analysis followed by quantification. The quantity of the mature ALDH was found to be about 80% that of the mutant pALDH. This implies that the activity of the mutant pALDH (21 units/mg) could be estimated to be about 80% that of the mature ALDH (25.3 units/mg) showing that both proteins had a similar specific activity consistent to what was found with the in vivo study.

The Mutant pALDH Was Degraded Faster than the Wild Type ALDH in Mitochondrial Matrix Space—Finding less pALDH mutant in the transformed yeast make it appear that the mutant enzyme was less stable in vivo than was the native enzyme. To test for this, a pulse-chase followed by an immunoprecipitation experiment was performed. The transformed HWTHL10 carrying the wild type pALDH or the mutant pALDH was initially radiolabeled with [35S]methionine in the presence or absence of CCCP (Fig. 5A), a proton ionophore that uncouples mitochondria and inhibits protein import (23). In the case of the wild type yeast pALDH gene, mature size ALDH was found in the absence of CCCP, presumably in the mitochondria, whereas the precursor size ALDH was found in the presence of CCCP, presumably in the cytosol. For the mutant pALDH, precursor size ALDH was obtained in the mitochondria or the cytosol in the absence or presence of CCCP, respectively. The radiolabeled ALDHs were then chased in the presence of unlabeled methionine (Fig. 5B). Almost no degradation of the yeast wild type pALDH or the mutant pALDH was found in the cytosol. However, around 60% of the mutant pALDH degraded whereas less than 20% of the wild type ALDH degraded after 100 min (Fig. 5C). It is concluded that the reason for finding a lower amount of mutant pALDH is that it was degraded in the mitochondria at a faster rate compared with the mature ALDH.

The Nonprocessed pALDH Was Thermally Less Stable in Vitro—Previously our laboratory showed that both rat pALDH and mature ALDH had a similar specific activity. However, the rat pALDH was less stable than the mature ALDH (8). To test if this was the case for the yeast ALDHs, we determined the stability of the mature and mutant yeast pALDH. The recombinantly expressed ALDHs were subjected to heat denaturation to assess their thermal stability. Yeast mature ALDH and nonprocessed pALDH were incubated at 52 or 30 °C, and their remaining catalytic activities were assayed as a function of time. The half-life of the mature ALDH at 52 °C was about 25 min
(Fig. 6). After a 5-min incubation at 52 °C, only 12% activity of the nonprocessed mutant pALDH remained. At 30 °C, the mature ALDH was very stable, whereas less than 50% activity of the nonprocessed mutant pALDH remained after a 30-min incubation (date not shown). The nonprocessed yeast pALDH remained after a 30-min incubation (date not shown). The nonprocessed mutant pALDH remained after a 30-min incubation (date not shown). The nonprocessed yeast pALDH was less stable both in vitro and in vivo when compared with mature ALDH.

The Fate of a Nonprocessed Yeast ALDH in HeLa Cell Mitochondria—It appeared from the above study that the presence of the leader caused yeast ALDH to be proteolytically unstable in the matrix space. A modified import protocol for HeLa cell mitochondria was used to verify the findings. The wild type and the mutant yeast pALDH were employed in an in vitro import study in isolated HeLa cell mitochondria for a time period longer than the usual 30 min (21). Here the TnT-synthesized proteins were incubated with HeLa cell mitochondria for 60 min. Because it is considered that the import is essentially completed within 30 min (11), incubations for more than 30 min will allow for the investigation of the degradation of the imported protein. As it was already shown that the mutant pALDH degraded faster compared with the mature ALDH in yeast mitochondria, we expected to find less mutant pALDH than the mature ALDH in HeLa cell mitochondria. Surprisingly, the mutant pALDH was processed after being imported into HeLa cell mitochondria (Fig. 7). The amount of imported proteins obtained at 30 or 60 min did not change nor did it change for the mutant form because both were processed. We have no explanation for why the mutant pALDH was processed in HeLa cell mitochondria, but it was not in yeast mitochondria.

Fate of a Nonprocessed pMDH in HeLa Cell Mitochondrial Matrix Space—Yeast mitochondrial pMDH was included in the study to investigate if a nonprocessed pMDH was processed like yeast mutant pALDH in HeLa cell mitochondria. The leader sequence of pMDH contains 17 amino acids, and it is processed by sequential actions of both MPP and mitochondrial intermediate peptidase (24). Two positively charged residues are present in the MDH leader sequence just upstream of the MPP-processing site and are suspected to be critical for processing (Fig. 1). These residues were mutated to glutamines, and the mutant protein, as expected, was found not to be processed by the purified yeast MPP (Fig. 8A). The pMDH and the mutant nonprocessed pMDH, synthesized in TnT, were incubated with isolated HeLa cell mitochondria. The wild type pMDH was imported and processed to mature MDH as expected. The mutant pMDH was imported but, unlike mutant yeast pALDH, was not processed inside HeLa cell mitochondria (Fig. 8B). The TnT-synthesized pMDH and the mutant pMDH were incubated with isolated HeLa cell mitochondria for up to 90 min. The amounts of imported proteins inside the matrix space were quantified after each 30-min time periods as summarized in Table 1.

The data show that with time the amount of mature MDH increases slightly, whereas the amount of mutant decreases slightly. The quantification of import is complex so the slight change may not be significant. We speculated that two events...
were going on simultaneously. One was very slow import after a 30-min incubation and the second was the degradation of imported proteins. The amount of proteins found was the combined results of import and degradation. Because it was difficult to investigate only the degradation of the imported protein, a slightly modified protocol was employed to examine only protein degradation inside the mitochondrial matrix space. The pMDH and the nonprocessed pMDH were incubated with isolated HeLa cell mitochondria as described. After a 30-min incubation, proteinase K was added to the import solutions, and incubation was continued. The proteinase K digested the proteins outside mitochondria preventing further import. This method allowed us to follow protein degradation inside the matrix space. The amounts of proteins imported at 30, 60, and 90 min were quantified (Fig. 8C). It appeared that mature MDH degraded very slowly inside mitochondria, and in contrast, mutant pMDH was degraded rapidly. To test that the finding of a lower amount of mutant pMDH was not because of leakage of protein from matrix space during incubation, a control experiment was run. The antibody against mitochondrial HSP60 was used to show that mitochondrial HSP60, a matrix located protein, remained intact in the matrix space during the incubation period (data not shown) showing no leakage occurred.

**Nonprocessed Rat pALDH Was Degraded in HeLa Cell Mitochondrial Matrix Space**—The mitochondrial import and processing of rat pALDH has been extensively investigated in our laboratory. The leader peptide is made of 19 amino acids and is processing by MPP (11). The sequence of the leader peptide is shown in Fig. 1. An R17Q mutation made previously was found to be importable but was not processable (11). This mutant protein was included in this study to examine whether this protein was also degraded inside the matrix space.

The import of wild type rat pALDH and the mutant R17Q pALDH was carried out as described for pMDH. After a 30-min incubation, proteinase K was added to the import reaction solutions. The amount of radiolabeled proteins at this time, 60 and 90 min, were determined (Fig. 9). Wild type rat pALDH was imported and processed to mature protein inside the matrix space. pALDH degraded very slowly after 60 or 90 min of incubation. However, the R17Q mutant after import remained nonprocessed but was rapidly degraded with time suggesting the mutant was less stable compared with the mature ALDH. Another mutant of rat pALDH, R3Q,R10Q, was also included in this study. This mutant was poorly imported to mitochondria but was processed as observed with rat mitochondria (11). The degradation of R3Q,R10Q was found to be much less than the degradation of R17Q (data not shown) in the matrix space consistent with the fact that the precursor is less stable than the mature form, independent of the nature of the processable leader. Although it was found that the mature form was more stable than the mutant precursor form, the stabilities of wild type precursor and the mutant precursor were not known. To test for this, the R17Q and the wild type precursor of pALDH were subjected to limited proteinase K digestion. It appeared from the figure that with time the intensity of a smaller band (indicated by an arrow) gradually increased for both pALDH and its mutant (Fig. 10). In addition, several smaller faint bands appeared with time for both proteins suggesting that the digestion profile for both was the same. This result showed that both pALDH and R17Q were equally sensitive to proteolysis (Fig. 10). We also used o-phenanthroline to inhibit MPP in intact mitochondria so that we could study the degradation of wild type precursor. o-Phenanthroline can enter into mitochondria and inhibit MPP (25). Purified mitochondria were incubated with 0, 2, 4, 6, and 8 mM o-phenanthroline on ice for 15 min to inhibit MPP. Import of wild type rat pALDH was then performed with mitochondria incubated with each concentration of o-phenanthroline. The data in Fig. 11A makes it appear that MPP was barely inhibited with 2 or 4 mM inhibitor. In contrast, at a concentration of 6 mM o-phenanthroline, the wild type rat pALDH was only 50% processed after 30 min. After the 60-min incubation, the precursor was completely degraded, but the mature (processed) protein remained intact. In the presence of 8 mM o-phenanthroline only 20% of the wild type precursor was processed after 30 min, but after 60 min the unprocessed precursor was completely degraded, whereas the processed mature form of the protein was not degraded (Fig. 11B).

### TABLE 1

Import of precursor proteins into HeLa cell mitochondria

| Precursor | 30 min | 60 min | 90 min |
|-----------|--------|--------|--------|
| pMDH      | 100 ± 8 | 80 ± 6  | 70 ± 8  |
| Mutant pMDH | 100 ± 8 | 50 ± 8  | 40 ± 8  |
| pALDH     | 100 ± 6 | 75 ± 8  | 70 ± 5  |
| Mutant pALDH | 100 ± 8 | 65 ± 7  | 45 ± 8  |

* Each import reaction was carried out at least three times.
* Import at 30 minutes was defined as 100%.
that wild type precursor was readily degraded in the matrix space compared with its mature form.

**The Rat RGP Linker-deleted pALDH Was Also Degraded Faster than the Mature ALDH in Yeast Mitochondria**—The wild type rat pALDH leader peptide forms helix-linker-helix conformation in the hydrophobic environment (Fig. 1) (11). If the linker region consisting of RGP is deleted, the leader forms a continuous helix. The linker-deleted leader is not processed by MPP (13) and was utilized in this study to investigate its fate in yeast mitochondrial matrix space. A pulse-chase followed by immunoprecipitation experiment was conducted using rat pALDH and the RGP linker-deleted form of pALDH. For the wild type, the mature size rat ALDH was found in the mitochondria, whereas for the mutant, the precursor size rat ALDH was obtained. Only 20% of the rat mutant pALDH remained, whereas over 50% of the wild type ALDH was found in the mitochondria after a 60-min chase (data not shown). These findings show that the nonprocessed linker-deleted rat pALDH was more prone to degradation in yeast mitochondria than was the mature ALDH. These results are consistent with what we found with HeLa cell mitochondria.

**DISCUSSION**

It is not known why the leader sequence is removed from the vast majority of precursor proteins after they are translocated into the mitochondrial matrix space. For a few of the proteins that possess a nonprocessed leader, there might be a structural advantage for retaining it. The three-dimensional structure of rhodanese (26), electron transfer flavoprotein (27), and adenylyl kinase (28), three mitochondrial matrix proteins with N-terminal presequence that are not processed after import, show that all possess a β-sheet in their leaders. This β-sheet region forms antiparallel β-sheets with part of the protein that possibly can contribute to the overall stability of the protein. Our laboratory showed that the first 23 residues of rhodanese serve as a leader peptide and carried other proteins to the mitochondrial matrix space (12). If the entire leader peptide was deleted, the enzyme was found to be inactive and unstable (29). If the first 9 residues from N terminus of rhodanese were deleted, the enzyme was found to be active but more susceptible toward dithiothreitol, urea, and heat denaturation compared with the full-length protein, suggesting that the leader peptide was critical for maintaining the three-dimensional structure of the enzyme. To our knowledge, there is no solved structure of the precursor form of a processed protein. Thus, it is not known where the leader would be located with respect to the structure of the mature portion of the protein. In the case of mammalian mitochondrial aldehyde dehydrogenase (15, 16), the initial seven residues from the mature form were not found in the crystal structure. This implies that they must have a high degree of mobility and possibly they have no defined conformation. Because the eighth residue is on the surface, it is highly likely that the positively charge leader segment would also be on the surface. The structure of the peptide corresponding to the mitochondrial leader solved by two-dimensional NMR showed that the leader formed an amphiphilic α-helix (30). This was also the structure found for other synthetically leaders solved by two-dimensional NMR studies, in the presence of trifluoroethanol or detergents (11, 31, 32). Unlike a β-sheet, the helix might not interact with the rest of the protein so the mere presence of the leader could increase the flexibility of the N termini, hence destabilizing the enzyme had it not been removed.

The precursor form of ALDH or aspartate aminotransferase can be expressed as an active enzyme in *E. coli* (8, 33). However, for mitochondrial fumarase (7) and mouse thymidine kinase (34), the precursor form proved to be essentially catalytically inactive when compared with the mature enzyme. Thus, no generalization can be made as to whether or not precursor proteins would be active had the leader not been removed. In the case of ALDH (15, 16) and aspartate aminotransferase (35), the initial 15 or more residues of the mature enzymes were found not to form secondary structure. In contrast, fumarase (36) has a 13-residue β-sheet secondary structure starting at the second residue of the mature form. The presence of leader possibly would disrupt this β-sheet structure hence affecting the folding and activity of fumarase.

To test why it was necessary for ALDH to be processed, we took advantage of the fact that it was possible to convert the processable precursor into a nonprocessable one. When the arginine residues in the rat liver leader peptide, located in the C-terminal helix of the helix-turn-helix structure, were changed to glutamines, an importable but nonprocessed precursor was produced. It was found that a comparable change could be made to the corresponding yeast ALDH leader, even though the primary sequences are different, as shown in Fig. 1.

Both the mature and precursor forms of rat liver ALDH were soluble after being expressed in *E. coli* (8). In contrast, the yeast wild type precursor was insoluble after being expressed, so it
was not possible to compare it with the soluble expressed mature enzyme form. The yeast nonprocessable mutant pALDH proved to be soluble and have essentially the same specific activity as did the recombinantly expressed yeast mature ALDH. Thus, like the rat liver enzyme, the presence of the leader did not affect the ability of the enzyme to fold into an active conformation. The mutant yeast pALDH expressed in E. coli was an active tetrameric enzyme that could be purified to homogeneity without being associated with heat-shock protein unlike what was found for the expressed mitochondrial aspartate aminotransferase precursor (37). The mutant pALDH expressed in S. cerevisiae had the same molecular size as the one purified from E. coli, showing that it did not strongly associate with the mitochondrial hsp70 in yeast. The yeast mutant pALDH was found to be thermally less stable just as was found for rat ALDH (8). At 52 °C, ~50% of the yeast mALDH activity was lost after a 25-min incubation, but 88% of the mutant pALDH activity was lost after just a 5-min incubation. Even at 30 °C, the temperature typically used for yeast growth and where the yeast mature ALDH is stable, the purified mutant pALDH lost 50% activity after a 30-min incubation. The presence of the modified leader dramatically affected the stability of the isolated yeast precursor ALDH.

To analyze for the in vivo proteolytic stability of the precursor protein, the yeast and rat mutant forms of pALDH were expressed in the triple ALDH-disrupted yeast strain we constructed previously (18). The yeast mutant pALDH was found in mitochondria as an expected unprocessed form. For comparative purposes, yeast was also transformed with the wild type pALDH that was processed to a mature form. Less mutant enzyme was found compared with the mature enzyme although both were placed on the identical plasmid. Finding less protein could be the result of a lower rate of translation or a more rapid rate of degradation. Pulse-chase analysis showed that it was the latter. The mutant enzyme was synthesized at essentially the same rate as was the wild type precursor, but it was destroyed in the cell at a faster rate. The faster rate of proteolysis could have been caused presumably by the altered structure of the precursor protein that was not stable, as shown by the in vitro study.

This laboratory has suggested that the import of a protein into mitochondria could be coupled with synthesis in HeLa cells (38), i.e. a co-translational import mechanism might occur. It was proposed that a co-translational import mechanism could also occur with some yeast mitochondrial proteins (23, 39). However, this has not yet been shown with yeast mitochondrial ALDH. It is possible that the precursor first accumulates in the cytosol and then is translocated in a post-translation manner into the mitochondria. If this were the case, it is possible that the mutant precursor could have been destroyed prior to entering the matrix space. To test for this, the yeast mitochondria were poisoned with CCCP, a chemical that uncouples mitochondrial membrane potential and inhibits the uptake of newly synthesized proteins into mitochondria (23). Under these conditions, the mutant precursor was found to be as stable as was the wild type one. The fact that the wild type was not processed in the presence of CCCP is indicative of the fact that indeed the precursors remained in the cytosol. This finding leads us to suggest that the proteolysis of the mutant precursor must have occurred in mitochondria because they were stable when left in the cytosol. Similar results were obtained when the yeast was transformed with the rat liver ALDH precursor. A digitonin fractionation study was included to show that proteins reached in the matrix space before degradation. No matter what was the mechanism for import of a mutant precursor, it was subjected to proteolysis after import into matrix space.

HeLa cell mitochondria were employed to determine how general were the findings we presented. Finding that the yeast mutant precursor protein was processed in HeLa cells was an unexpected finding. No explanation for this can be offered for there are few, if any, reports of MPP from one species having different specificity from that of another. It is possible that actual processing did not occur but simply a proteolytic step took place that happened to produce a mature-like sized protein. No attempt was made to further use the yeast protein with HeLa cell mitochondria. Instead, pMDH was used. The crystal structure of the leader from yeast pMDH bound to yeast MPP was determined (40). It, like pALDH, was converted into a nonprocessed precursor by changing the positive charged residues to neutral glutamines. The wild type pMDH and the mutant pMDH were imported with the wild type being processed, whereas the mutant pMDH was not processed in HeLa mitochondria. The mutant pMDH proved to be degraded at a faster rate in the matrix space than was the mature form of the enzyme. In addition, a mutated form of rat liver pALDH that was not processed was found to be also degraded at a faster rate than was the mature form of the enzyme. Thus, we obtained essentially the same results using a few different precursor proteins. We find all the nonprocessed precursors to be degraded at a much faster rate than the mature protein. The nonprocessed precursors we have used here were all mutant proteins, and this raises the possibility that the mutant precursor might be less stable than the wild type precursor. By using limited proteinase K digestion, we showed that the wild type rat pALDH and mutant R17Q were equally sensitive to proteinase K. In addition, we used o-phenanthroline, an inhibitor of MPP, to study the degradation of wild type precursor protein in the matrix space. Finding that the wild type precursor was unstable when MPP was inhibited shows that a precursor protein will be degraded whether or not it was a mutant variant.

Mitochondria have evolved to degrade misfolded proteins in the organelle. The degradation is carried out by different mitochondrial proteases including the mitochondrial processing peptidase, oligopeptidase, and the ATP-dependent proteases (41). The leader peptides are cleaved by MPP, whereas most of the unfolded proteins are degraded by ATP-dependent proteases. The ATP-dependent proteases are located in matrix space and also in intermembrane space (42). Because the mutant nonprocessed precursors are degraded in matrix space, it is anticipated that matrix Lon-like protease could be involved in proteolysis of mutant proteins.

It is concluded that the leader peptide must be removed by the action of the mitochondrial processing peptidase after import for stability purposes. It is possible that for some proteins leaders are needed to be removed for assembly or membrane binding. Obviously, if the precursors were inactive, then processing must occur to allow an active conformation to form.
Finding less of a mitochondrial protein in a disease state could reflect a lower rate of synthesis, but as we show here, it could be a result of a mutation occurring in the leader that converted the precursor into a form that is not processed.

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