Protein Folding in a Cell-free Translation System

THE FATE OF THE PRECURSOR TO MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE*

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The precursor to rat mitochondrial aspartate aminotransferase (pmAspAT) can be expressed in and purified from Escherichia coli as a fully active enzyme with remarkable trypsin resistance. Only two sites within the presequence are readily hydrolyzed (Martinez-Carrion, M., Altiere, E., Iriarte, A., Mattingly, J., Youssef, J., and Wu, T. (1990) Ann. N. Y. Acad. Sci. 585, 348–356). In contrast, pmAspAT freshly synthesized in rabbit reticulocyte lysate is significantly less resistant to proteolysis and is completely digested by trypsin. Extended incubation of the pmAspAT translation product slowly converts it to a species with qualitatively the same trypsin resistance as the purified pmAspAT. In addition, this species binds pyridoxal 5'-phosphate, exhibits catalytic activity, and loses its ability to be imported into mitochondria. This process appears to reflect protein folding. The rate of folding is unaffected by the addition of cofactor or the depletion of endogenous cofactor and is not significantly affected by the concentration of translation product in the reaction. Agents that decrease the availability of ATP partially inhibit the folding, whereas the sulfhydryl alkylating reagent N-ethylmaleimide and the detergent Triton X-100 completely prevent the conversion. Although the folding of pmAspAT in reticulocyte lysate is slow, folding is rapid once the translation product is sequenced into the mitochondria as the mature form of the enzyme. These results are presented as a model for the in vivo folding of pyridoxal-dependent, oligomeric mitochondrial precursors in the presence of cytoplasmic components and for the fate of true mitochondrial precursor proteins when not imported.

A number of denatured proteins are capable of unassisted refolding in vitro, clearly demonstrating that the primary sequence of a polypeptide contains sufficient information to determine its final conformation (Seckler and Jancicke, 1992). However, the frequent inability to find suitable in vitro conditions to make this a kinetically feasible event suggests that external factors may, for many proteins, be at least equally important in directing the folding process. A rapidly increasing number of reports suggests that molecular chaperones are among those factors, acting both in vitro and in vivo to accelerate the formation of functional proteins from disorderd polypeptides (Gething and Sambrook, 1992). Much recent work on facilitated refolding in defined systems has focused on the Escherichia coli chaperones, GroEL and GroES (Bacoe et al., 1991; Langer et al., 1992; Martin et al., 1991; Mendoza et al., 1991; Viitanen et al., 1991; Zahn and Pluckthun, 1992). These chaperonins, sometimes referred to more generally as chaperonin 60 (cpn60)* and chaperonin 10 (cpn10), appear to have complementary roles with GroEL preventing improper protein folding by interacting with the unfolded polypeptide and GroES facilitating release of a properly folded peptide by participating in a GroEL-GroES-polyopeptide complex. Analogies of these proteins have been identified in a variety of intracellular organelles (Ellis and van der Vies, 1991) where they are required for proper folding of imported proteins (Cheng et al., 1989, 1990; Smith and Yaffe, 1991).

A proposed requirement for import of proteins into the mitochondrial matrix is a partially folded or flexible conformation (Glick and Schatz, 1991; Neupert et al., 1990). A number of studies have suggested that members of a different class of the molecular chaperones, the 70-kDa heat-shock proteins, may act in concert with other cytosolic factors to maintain this requisite import competent conformation (Becker et al., 1992; Deshaies et al., 1988; Langer et al., 1992; Murakami et al., 1988, 1990; Murakami and Meri, 1990; Sheffield et al., 1990). The observation that many newly synthesized polypeptides in the cytoplasm bind to hsp70 under conditions of stress (Beckmann et al., 1990) corroborates this model. In vitro interaction of hsp70 with denatured proteins has been demonstrated as well (Palleros et al., 1991). A mitochondrial hsp70 protein seems to bind newly translocated proteins and then release the proteins to allow interaction with the mitochondrial cpn60-cpn10 system which, in turn, facilitates complete folding (Manning-Krieg et al., 1991; Mizzen et al., 1991; Baker and Schatz, 1991).

In contrast to ample evidence for similar chaperone-mediated mechanisms both in mitochondrial and bacterial protein folding, direct evidence for chaperone-assisted folding of proteins synthesized in the eukaryotic cytosol is scarce. Initial attempts to identify eukaryotic cytosolic molecular chaperones were limited to sequence homology searches based on known chaperones and suggested the t-complex polypeptide

* The abbreviations used are: cpn60, chaperonin 60; cpn10, chaperonin 10; hsp70, 70-kDa heat-shock protein; pmAspAT, precursor to mitochondrial aspartate aminotransferase; DTT, dithiothreitol; HEPPS, N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid; NEM, N-ethylmaleimide; PBF, presequence binding factor; pmAspAT, precursor to mitochondrial aspartate aminotransferase; pyridoxal-P, pyridoxal 5'-phosphate; pyridoxamine-P, pyridoxamine 5'-phosphate; RRL, rabbit reticulocyte lysate; PAGE, polyacrylamide gel electrophoresis; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

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1 as a candidate (Ellis, 1990; Gupta, 1990; Ursic and Culbertson, 1992). More recent results have strengthened that hypothesis by showing a high degree of sequence homology between t-complex polypeptide X and molecular chaperone found to function in a thermophlic archaebacterium (Trump et al., 1991). An alternative approach has led to the purification of a protein from reticulocyte lysate which promotes refolding of a denatured cytosolic protein (Gao et al., 1992) and appears functionally analogous to the biochemically defined chaperones from bacteria, mitochondria, and chloroplasts. It thus appears that macromolecular agents for impeding and facilitating protein folding coexist in the cytosol. In an attempt to develop a model for studying how proteins targeted to mitochondria might be prevented from folding in the cytosol or might fold should import not occur, we have studied the precursor to mitochondrial aspartate aminotransferase (pmAspAT) after its in vitro synthesis in rabbit reticulocyte lysate (RRL). We propose that RRL is a suitable model system for studying in vivo protein folding, since this medium retains some of the complexity of the eukaryotic cytosol and yet is amenable to in vitro manipulation. Despite the widespread use of in vitro translation in RRL to produce passenger proteins for translocation into mitochondria, little information is available concerning events that might occur during the time which could elapse between synthesis of the import substrate and its use in the import reaction. This is particularly true for authentic mitochondrial proteins, as most studies which examine the conformation of in vitro translation products have focused upon artificial proteins: chimera constructed from mitochondrial targeting presequences and cytosolic or bacterial proteins (Endo et al., 1988; Skerjanc et al., 1990). Equally limited are studies of the antifolding properties of RRL based on the use of chemically denatured precursors, whether real or constructed (Murakami and Mori, 1990; Shefield et al., 1990).

The model protein we have chosen has, in addition to being an authentic precursor protein, several novel features which may allow to elucidate particular steps in the mechanism of in vivo protein folding; namely, the functional enzyme is dimeric with two symmetric active sites composed of residues from each of the two identical subunits and requires pyridoxal 5′-phosphate as a cofactor. Furthermore, pmAspAT from rat can also be expressed in E. coli as a soluble and fully catalytically active enzyme (Alieri et al., 1989), indicating that, as observed for another authentic precursor protein (Murakami et al., 1990), the presence of this protein (mAspAT) has been characterized extensively (Christen and Metzler, 1985), including a detailed crystallographic structure (Jansomius and Vincent, 1987), and, in principle, is well suited for studying the mechanism by which its conformation is achieved.

This work shows that whereas the purified recombinant pmAspAT is largely resistant to trypsin hydrolysis, with only two sites within the precursor being hydrolyzed (Martinez-Carrion et al., 1990), the same pmAspAT protein synthesized in vitro in reticulocyte lysate is initially very susceptible to extensive protease digestion. Over time, the latter species undergoes a slow conversion from a protease-susceptible, import-competent conformation, to a protease-resistant, import-incompetent protein which is also catalytically functional. The conversion process is inhibited by diminished ATP concentrations and is completely prevented by the alkylating reagent N-ethylmaleimide or the nonionic detergent Triton X-100. Furthermore, although trypsin resistance is acquired slowly in RRL, the translation product which is sequestered within mitochondria becomes trypsin-resistant almost immediately. We propose that these observations reflect the folding of the precursor to a native-like conformation. Thus, the precursor can fold properly to a protease-resistant conformation of the cytosolic protein, probably having a chaperone-like function, slow down the process thereby maintaining the protein in a translocation-competent conformation.

**EXPERIMENTAL PROCEDURES**

**Preparation of Purified pmAspAT—** pmAspAT was expressed in the E. coli strain BL21 (DE3) (Studier and Moffatt, 1986) using the pET3a vector (Rosenberg et al., 1987). In the process of cloning the originally isolated cDNA into this vector, most of the 3′-encoding region of the cDNA was deleted, and an Ndel site was created as follows. The Bluescript KS plasmid containing the pmAspAT cDNA inserted at the EcoR1 site (Mattingly et al., 1987) was digested with Smal, partially digested with DraIII, and the ends blunt with T4 DNA polymerase. The appropriate fragment containing the coding region for pmAspAT was isolated and religated to give the pBSKS-3 plasmid. An Ndel site was introduced by oligonucleotide-directed site-specific mutagenesis while still maintaining the proper reading frame (pBSKS-4). An Ndel BamHI fragment of pBSKS-4 was isolated and cloned into pET3a. pmAspAT can be purified from this expression system by a procedure similar to the one reported previously (Alieri et al., 1989). The yield is about 0.3 mg of purified precursor per liter of culture.

**Synthesis of pmAspAT in Reticulocyte Lysate—** pmAspAT was synthesized in an in vitro reaction using RRL essentially according to the protocol provided by the supplier (Promega) with [35S]methionine as the labeled amino acid. The reaction was optimal with 25 µg/ml pmAspAT mRNA, which was prepared in vitro T7 RNA polymerase transcription of BamHI-linearized pBSKS-4 template using the mCAP kit from Stratagene.

**Determining the Time Course of Trypsin Digestion of In Vitro Synthesized pmAspAT—** pmAspAT was synthesized in vitro, and the reaction was terminated after 20 min of incubation by chilling on ice and adding cycloheximide from a 100-fold concentrated stock solution to a final concentration of 50 µM. A 2.6-µl aliquot was removed and diluted into 23.4 µl of ice-cold 20 mM HEPPS, 150 mM NaCl, 0.1 mM EDTA, pH 8.3. 2.5 µl of the diluted translation product was removed and added to 22.5 µl of SDS-PAGE sample buffer and corresponds to the i = 0 sample. 19 µl of the diluted translation product was then added to 1 µl of 0.4 mg/ml TPCK-treated trypsin to start the digestion. The trypsin digestion was kept at 6°C. When the appropriate lengths of time had elapsed, 2.5-µl aliquots were likewise removed and diluted into 22.5 µl of room temperature SDS-PAGE sample buffer to stop the trypsin digestion. 5-µl aliquots of the SDS-denatured samples were electrophoresed on a 10% polyacrylamide gel, which was fixed to a 10% acetic acid, 20% methanol, dried, and radioisob was detected by an overnight exposure using the Molecular Dynamics PhosphorImager.

**Immunoprecipitation of pmAspAT from the Translation Reaction—** Antibodies against pmAspAT were raised in rabbits by hyperimmunization with purified, native recombinant pmAspAT emulsified with Freund's complete (primary injection only) or incomplete adjuvant. Antibodies against phosphopyridoxylsine were prepared as described previously (Perez-Ramirez and Martinez-Carrion, 1989). IgG was purified from serum by protein A-agarose affinity chromatography (Repligen). pmAspAT was synthesized in vitro as described above, and samples for immunoprecipitation of the "fresh" translation product were then removed. 147.9 µl of the ice-cold buffer (sterile 20 mM HEPPS, 150 mM NaCl, 0.1 mM EDTA, pH 8.3). Duplicate samples were removed for immunoprecipitation with anti-pmAspAT IgG (samples A and B, respectively). Another 21.2 µl of diluted 75-fold by adding p 1.5, 569 µl of buffer containing 0.5% sodium borohydride in 0.01 N NaOH was added to samples A and B, and the reaction was allowed to continue on ice for 20 min when an additional 1.5 µl of a fresh 0.5 M sodium borohydride stock solution was added and allowed to react another 20 min. 13.9 µl of 20% SDS was then added to each final concentration of 0.88% or ~38 µg/ml SDS to 0.5 µg/ml pmAspAT protein. The solution was briefly (2 min at 55°C) and then returned to the ice bath. Unless otherwise indicated, every...
effort was made to keep the immunoprecipitation reaction samples ≤ 4 °C.

After incubating the remainder of the translation reaction for 240 min at 15 °C, three more aliquots were removed and treated just like the aliquots of the fresh translation product. When the reductions and denaturations were complete, the samples were diluted with 1,200 μl of Trition X-100 (w/v) to lower the concentration of SDS to one compatible with immunoprecipitation.

5-μl aliquots of dilutions of anti-pyridoxal-P and anti-pmAspAT IgG were put in 1.5-ml siliconized microtubes, and then a 187-μl aliquot of variously treated translation products was added to each tube. The reactions were incubated overnight in the cold. The next morning, 10-μl aliquots of a 1:1 suspension of Repligen protein A-agarose were added and incubated with vortexing for at least 2 h in the cold. The suspensions were centrifuged about 30 s at maximum speed in a microcentrifuge in the cold, and a 20-μl aliquot of the supernatant was put in 1.5-ml siliconized microtubes, and then a 187-μl sample buffer (without bromphenol blue) was added, and the samples were heated at 75 °C for 5 min and then stored at 4 °C until analysis. 5 μl of the SDS extract was counted by liquid scintillation counting.

Miscellaneous Methods—Transaminase activity was assayed in a reaction consisting of 0.5 ml of 10 mM L-aspartate, 6 mM 2-oxoglutarate, 100 mM HEPES, pH 7.5, 0.2 mM NADH, and 7 units of mitochondrial aspartate aminotransferase (Boehringer Mannheim, glycol suspension, from pig heart). The reaction, thermostatted at 25 °C, was started by the addition of pmAspAT, and the rate of decrease in absorbance at 340 nm was determined using a Hewlett-Packard HP8452A spectrophotometer and associated kinetics software. Mitochondria were prepared by a simple differential centrifugation procedure (Arison et al., 1966) in a slightly modified buffer (MESH): 220 mM mannitol, 70 mM sucrose, 20 mM HEPES, 0.1 mM, EDTA pH 7.4. SDS-PAGE employed the Bio-Rad MiniProtein II apparatus using the discontinuous buffer system of Laemmli (1970) and, unless otherwise noted, 12% polyacrylamide separating gels approximately 5 cm in length. The gels were fixed for at least 60 min in 10% acetic acid, 20% methanol, dried, and radiolabel was detected and quantitated after overnight exposure by using the Molecular Dynamics PhosphorImager or by autoradiography and densitometry using the ISCO 1312 densitometer in conjunction with Hoofer densitometry software.

RESULTS

Instability of the pmAspAT in Vitro Translation Product—Synthetic pmAspAT mRNA is translated in RRL into primarily a single polypeptide with the same mobility with SDS-PAGE as purified pmAspAT. Analysis of a 5-6 min, the amount of pmAspAT translation product increases until a maximum is reached after about 20 min. On the basis of the amount of trichloroacetic acid-precipitable radiolabel incorporated into protein, the specific activity of the added [35S]methionine, the concentration of endogenous methionine in RRL, the presence of 12 methionines/pmAspAT subunit, and the observation that significant amounts of radiolabel are incorporated only into pmAspAT, we estimate that the maximum concentration of translation product in a typical reaction is about 100 ng/ml or 2.1 pm. However, this protein is unstable; continued incubation at 30 °C causes the amount of translation product to diminish (t1/2 ≈ 200 min) until only 10–15% of the maximum remains (Fig. 1). Close inspection of the SDS-PAGE profile of the translation reaction shows some minor intermediate species formed during the synthetic phase of the reaction (data not shown). The relative abundance of these minor bands, most of which are of a higher apparent molecular weight, changes during the degradative phase of the reaction, producing even slower migrating species. If the translation reaction is shifted to lower temperatures after 20 min of incubation at 30 °C, less protein is lost. After incubation for 20 h at 15 °C, approximately 80% of the translation product remains, and virtually no translation product is lost with extended incubation on ice. Dilution of translation product either into RRL or 20 mM HEPPS, 150 mM NaCl, 0.1 mM EDTA, pH 8.3, supplemented with potas-

Sensitivity of pmAspAT to Exogenous Protease—Purified pmAspAT is a remarkably trypsin-resistant protein with the exception of two sites within the 29-residue presequence which are readily hydrolyzed. A low concentration of trypsin progressively hydrolyzes the peptide bonds, first after Arg-22 and then after Arg-2, but proceeds no further (Martinez-Carrion et al., 1990). (The sequence is numbered relative to the start of the mature protein, hence residues at negative positions are in the presequence (Mattingly et al., 1987).) Hydrolysis at the latter site mimics the amino-terminal proteolytic processing that would occur if the precursor were to be processed by the mitochondrial protease during normal import into the organelle. The resulting protein (∆1-28 pmAspAT) is just as resistant to further proteolysis as is the pmAspAT isolated from chicken heart (Sandmeier and Christen, 1980).

pmAspAT, freshly synthesized in RRL and digested at 0 °C with 20 μg/ml trypsin, demonstrates a significantly greater sensitivity to added protease than purified pmAspAT. Aliquots of the trypsin reaction were removed after increasing intervals of time and analyzed by SDS-PAGE. Fig. 24 shows the distribution of radiolabeled bands revealed on those gels. In less than 1 min, almost 40% of the initial amount of radiolabel present in the pmAspAT band is lost from the gel, presumably as peptides too small to be retained within a 15% acrylamide gel. It should be noted that methionine residues are more or less uniformly distributed throughout the pmAspAT sequence. Of the remaining radiolabel seen on the gel, a small amount is in the intact pmAspAT band, but most is distributed in several smaller peptides. When the digestion is complete, after about 30 min of incubation, only 1–2% of the radiolabel originally present remains as an apparent ∆1-28 pmAspAT species, and the intermediate species have largely disappeared. The trypsin digestion of freshly synthesized translation product thus appears to proceed through a series of intermediate species on the way to complete digestion.

If the freshly synthesized translation product is incubated at 15 °C for 4 h before digesting with 20 μg/ml trypsin at 0 °C, a quite different trypsin digestion pattern is observed (Fig. 2B). The radiolabeled pmAspAT, rather than being completely digested, is merely converted to a species which migrates the same as the ∆1-28 pmAspAT species produced by trypsin digestion of purified pmAspAT (Martinez-Carrion et al., 1990). The time course for conversion of the translation product to a trypsin-resistant state is shown in Fig.3 and is expressed as the proportion of radiolabel present in the ∆1-28 pmAspAT band after digestion relative to the amount of radiolabel present in the pmAspAT band before digestion. To calculate how much of the translation product is trypsin-resistant from the amount of radiolabel present in the ∆1-28 pmAspAT band, the distribution of methionine residues in the protein must be considered. The presequence contains 2 of the 12 methionine residues in pmAspAT (Mattingly et al., 1987). If all of the translation product were trypsin-resistant, 2 of the 12 methionine residues in pmAspAT would be removed with the presequence during the trypsin digestion. In other words, about 80% of the radiola-
The rate of pmAspAT synthesis in a rabbit reticulocyte lysate in vitro translation system. At the indicated times, a 1-μl aliquot of the translation reaction was removed and added to 49 μl of SDS-PAGE sample buffer. The radiolabel incorporated into the pmAspAT band was then measured by analyzing an SDS-PAGE minigel using the Molecular Dynamics PhosphorImager.

bel in the Δ1-28 band after trypsin digestion of the fully "aged" translation product suggests that almost 90% of total translation product has become trypsin-resistant.

The process of conversion to a trypsin-resistant state is temperature-dependent and is optimal at about 15 °C. As indicated above, incubation of the freshly synthesized translation product at 30 °C causes the disappearance of most of the translation product, but some trypsin-resistant material (about 20%) does form at this temperature. This conversion proceeds at a significantly lower rate at 4 °C.

Effect of Translation Product Concentration, EDTA, ATP Depletion, NEM, and Triton X-100 on Conversion of the Translation Product to a Trypsin-resistant Species—One way of manipulating the concentration of translation product is by varying the concentration of RNA added to the translation reaction. Decreasing the translation product concentration 10-fold has no significant effect on the percentage of the translation product which becomes trypsin-resistant (data not shown). Decreasing the concentration of translation product by simply diluting the translation reaction 10-fold into 20 mM HEPPS, 150 mM NaCl, 0.1 mM EDTA, pH 8.3, before incubation at 15 °C reduces the proportion of translation product which is trypsin-resistant material by about two-thirds throughout the time course of the reaction (data not shown). Dilution into that buffer supplemented with potassium, magnesium, ATP, and DTT has a less severe effect and reduces the proportion of trypsin-resistant material by only about one-half (Fig. 3). Dilution into RRL appears to reduce somewhat the rate at which trypsin resistance is acquired but does not significantly reduce the ultimate amount of protease-resistant material produced.

Fig. 3 also shows the effect of several other perturbants. EDTA has a concentration-dependent, inhibitory effect on the conversion of the translation product to a trypsin-resistant state. 1 mM EDTA has a small effect, whereas 5 and 10 mM EDTA are equivalent in reducing the maximum proportion of trypsin-resistant material by about one-half. The rate at which trypsin resistance is acquired is apparently unaffected. Alkaline phosphatase and apyrase, which hydrolyze ATP phosphate groups, have approximately the same effect as EDTA and reduce the amount of the trypsin-resistant radiolabel by about one-half.

Sufficiently high concentrations of NEM completely prevent conversion of translation product to a trypsin-resistant species. 2 mM NEM reduces the amount of trypsin-resistant material by almost two-thirds, even though DTT is still in excess at a nominal concentration of 2 mM in the translation reaction. 4 and 6 mM NEM are equivalent in completely stopping any time-dependent appearance of trypsin-resistant species. The effect of NEM appears to be irreversible, since adding more DTT subsequent to NEM does not restore the ability of the translation product to become trypsin-resistant.

Addition of Triton X-100 to 0.1% w/v also completely blocks conversion to trypsin-resistant species.

Immunoprecipitation of Fresh and Aged Translation Product—Polyclonal antibodies raised against the native pmAspAT can be used to probe the exposure of antigenic determinants within the translation product, possibly including conformational epitopes which would be present in the native protein. The amount of anti-pmAspAT IgG required to precipitate the maximal amount of fresh and aged pmAspAT translation product is shown in Fig. 4A. At least 15-fold more anti-pmAspAT IgG is required to precipitate all of the fresh pmAspAT translation product than is required to precipitate all of the aged pmAspAT translation product. The slightly greater amount of fresh translation product which can be immunoprecipitated with higher concentrations of antibodies reflects the loss of translation product observed during the aging at 15 °C. Denaturation of the two translation products abolishes any difference in the amount of IgG necessary for precipitation of either, but the amount of IgG required to precipitate the denatured translation product is much greater than that needed to precipitate nondenatured translation product. Analysis of the immunoprecipitation reaction supernatants by SDS-PAGE shows that the amount of radiolabel precipitated is proportional to the amount of translation product removed from the supernatant and that pmAspAT is quantitatively precipitated. These changes in antibody titer, together with the increase in trypsin resistance already described, could indicate that during aging the translation product acquires a conformation resembling that of purified, native pmAspAT.

Interpretation of the changing antibody titers is somewhat complicated by the heterogeneity inherent in polyclonal antibodies. The increase in titer that accompanies the increase in trypsin resistance could result from greater accessibility of the antibody to an unchanging number of epitopes on the translation product, an increase in the actual number of
FIG. 2. The time course of trypsin digestion of in vitro synthesized pmAspAT. Panel A, pmAspAT was synthesized in vitro and immediately after completion of the reaction, the translation product was digested with TPCK-treated trypsin and analyzed by SDS-PAGE as described under "Experimental Procedures." Traces a-h represent the distribution of radiolabeled electrophoretic bands after 0, 0.6, 1.7, 3.5, 7.3, 15, 20.0, and 30.0 min of trypsin digestion, respectively. Panel B, the remainder of the translation reaction, which was used to produce the pmAspAT used in panel A, was incubated at 15 °C for 4 h and then digested with trypsin in the same fashion. Traces a-h correspond to 0, 0.6, 1.8, 3.4, 7.7, 15, 20, and 30 min of trypsin digestion, respectively.

epitopes present, or from a combination of both events. Our IgG preparation has a very similar titer against both native and denatured antigen (data not shown). However, preabsorption with urea-denatured pmAspAT eliminates the reactivity against denatured antigen while retaining 20–25% of the titer against native protein. Thus, although our polyclonal antibodies recognize mainly sequential determinants, they should also be able to detect changes which produce new conformational epitopes. However, much of the change in antibody titer most likely reflects increasing accessibility of the external surface of the translation product, possibly after its release from a molecular chaperone or other macromolecular component(s) present in RRL.

Although we have no direct evidence of large scale conformational changes accompanying the "aging" process, consideration of the apparent changes in accessibility to antibody probes combined with the observation regarding protease susceptibility does suggest such changes. The decreased trypsin susceptibility we see in aged pmAspAT translation product could be explained by a sequestering of the scissile peptide bonds in the interior of the protein in a protein folding reaction or by an accretion of factors which impede trypsin's access to a translation product with an invariant conformation. This latter explanation for the acquisition of trypsin resistance would contradict possible explanations of the antibody results. Therefore, it seems that these two probes of translation product conformation agree in suggesting that the aging process involves the folding of the polypeptide to a conformation resembling that of purified pmAspAT possibly after release from a stERICALLY protective environment.

Immunochemical Determination of Cofactor Binding and Catalytic Activity of the Translation Product—Pyridoxal-P covalently binds at the active site of aminotransferases through an aldimine bond with a lysyl residue. This form of the enzyme can react with amino acid substrates to produce a corresponding keto acid and pyridoxamine-P. This reaction is readily reversible, depending upon the relative concentrations of amino acid and keto acid. Pyridoxamine-P is not attached covalently, but is still tightly bound to the protein through a variety of non-covalent interactions. Denaturation of either form of the transaminase results in release of the cofactor. (The aldimine bond is hydrolyzed when the integrity of the active site pocket is lost.) Sodium borohydride reduction of the aldimine bond traps pyridoxal-P at the active site so
that cofactor remains associated with the protein even after denaturation. IgG recognizing the product of this reduction, phosphopyridoxyllysine, can be used to determine whether the pyridoxal-P cofactor is bound to the fresh or aged translation product, provided the cofactor can first be trapped by reduction of the internal Schiff base with sodium borohydride. Fig. 4B shows that more of the aged than fresh translation product can be precipitated with anti-phosphopyridoxyllysine IgG, indicating that the amount of pyridoxal-P bound to the translation product increases when the protein becomes trypsin-resistant. However, the maximum amount of translation product that can be precipitated is less than that precipitated by IgG against the total protein, suggesting that not all of the pyridoxal-P cofactor is bound to the fresh or aged translation product.

Anti-phosphopyridoxyllysine antibodies can also be used to monitor whether the translation product becomes catalytically competent. The pyridoxamine-P form of the cofactor cannot be detected by anti-phosphopyridoxyllysine IgG. Since there is a high concentration of L-aspartate (≈1 mM) and a lesser, but still significant, amount of L-glutamate (≈0.3 mM) in RRL, part of the cofactor bound to the translation product should be present in the pyridoxamine form if the enzyme is catalytically active. This could account for the observation that not all of the aged translation product can be precipitated with anti-phosphopyridoxyllysine IgG. The addition of a high concentration of keto acid should reverse the reaction, transforming bound pyridoxamine-P back to active site-bound pyridoxal-P and thereby increasing the amount of translation product precipitated by anti-phosphopyridoxyllysine IgG. Fig. 4C shows that this is the case. Adding 10 mM 2-oxoglutarate increases the amount of aged translation product precipitated. Conversely, adding an excess of amino acid substrate to aged translation product should decrease the amount of radiolabel precipitated and that expected result is also shown in Fig. 4C. Cysteine sulfinate is more effective for this purpose than the endogenous, normal substrates because it drives the reaction toward pyridoxamine-P by producing an unstable keto acid which decomposes to give pyruvate which does not react well in the reverse reaction (Jenkins and D’Ari, 1966). Substrates have no effect on the immunoprecipitation of fresh translation product by anti-pmAspAT IgG.

The effects of substrates on the immunoprecipitation of aged translation product by anti-phosphopyridoxyllysine IgG clearly demonstrate that all of the cofactor bound to the translation product is catalytically active. How much of the aged translation product is both trypsin-resistant and actually has bound cofactor? Based upon the maximum amount of radiolabel precipitated by the two antibodies, a maximum of about 35% of the total translation product has pyridoxal-P bound; the maximum amount of the translation product which is trypsin-resistant is about 90%. Since pretreatment of the translation product with trypsin has no effect on the amount of radiolabel which can be immunoprecipitated by anti-phosphopyridoxyllysine IgG (data not shown), it is likely that only trypsin-resistant translation product has cofactor bound. We calculate that about 40% of the trypsin-resistant, aged translation product has bound pyridoxal-P which can be trapped by reduction with sodium borohydride, i.e. cofactor bound in a way similar to that found at the active site of the fully
The relatively high concentration of amino acid form. Another possible explanation for a deficit in bound in RRL may prevent the added keto acid from completely product (measured by the amount of radiolabel precipitated with anti-phosphopyridoxyllysine IgG) was compared with the rate at which trypsin resistance was acquired during incubation at 15 °C. In addition, the rate at which the translation product became more easily precipitated by anti-pmAspAT IgG was also determined by measuring the amount of radiolabel precipitated by a concentration of anti-pmAspAT that preferentially precipitates the trypsin-resistant translation product.

The amount of radiolabel precipitated by anti-phosphopyridoxyllysine or anti-pmAspAT IgG increases with time in direct proportion to the fraction of the translation product which is trypsin-resistant (Fig. 5); that is, the rate of cofactor binding to the translation product, the rate of conversion to a more accessible antigen, and the rate of acquiring trypsin resistance are all the same. One possible explanation of these similar rates is that one of the processes is rate-limiting.

Endogenous Cofactor Concentration in RRL and Its Effect on the Conversion of Translation Product to a Trypsin-resistant Species—To investigate the potential role of the cofactor in the efficient folding of the pmAspAT translation product, we analyzed the effect of altering the concentration of cofactor present in the reticulocyte lysate on the aging process. We attempted to accomplish this by adding cofactor or by depleting its concentration through incubation with apotransaminase, a possible competitor in cofactor binding. Prior to undertaking these experiments, we first set out to determine the amount of endogenous cofactor in RRL and whether it could actually be removed by the addition of apotransaminase. The concentration of cofactor in an inert buffer can be determined by measuring the transaminase activity which results from the reaction of cofactor with the apotransaminase, provided one knows the specific activity of the transaminase with a known amount of bound cofactor. These latter values can be easily determined in a defined system, and we reasoned that the same might also be accomplished in RRL. Upon addition of apotransaminase to RRL at a final apo-enzyme subunit concentration of 2.1 μM, the transaminase ac-

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**Fig. 4.** Immunochemical analysis of changes in conformation, cofactor binding, and catalytic activity of pmAspAT translation product during incubation at 15 °C. Panel A, immunoprecipitation of the pmAspAT translation product with antibodies against the native, purified, recombinant pmAspAT. Freshly synthesized pmAspAT, native (■) or denatured with SDS (▲), and aged pmAspAT, native (●) or denatured (●), were immunoprecipitated with anti-pmAspAT IgG as described under "Experimental Procedures." Panel B, immunoprecipitation of the pmAspAT translation product with antibodies against phosphopyridoxyllysine. Freshly synthesized pmAspAT (■) or aged pmAspAT (●) were reduced, denatured, and immunoprecipitated with anti-phosphopyridoxyllysine IgG. Prior to reduction, denaturation, and immunoprecipitation with anti-phosphopyridoxyllysine IgG, aliquots of a translation reaction were allowed to react with cysteine sulfinate (as well as 2-oxoglutarate in the case of the aged translation product) for 20 min at 0 °C either immediately after completion of the synthesis reaction or after aging for 4 h at 15 °C. The substrates were added from a 100-fold concentrated stock solution. The amount of radiolabel precipitated is expressed as a percentage of the maximum amount of reduced, denatured translation product precipitated by anti-pmAspAT IgG as shown in panel A. Symbols: ○, fresh translation product with no addition; □, fresh translation product with 1 mM cysteine sulfinate; ●, aged translation product with no additions; ▲, aged translation product with 1 mM cysteine sulfinate; and ■, aged translation product with 1.9 mM 2-oxoglutarate.

The actual amount of bound cofactor, either as pyridoxal-P or pyridoxamine-P, is likely greater than 40% since our assay can only detect one of the forms of the bound cofactor, pyridoxal-P. The relatively high concentration of amino acid substrates and the unknown metabolic fate of 2-oxoglutarate in RRL may prevent the added keto acid from completely converting any pyridoxamine-P cofactor to the pyridoxal-P form. Another possible explanation for a deficit in bound cofactor could be found in the reduction reaction used to stabilize the enzyme-cofactor bond. The efficiency of sodium borohydride trapping of the cofactor on relatively high concentrations of properly folded, purified holo-pmAspAT added to RRL is near 100%; however, competing reactions in a complex mixture such as RRL may reduce that efficiency especially since the translation product is much more dilute. The possibility that some of the aged translation product is present as apoenzyme must still be recognized, although this may result, not from an inability to bind cofactor, but from a slow rate of cofactor binding such as suggested in experiments described below.

RRL contains a small amount of intrinsic aspartate transaminase activity; but relative to the amount of translation product synthesized, it is very large. Consequently, it is not possible to assay the translation product directly for activity, nor is it possible to determine the specific activity of translation product with bound cofactor. However, we propose that the catalytic activity observed in the half-transamination reactions described above indicates that the trypsin-resistant translation product has a conformation which closely resembles that of the nature, dimeric enzyme. We justify this proposition by recalling that formation of the active site requires the proper juxtapositioning of some residues from both subunits and many residues which are not adjacent in the protein's primary structure (Jansonius and Vincent, 1987).

Rate of Cofactor Binding to the Translation Product and of Its Conversion to a Trypsin-resistant Species—The rate of pyridoxal-P cofactor binding to the translation product (measured by the amount of radiolabel precipitated with anti-phosphopyridoxyllysine IgG) was compared with the rate at which trypsin resistance was acquired during incubation at 15 °C. In addition, the rate at which the translation product became more easily precipitated by anti-pmAspAT IgG was also determined by measuring the amount of radiolabel precipitated by a concentration of anti-pmAspAT that preferentially precipitates the trypsin-resistant translation product.

The amount of radiolabel precipitated by anti-phosphopyridoxyllysine or anti-pmAspAT IgG increases with time in direct proportion to the fraction of the translation product which is trypsin-resistant (Fig. 5); that is, the rate of cofactor binding to the translation product, the rate of conversion to a more accessible antigen, and the rate of acquiring trypsin resistance are all the same. One possible explanation of these similar rates is that one of the processes is rate-limiting.
activity increases very slowly, reaching an apparent maximum after 4-5 days (Fig. 6A). To estimate the specific activity of the fully reconstituted apoenzyme in RRL, an excess of pyridoxal-P or pyridoxamine-P (final concentrations of 20, 40, or 80 μM cofactor) was added to the same 2.1 μM concentration of apoenzyme subunits in RRL, and the activity was measured over time (Fig. 6A). The reactions with added cofactor were apparently first order, and all reached the same final activity level. From the maximum activity of the apoenzyme plus added cofactor, the assumption that all of the cofactor binding sites were occupied by the added cofactor, and the transaminase activity measured in the absence of added cofactor, a minimum concentration of 0.2 μM can be calculated for the endogenous cofactor present as either pyridoxal-P or pyridoxamine-P. As mentioned previously, the concentration of translation product is 2.1 pM. Thus, RRL contains a 10,000-fold excess of cofactor relative to potential binding sites in the pmAspAT translation product.

Reconstitution of 2.1 μM apoenzyme with cofactor in the absence of RRL is complete in less than 1 min for all but the lowest concentration of pyridoxamine-P and reaches a final value approximately 1.5-fold greater than that achieved in RRL, suggesting that apoenzyme added to RRL cannot be fully reconstituted to holoenzyme or that the reconstituted holoenzyme is inhibited in some fashion. The pseudo-first order rate constants for reconstitution of apoenzyme in RRL were linearly proportional to the concentration of the cofactors added (Fig. 6B); however, the lines do not pass through the origin as would be predicted if the reaction mechanism in RRL was the simple bimolecular reaction observed in the absence of RRL (Michuda and Martinez-Carrion, 1969). This anomalous behavior and the very slow rate of reconstitution could arise from either the presence of inhibitors of reconstitution or the limited availability of pyridoxal-P because of binding to other protein lysyl residues.

Increasing the intrinsic concentration of cofactor by adding either pyridoxal-P or pyridoxamine-P to 80 μM after translation produced only a minor (less than 5%) increment in the amount of trypsin-resistant material. This increase is comparable to our experimental error. This observation lessens
the possibility that the rate-limiting step in the conversion to a trypsin-resistant and antibody-accessible state is a bimolecular reaction between cofactor and translation product. However, cofactor binding could still be a prerequisite for this process. If the rate-limiting step involves a rearrangement of a previously formed translation product-cofactor complex, increasing the concentration of cofactor would not significantly increase the amount of translation product with bound cofactor, since there is already a large excess of endogenous cofactor relative to pmAspAT in the translation reaction mixture.

An alternative way of evaluating the role of cofactor in the folding of pmAspAT is to eliminate as much free cofactor as possible prior to synthesis of the pmAspAT translation product to prevent the formation of a pmAspAT-cofactor complex. Under these conditions, no trypsin-resistant pmAspAT would be formed if the so-called folding process required cofactor binding. We attempted to reduce the concentration of endogenous pyridoxal-P or pyridoxamine-P in the RRL by adding 1 mg/ml of either purified apocytoplasmic pig AspAT (cAspAT) or apompmAspAT prior to translation, concomitant with translation, or after translation. Pretreatment with apocAspAT or cotranslational treatment with either apocytoplasmic AspAT or apompmAspAT decreases the total amount of translation product obtained and slightly reduces the proportion of the translation product which becomes trypsin-resistant, particularly at early time points in the aging process (data not shown). Adding apoenzyme after translation has no significant effect on the conversion to trypsin resistance. We were not able to measure how much of the endogenous cofactor was bound by the added apoenzymes because of the limited sensitivity of our assay for cofactor. Even if our assay indicated that all of the cofactor had been removed, sufficient cofactor could still remain so as to provide as much as a 100-fold excess over the amount of translation product. Nor did we attempt to assess the effectiveness of the depletion of cofactor by immunochemically measuring the amount of cofactor bound to the resultant trypsin-resistant pmAspAT for two reasons. First, the potentially large relative amount of holotransaminase now present in the translation reaction would interfere; and second, its effect on the pyridoxal-P/ pyridoxamine-P ratio of the translation product would be difficult to assess. Nevertheless, the lower yield of stabilization product obtained in reactions with an excess of apotransaminase might reflect a decrease in stability of pmAspAT in its earliest stages of folding when synthesized in the presence of limiting concentrations of cofactor. In this regard, it is known that pyridoxal-P increases the yield of refolding of cytosolic AspAT (West and Price, 1989) and mitochondrial AspAT2 from their denatured states in guanidinium HCl. These observations suggest that, even if not absolutely required for folding, pyridoxal-P may stabilize some early folding intermediate(s) which could lead to higher final yields of the stable, folded protein.

Correlation of the Proportion of Trypsin-resistant Translation Product with Import Competence—Freshly synthesized translation product is readily translocated in vitro into mitochondria. Imported translation product is distinguished from external translation product by the appearance of a truncated polypeptide with higher electrophoretic mobility associated with the mitochondrial pellet. The intact translation product band associated with the reisolated mitochondria is external to the mitochondria, either bound on the mitochondrial surface or in the residual supernatant. This band can be removed by hydrolisis with trypsin while the higher mobility band is unaffected, being made inaccessible to trypsin by the mitochondrial membranes. In most experiments we did not treat the reisolated mitochondria with protease, but rather simply quantitated the amount of radiolabel in the lower band as a measure of import. Under optimal conditions, approximately 70–80% of the fresh translation product can be imported into mitochondria. As the translation product becomes more resistant to trypsin as a result of incubation at 35 °C, less translation product is bound to mitochondria and imported. There is an inverse linear relationship between the fraction of translation product imported and the fraction of translation product resistant to trypsin (Fig. 7), and it appears that with the best mitochondria preparations, the maximum amount of translation product which can become trypsin-resistant is also the maximum amount of translation product which can be imported immediately after being synthesized (data not shown).

These results indicate that translation product conformations which are similar to that of the purified recombinant pmAspAT, i.e. trypsin-resistant and able to bind cofactor, prohibit import. This is consistent with many other studies of the import of precursors and precursor-like proteins which conclude that proteins are not imported into mitochondria in a tightly folded conformation (Glick and Schatz, 1991; Neupert et al., 1990). However, those observations are at variance with other reports which seem to show that the fully folded, mature form of holo-mAspAT can be translocated into mitochondria (Marra et al., 1977) and that this folded protein can compete with a pmAspAT translation product in import reactions (Giannattasio et al., 1991). This contradiction draws attention to the importance of assessing the physiological relevance of different in vitro model systems, of quantitatively evaluating the rates and yields of the import processes being compared, and of continuing investigation in this area.

Trypsin Resistance of Freshly Synthesized Translation Product after Import into Mitochondria—To determine how quickly translation product imported into mitochondria becomes trypsin-resistant once inside is of interest for the analysis of the overall folding process of the protein from its synthesis in the cytoplasm to the acquisition of the mature conformation in the mitochondrial matrix. The rationale for this experiment is based on the fact that, as already discussed, properly folded translation product of pmAspAT is resistant to complete hydrolysis under specific trypsin digestion conditions, whereas incompletely folded translation product is not, and that lysis of the mitochondria with a nonionic detergent makes internalized translation product, whether properly folded or not, inaccessible to trypsin with no change in its conformation (data not shown). At the earliest time point measured, there is no trypsin-resistant translation product in the import reaction either with or without detergent (Fig. 8). After incubation for 3 min, there is some translation product which resists trypsin digestion in the absence of detergent but not in the presence of detergent. As the import reaction approaches completion, the amount of trypsin-resistant translation product is the same regardless of the presence or absence of detergent. Mitochondria pretreated with valinomycin before addition to the import reaction never produce a trypsin-resistant species. These results suggest that there is at most only a small lag in the proper folding of imported translation product; that is, as opposed to what is observed for pmAspAT in the cytoplasmic-like environment of RRL, folding is rapid once the protein reaches the mitochondrial matrix as a processed mature form.

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Fig. 7. The correlation between trypsin resistance and import of the pmAspAT translation product into mitochondria. Mitochondrial import reactions contained 45 μl of translation reaction which had been incubated for various times at 15 °C and 5 μl of mitochondria with 3 mg of protein/ml. The reactions were allowed to proceed for 30 min at 30 °C and then chilled on ice. The reaction was centrifuged, and the supernatant was removed and added to an equal volume of 2 × SDS sample buffer. The pelleted mitochondria were washed twice by re suspending in 190 μl of MESH and recovered by centrifugation and finally suspended in 45 μl of MESH. The resolated mitochondria were denatured with an equal volume of 2 × SDS sample buffer. Aliquots of the supernatant and mitochondria were analyzed by SDS-PAGE and PhosphorImager autoradiography. The ratio of the counts in the mature sized AspAT band associated with the mitochondria (presumed to be imported protein) to the sum of the counts in the pmAspAT band in the import reaction supernatant plus the counts in the pmAspAT and mature AspAT bands associated with the mitochondria was used to calculate the percentage of pmAspAT imported.

Fig. 8. The acquisition of trypsin resistance after mitochondrial import of pmAspAT. At the indicated times, an 8-μl aliquot was removed from an import reaction (comprised of equal volumes of translation reaction and mitochondria at 6 mg of protein/ml) and simultaneously added with 2 μl of 0.6 mg/ml trypsin to 70 μl of an ice-cold quenching solution consisting of 10 μM valinomycin in MESH. Duplicate 20-μl aliquots were removed and added to 1 μl of 1.4% Triton X-100, and all of the trypsin reactions were allowed to continue for 20 min on ice. The reactions were terminated by addition of 0.5 μl of phenylmethylsulfonyl fluoride. Upon removal and digestion of all aliquots of the import reaction, 20 μl of each of the quenched trypsin digestions was denatured by adding an equal volume of SDS-PAGE sample loading buffer, and aliquots were electrophoresed. The amount of translation product was quantitated by autoradiography and densitometry. The symbols are: □, the amount of translation product in the import reaction which survives trypsin digestion in the absence of detergent; and ○, the amount which resists digestion in the presence of detergent.

DISCUSSION

There are some examples of proteins which have apparently folded properly after in vitro synthesis in RRL. 4-Aminobutyrate aminotransferase (Choi and Churchich, 1986) and glutamate dehydrogenase (Felipo et al., 1983) are two authentic mitochondrial precursor enzymes which have catalytic activity after cell-free translation. A structural protein of the cytosol, the β-subunit of tubulin, seems to undergo a slow
conversion to a functional conformation after synthesis in RRL as well (Yaffe et al., 1989). Unfortunately, these studies did not examine the process which led to these functional states. Most of the pertinent information obtained to date on protein folding after in vitro synthesis has originated from studies related to the mechanism of protein translocation into mitochondria and to the effect of the prescence on the folding of the protein. In one of the first such investigations, an artificial mitochondrial precursor, produced by attaching the cytochrome oxidase subunit IV pressequence to mouse dihydrofolate reductase, was shown to be able to bind a ligand after its synthesis in RRL thereby indicating that it had rapidly attained a high degree of folding (Eilers and Schatz, 1986). These results were confirmed by the protease resistance of another newly synthesized dihydrofolate reductase construct and by finding catalytic activity in a newly synthesized translation product, formed by fusion of the pressequence of ornithine carbamyl transferase to bacterial chloramphenicol acetyltransferase, immediately after being released from the ribosome (Skerjanc et al., 1990). In this work we show that pmAspAT synthesized in the RRL cell-free translation system can acquire a conformation resembling that of the purified native protein, and we conclude that this is a process of protein folding, having a half-life of about 100 min. This folding proceeds at a rate significantly lower than that observed for the spontaneous refolding of chemically denatured pmAspAT in the absence of other cellular components. The reluctance of pmAspAT to assume a folded conformation rapidly after in vitro synthesis is in distinct contrast to the rapid folding observed in the previously described model systems and suggests that the behavior of a protein in the presence of cytosolic factors may be determined by the unique information contained in its complete amino acid sequence. Indeed, there are proteins which are unable to acquire an active conformation after synthesis in RRL (Kalousek et al., 1984). Thus, this work emphasizes that an authentic mitochondrial precursor protein may provide a more realistic model for protein import.

On the basis of the considerable information regarding factors required for import and the postulated mechanisms for molecular chaperone action, we can propose an explanation for the behavior of pmAspAT synthesized in RRL. There is ample prior evidence that mitochondrial precursor proteins interact with cytosolic factors. Chemically denatured, purified mitochondrial precursors can be efficiently imported into mitochondria without accessory cytosolic factors, only if the proteins do not have an opportunity to refold or aggregate (Glick and Schatz, 1991). However, cytosolic factors are required to maintain import competence under conditions favoring refolding. We can reasonably conclude that similar factors seem to interact with the newly synthesized pmAspAT and thus account for its incomplete folding and attendant trypsin susceptibility, its poorer reactivity with anti-native pmAspAT, and its capacity to interact with the mitochondrial import receptor system. The full identity of these protein factors is uncertain, although two different classes of import factors appear to exist.

Genetic manipulations which led to conditionally lethal import defects in yeast implicated members of the hsp70 family of chaperones as one type of import factor (Deshaies et al., 1988). Further studies demonstrated that hsp70 chaperones were not sufficient to impart import competence to a mitochondrial protein synthesized in wheat germ extract and that yeast cytosol contained an additional, NEM-sensitive import factor (Murakami et al., 1988). This factor may be related to a pressequence binding factor isolated from RRL and proposed to act in concert with yeast hsp70 to stimulate import of a purified, denatured mitochondrial precursor protein (Murakami and Mori, 1990) and to be required for import of mitochondrial proteins synthesized with a cleavable pressequence (Murakami et al., 1992). Another pressequence binding factor with a significantly different subunit molecular weight was purified by a subtly different procedure (Ono and Tuboi, 1990b). Although the addition of this PBF was shown to enhance import of an RRL in vitro translation product and depletion was shown to inhibit that import (Ono and Tuboi, 1990a), it is unclear whether it acts synergistically with hsp70 to restore or maintain import competence for proteins otherwise unable to be imported. The ability of hsp70 chaperones to bind unfolded proteins has been demonstrated in vitro and in vivo and that is likely their function in facilitating mitochondrial import, since hsp70 may maintain a denatured purified precursor in an unfolded or nonaggregated state (Sheffield et al., 1990). This same study confirmed, though, that a cytosolic fraction can maintain import competence, whereas hsp70 alone cannot. PBFs have a less clear role. Although it has been suggested that the PBF may function analogously to the endoplasmic reticulum signal recognition factor in targeting translocated polypeptides, more recent results (Becker et al., 1992) infer a chaperoning role. However, all current results indicate that the PBF need not be present in the early stages of protein synthesis (Murakami et al., 1992). This observation lends credence to an alternative function for PBFs, namely, one of facilitating release of the precursor from a hsp70 chaperone to allow import. This function also seems more consistent with our observations and suggestion that the folding of pmAspAT requires dissociation from a chaperone such as hsp70, particularly since NEM prevents both the import-facilitating function of the PBF (Murakami et al., 1988) and the aging process we describe (Fig. 3). The hypothesis that the rate-limiting step in the aging process is dissociation from a chaperone complex is further supported by the effect of agents which diminish the availability of ATP, since hydrolysis of ATP may be required for release of bound peptides from chaperones (Flynn et al., 1991). This putative function for the PBF may be analogous to the role of cpn10 in aiding the release of peptides from cpn60 or the recently reported GroE-dependent transfer of incompletely folded rhodanese from a DnaK-DnaJ complex to a GroEL-GroES complex which allows folding (Langer et al., 1992).

The non-random accessibility of potential trypsin hydrolysis sites in pmAspAT, indicated by the intermediate species observed during the course of the digestion, could be consistent with an extended peptide conformation such as thought to bind to hsp70 chaperones (Puller et al., 1991). However, if the entire polypeptide possessed this conformation, it would imply that many hsp70 chaperone molecules would be bound to each pmAspAT polypeptide since the major proteolytic intermediates are rather large compared with the length of the peptide which could be accommodated by the proposed peptide binding region of hsp70 (Flynn et al., 1991). We favor an alternative interpretation of these results which would suggest the presence of regions with a more compact peptide conformation (i.e. molten globule) in the protease-susceptible pmAspAT such as those thought to bind to hsp60 chaperones. This predilection is based upon a supposition that the hydrophilic residues recognized by trypsin would not be sequestered within the hsp70 peptide binding cleft since hsp70 is thought to bind preferentially to hydrophobic regions. This point illustrates the need for additional assessments of the confor-
motions of peptides bound to the various types of molecular chaperones.

In contrast to the behavior of pmAspAT in the presence of the cytosolic components in RRL, folding of the protein in the mitochondrial matrix is very rapid. The small lag observed in the appearance of trypsin-resistant species following import might result from the lack of enough folded monomers needed for dimerization or from some other as yet unknown event in the folding of a protein emerging from the mitochondrial membrane environment. The acceleration of the folding inside mitochondria cannot be solely ascribed to the removal of the presequence during import since the precursor and mature forms of AspAT show very similar in vitro refolding rates after denaturation in guanidinium HCl. Most likely, the coordinated action of the intramitochondrial chaperonins cpn60/cpn10 (Vitale et al., 1992; Osterman et al., 1989; Cheng et al., 1989) with the possible participation of mt-hap70 (Manning-Krieg et al., 1991) facilitates the assembly of the emerging processed protein, perhaps already containing significant amounts of structure. Whether the removal of the presequence alone has significant effects or just the presence of specific molecular chaperones in different cellular compartments determines the rate of protein folding remains to be elucidated.

The instability of newly synthesized pmAspAT in RRL is consistent with a previous observation concerning its fate in vivo. Just as the pmAspAT translation product disappears when incubated in reticulocyte lysate at 30 °C, the in vitro existence of pmAspAT is transient with a half-life of about 5 min in fibroblasts rendered unable to import mitochondrial proteins by treatment with carbonyl cyanide m-chlorophenylhydrazone (Jaussi et al., 1982). Our data indicate that in RRL the proportion of pmAspAT which is converted to a trypsin-resistant conformation decreases as the incubation temperature is increased from 15 to 30 °C; thus, it is unknown how much of the pmAspAT which is not imported in vivo would fold to a functional conformation in the cytoplasm of an intact cell. The mechanism through which pmAspAT is apparently lost during extended incubation of the translation reaction is not clear. The small amounts of higher molecular weight species which are observed in SDS-PAGE gels during the course of the incubation could indicate a ubiquitin-dependent proteolysis or could arise from an aggregation process. The removal of the presequence during import since the translation product. Such studies are now in progress in our laboratory.

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The deleterious effect of increasing temperature on the efficiency of the holding of pmAspAT in RRL is reminiscent of the tendency of purified, chemically denatured proteins to refold poorly as the renaturation temperature increases. This may suggest that the folding process we observe does not proceed through direct participation of a molecular chaperone at all and is actually just the result of a slow release from a chaperone which prevents folding. However, the inhibition of pmAspAT folding in RRL by Triton X-100 is consistent with a chaperone-mediated mechanism since hydrophobic interactions are believed to be at least partially responsible for the interaction of molecular chaperones with unfolded polypeptides. Furthermore, although we have no data concerning the effect of detergents on the refolding of chemically denatured, purified pmAspAT, the in vitro refolding of another mitochondrial protein, rhodanese, is facilitated by lauryl maltoside micelles (Zardeneta and Horowitz, 1992). These micelles probably assume the role of chaperonins in assisting folding by preventing aggregation. Nonetheless, the nature of the folding process for pmAspAT in RRL must await the direct determination of the actual structure of the protease-susceptible conformation of the partially folded translation product and the identification of the chaperone(s) involved in any complexes with the translation product. Such studies are now in progress in our laboratory.

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