Structural Features of the Precursor to Mitochondrial Aspartate Aminotransferase Responsible for Binding to hsp70*

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The precursor (pmAspAT) and mature (mAspAT) forms of mitochondrial aspartate aminotransferase interact with hsp70 very early during translation when synthesized in either rabbit reticulocyte lysate or wheat germ extract (Lain, B., Iriarte, A., and Martinez-Carrion, (1994) J. Biol. Chem. 269, 15588–15596). The nature of the structural elements responsible for recognition and binding of this protein to hsp70 has been studied by examining the folding and potential association with the chaperone of several engineered forms of this enzyme. Whereas pmAspAT and mAspAT bind hsp70 very early during translation, the cytosolic form of this enzyme (cAspAT) does not interact with hsp70. A fusion protein consisting of the mitochondrial presequence peptide attached to the amino terminus of cAspAT associates with hsp70 only after the protein has acquired its native-like conformation, apparently through binding to the sequence exposed on the surface of the folded protein. Deletion of the amino-terminal segment of mAspAT or its replacement with the corresponding domain from the cytosolic isozyme eliminates the cotranslational binding of hsp70 to the mitochondrial protein. We conclude that both the presequence and NH₂-terminal region of pmAspAT represent recognition signals for binding of hsp70 to the newly synthesized mitochondrial precursor. Results from competition studies with synthetic peptides support this conclusion. The ability of hsp70 to discriminate between these two highly homologous proteins probably involves the recognition of specific sequence elements in the NH₂-terminal portion of the mitochondrial protein and may relate to their separate localization in the cell. A slower folding rate and higher affinity for cytosolic chaperones may represent evolutionary adaptations of translocated mitochondrial proteins to ensure their efficient importation into the organelle.

Many denatured proteins are able to refold spontaneously which clearly demonstrates that the functional three-dimensional structure of proteins is solely determined by their amino acid sequences (Anfinsen and Scheraga, 1975; Jenicke, 1991). In order to avoid the competing reactions of aggregation, low temperatures and low protein concentrations are used in most of these in vitro refolding studies. However, in vivo, proteins manage to fold at high protein concentrations and at physiological temperatures (37 °C). Apparently, this is accomplished with the collaboration of external factors such as molecular chaperones, which control the process by binding to incompletely folded polypeptides and thereby preventing their aggregation, misfolding or premature folding (for recent reviews, see Gething and Sambrook (1992) and Hendrick and Hartl (1993)). Furthermore, in the intact cell, proteins may initiate folding during biosynthesis on the ribosome and before the polypeptide chain is released into the cytoplasm (Freedman, 1992; Fedorov et al., 1992; Kolb et al., 1994; Fedorov and Baldwin, 1995). This cotranslational folding is probably assisted by molecular chaperones. Chaperones DnaJ (Hendrick et al., 1993) and hsp70† (Hansen et al., 1994) have been found associated with newly synthesized proteins still attached to the ribosome. Frydman et al. (1994) recently proposed that firefly luciferase synthesized in rabbit reticulocyte lysate (RRL) interacts sequentially with hsp40 (a homologue of bacterial DnaJ), hsp70, and TCP-1 containing ring complex. These interactions allow the cotranslational formation of protein domains and contribute to the rapid folding of the newly synthesized enzyme once released from the ribosome (Fedorov and Baldwin, 1995).

The extent of folding achieved before the polypeptide chain is released from the ribosome may, however, vary for different proteins. For instance, the precursors of translocated mitochondrial proteins must be maintained in a partially unfolded, loose conformation in the cytosol in order to be imported into the organelle (Verrier and Schatz, 1987; Pfanner et al., 1987). Such a delayed folding may not be required for proteins that stay in the cytosol, and therefore they might afford to fold rapidly after synthesis. The mechanism by which cells prevent the "premature" folding of proteins before being translocated across the mitochondrial membranes is not fully understood, but various proteins, including cytosolic hsp70, have been implicated in the process (Chirico et al., 1988; Deshaies et al., 1988; Murakami et al., 1988; Sheffield et al., 1990).

The cytosolic and mitochondrial isoforms of aspartate aminotransferase (AspAT) constitute a well suited system to investigate the factors responsible for the selective control of the folding of proteins according to their final destination in the cell. Both enzymes are encoded by the nuclear DNA and synthesized in cytosolic polysomes. The two dimeric proteins share

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†The abbreviations used are: hsp70, 70-kDa heat-shock protein; hsp40, 40-kDa heat-shock protein; AspAT, aspartate aminotransferase; mAspAT, mitochondrial aspartate aminotransferase; pmAspAT, precursor to mitochondrial aspartate aminotransferase; cAspAT, cytosolic aspartate aminotransferase; pAspAT, precursor chimera to cytosolic aspartate aminotransferase; pmAspAT, precursor chimera to mitochondrial aspartate aminotransferase; mAspAT, chimera to cytosolic aspartate aminotransferase; Δ3-30 mAspAT, mature mitochondrial aspartate aminotransferase deletion mutant; DEPC, diethyl pyrocarbonate; PAGE, polyacrylamide gel electrophoresis; RRL, rabbit reticulocyte lysate.
a high degree of sequence homology, an almost superimposable three-dimensional structure, and a basically identical catalytic mechanism (Janssou and Vincent, 1987). However, they show differences in overall stability (Iriarte et al., 1984c) and isoelectric point (pI). The more stable cystolic enzyme (cAspAT) has a slightly acidic pI, whereas the mitochondrial form (mAspAT) has a pI of around 9.0 (Martinez-Carrion and Tiemeyer, 1967). Furthermore, the folding process of the two isoymes synthesized in cell-free extracts is clearly distinctive. The mitochondrial enzyme, which is synthesized in the cytosol as a precursor protein with a 29-residue amino-terminal extension or presequence peptide (pAspAT; Altieri et al., 1989; Mattingly et al., 1987), folds relatively slowly after in vitro synthesis in RRL (half-life of ~100 min). By contrast, the highly homologous cystolic isozyme rapidly acquires a protease-resistant conformation after synthesis in the same cell-free extract (Mattingly et al., 1993a, 1993b). Yet, the spontaneous in vitro refolding of the cystolic enzyme after denaturation with guanidine hydrochloride is only 2-3-fold faster than the mitochondrial enzyme (Iriarte et al., 1994; Mattingly et al., 1995). It is thus apparent that components of the cell-free extract affect differently the folding rate of each isozyme.

We recently reported that hsp70 interacts with both the precursor and mature forms of mitochondrial AspAT very early during translation in either RRL or wheat germ extract (Lain et al., 1994). In this work we have continued these studies using the cystolic isozyme of AspAT and several chimeric constructs prepared by exchanging selected sequence fragments between the two isoymes. Both the rate of folding and the interaction with chaperones were analyzed for these diverse AspAT forms synthesized in cystolic-like environments. We found that the distinct folding rate of the cystolic and mitochondrial members of this isozyme pair may be determined by the exclusive interaction of chaperones with the mitochondrial form. In addition, this study provides information regarding the targeting signals that allow molecular chaperones to discriminate between highly homologous proteins destined to reside in different intracellular compartments.

**EXPERIMENTAL PROCEDURES**

**Materials**—The peptides and oligonucleotides used in this work were chemically synthesized at the Protein and DNA Core facility of the School of Biological Sciences, University of Missouri-Kansas City. Antibodies against hsp70 were raised in rabbits by hyperimmunization with purified bovine brain hsp70 (Welch and Feramisco, 1985) emulsified with Freund's complete (primary injection) or incomplete adjuvant. IgG were purified using a protein A-agarose (Repligen) column or a DEAE-Affi-Gel blue column (Bio-Rad). Antibodies were used either as whole serum or as purified IgG.

**Construction of Plasmids Encoding pmAspAT, mcAspAT, and pmcAspAT**—A complete cDNA-encoding pmAspAT was assembled from two previously isolated plasmids, pBSKS-5 and pBSKS-6, encoding pmAspAT and mcAspAT, respectively. Construction of pBSKS-5 and pBSKS-6 was as described previously (Mattingly et al., 1993a, 1993b). pBSKS-6 was digested with HpaI and SalI, and the resulting 4787-bp fragment was purified by electroelution. This DNA fragment corresponds to the pbScript vector and a core of cAspAT lacking the first 33 NH2-terminal residues. pBSKS-5 was used as a template to generate by PCR a cDNA sequence spanning the presequence and the first 30 residues of the NH2-terminal region of pmAspAT. We used the ULTma sequence spanning the presequence and the first 30 residues of the mature protein (pBluescript vector and a core of cAspAT lacking the first 30 amino acids. Two reverse primers were used, the underlined nucleotide sequences were used as a template for the PCR reaction, and the resulting PCR product included the presequence encoding the first 30 amino acids (residues 1-33) from the NH2-terminal region of the mitochondrial mAspAT protein. After digestion with SalI, the 221-bp DNA fragment was purified by electroelution and ligation to the 4787-bp HpaI-SalI fragment from pBSKS-6. The desired recombinant plasmid, pBSKS-10, was selected by PCR screening from randomly selected transformants and was subsequently sequenced.

The pmcAspAT chimerica was constructed by combining segments of pBSKS-6 and pBSKS-5 cDNAs using recombinant PCR. The primer PCR1 (TGATGATCGTATTCCGAAAGGTTAACCTCGAGTTTGCCCTTA) defines the splice point between the two proteins. It encodes the last 3 amino acids of the 63-residue amino-terminal pAspAT peptide to be encoded in the mAspAT sequence. The primers for encoding pmcAspAT, beginning at nucleotide 276 which begins the codon for amino acid 63. Primer PCR2 (ATGTCAAGAAGTCCAGAGAAT) is complementary to nucleotides 826-865 in the coding strand for pmAspAT. As PCR3 we used the primer corresponding to the T3 promoter region of pbScript KS5 indicated before; PCR4 (AACCTTGGGGATCCGAGTAGC) is complementary to nucleotides 314-291 which encodes the last 8 amino acids of the peptide to be attached to the mAspAT core. The two fragments were freed of excess primers by ultrafiltration, aliquots of each were combined, and another amplification using PCR3 and PCR2 was performed. The resulting product was digested with NdeI and PstI and the desired 340 bp was gel-purified. This fragment was ligated to NdeI- and PstI-digested pBSKS-4 to yield pBSKS-8. Further information on these constructs is available from the authors upon request.

**In Vitro Transcription and Translation—**mRNAs were prepared by in vitro T3 RNA polymerase transcription of BamHI-linearized pBSKS templates, with the exception of cAspAT, where T7 RNA polymerase was used, using the mCAP kit from Stratagene. The same amount of radiolabeled protein was synthesized in each reaction, and the resulting 35S-labeled purified pmAspAT. Protein folding of mAspAT in vitro translation products was assessed by monitoring the acquisition of trypsin resistance as described previously (Lain et al., 1994)). The amount of radiolabeled translation product was chosen based on appropriate titrations (Lain et al., 1994). The same amount of preimmune serum was used as control. Immunoprecipitation of the translation products was performed as described before (Lain et al., 1994)). The amount of radiolabeled translation product immunoprecipitated is expressed as percentage relative to the total amount of protein that is synthesized and is the average of two determinations.

**Miscellaneous**—SDS-PAGE was performed with the Bio-Rad Mini-PROTEAN II apparatus using the discontinuous buffer system of Laemmli (1970). Unless otherwise noted, 12% polyacrylamide separating gels approximately 5 cm in length were used. The gels were fixed for 30 min in 10% acetic acid, 20% methanol, dried (2 h, 80 °C), and radiolabeled bands were detected by autoradiography after an at least overnight exposure using the Molecular Dynamics Phosphorimager II. The Phosphorimager response is proportional to the concentration of radiolabeled protein present as independently assessed using a standard curve of PS-labeled purified pmAspAT. Protein folding in vitro synthesized proteins was assessed by monitoring the acquisition of trypsin resistance as described previously (Lain et al., 1994)). Data are expressed as the percentage of total labeled protein present (aliquot treated with trypsin) that has become trypsin-resistant (aliquot treated with trypsin).
NH₂-terminal Sequence in a Precursor Protein Binds hsp70

RESULTS

Folding and Interaction with hsp70 of mAspAT and cAspAT Engineered at Their NH₂-terminal Region—Earlier studies on the folding of the two isozymes of AspAT in cell-free extracts (Mattingly et al., 1993a, 1993b; Lain et al., 1994) suggested that both the presequence peptide and amino-terminal regions of pmAspAT might be involved in the control of its folding and interaction with hsp70. To test this hypothesis, we prepared different protein constructs in which the structural elements in the mitochondrial enzyme that we suspected were responsible for hsp70 recognition were either deleted or replaced with the corresponding segments from the cytosolic isozyme. Conversely, the presequence and the NH₂-terminal region of the mitochondrial enzyme were introduced into the structure of the cytosolic counterpart. Fig. 1 shows a box diagram of the eight different proteins we have used in this study: pmAspAT, the authentic mitochondrial precursor, and mAspAT, the mature-like protein; Δ3–30 mAspAT, a deletion mutant lacking the first 28 residues of the NH₂-terminal region; cAspAT, the cytosolic isozyme and pcAspAT, an artificial precursor protein consisting of the cytosolic isozyme with the mitochondrial presequence fused to its amino terminus; pcmAspAT, a chimera in which the NH₂-terminal region of the mature mitochondrial protein has been replaced with the corresponding NH₂-terminal region from its cytosolic counterpart; and pmcAspAT, a cytosolic enzyme containing the presequence and the 30-residue NH₂-terminal segment from the mitochondrial protein. mAspAT is the mature-like form of pmcAspAT, that is, the cytosolic enzyme with the amino-terminal segment from the mitochondrial isozyme.

The various protein forms were synthesized in RRL at 30 °C, as previously reported (Lain et al., 1994), using [35S]methionine as the radiolabeled amino acid. All of them migrated in SDS-PAGE gels according to their expected molecular weights (data not shown). The constructs which contain the core component from the cytosolic isozyme, cAspAT, pcAspAT, pmcAspAT, and mcAspAT, are more stable in RRL after synthesis at 30 °C than their mitochondrial counterparts (Mattingly et al., 1993b; data not shown). Furthermore, the yield of protein synthesis for pmcAspAT and mcAspAT is about 3-fold higher than for the mitochondrial and cytosolic wild type proteins. The reasons for this difference in translation efficiencies are unknown.

The association of the different constructs with hsp70 during protein synthesis in RRL (Fig. 2) or upon further incubation following termination of the translation reaction (Fig. 3), was followed by analyzing at different times the total amount of protein present and the amount of radioactive translation products coimmunoprecipitating with anti-hsp70 antibodies. As shown before (Lain et al., 1994), both the precursor and mature forms of mitochondrial AspAT coimmunoprecipitate with hsp70 at the early stages of the translation reaction. The fraction of pmAspAT and mAspAT associated with hsp70 decreases rapidly as translation progresses (data included in Fig. 2A for comparison purposes), suggesting a “transient” association of the translation product with the chaperone. The amount of pmAspAT coimmunoprecipitating with hsp70 increases again when the translation reaction is incubated at 20 °C following completion of protein synthesis (Fig. 3B). This “stable” reassociation of hsp70 with pmAspAT is dependent on the presence of the presequence and parallels the acquisition of protease resistance by the translation product (Fig. 3A). Apparently, hsp70 binds tightly to the presequence exposed on the surface of the folded protein. By contrast, no coimmunoprecipitation of...
A percentage of total labeled pmcAspAT present (aliquot not treated with diethyl pyrocarbonate (DEPC)-treated water. As shown in Fig. 2A, deletion of the 28-residue NH$_2$-terminal fragment of mAspAT (Δ3-30 mAspAT), or its substitution with the corresponding sequence from cAspAT (pcmAspAT), dramatically decreased the amount of translation product immunoprecipitating with hsp70 antibodies during the initial stages of the translation reaction. Binding of hsp70 to the presequence-containing pcmAspAT did not increase either upon incubation at 20 °C after arresting translation (Fig. 3B), probably because this protein, like Δ3-30 mAspAT, is unable to acquire a folded, protease-resistant conformation (Fig. 3A). According to these results, the NH$_2$-terminal peptide of mAspAT appears to be essential not only for binding to hsp70 but also for productive folding of the newly synthesized polypeptide chain.

In order to clarify the involvement of the NH$_2$-terminal region of mAspAT on its interaction with hsp70, we examined the consequences of introducing this mAspAT region into the NH$_2$-terminal end of cAspAT. The hypothesis underlying the design of these experiments was that if that sequence constitutes a linear recognition site for hsp70, it should be able to render the otherwise inert cAspAT a “substrate” for the chaperone, as the presequence does for the folded protein. Yet, the data presented in Fig. 2B show that the level of immunoprecipitation of pcmAspAT early during translation is identical to that of wild type cAspAT. When the NH$_2$-terminal sequence was inserted together with the presequence peptide (pcmAspAT), the levels of immunoprecipitation at the initial translation times were still identical to the corresponding wild type control (pcmAspAT in this case). Only after incubation following the end of translation, this presequence-containing protein appeared in a complex with hsp70 (Fig. 3B). This association correlated once more with the appearance of trypsin resistant species (Fig. 3A).

Thus, the presequence and the NH$_2$-terminal peptides of pmcAspAT by themselves do not contain enough information to transform the cAspAT chains emerging from the ribosome into a target recognizable by hsp70. Either additional targeting signals are required for binding or the rate of folding of the protein being synthesized determines the accessibility of the potential binding sites to interaction with the chaperone.

Effect of Synthetic Presequence and NH$_2$-terminal Peptides on AspAT Synthesis, Folding, and Binding to hsp70—The involvement of the presequence and NH$_2$-terminal peptide of pmcAspAT on its interaction to hsp70 was also investigated by analyzing the effect of synthetic peptides corresponding to these sequence regions on the binding of in vitro translated protein to the chaperone. As a control, we determined first if protein translation itself was affected by the synthetic peptides. To account for the small dilution factor introduced in the reaction mixture by addition of the peptide aliquot, we ran as a control a reaction that had received an equivalent volume of diethyl pyrocarbonate (DEPC)-treated water. As shown in Fig. 4, some very interesting and somehow unexpected results were
obtained. First of all, the 1–19 NH²-terminal peptide from the mitochondrial presequence and NH²-terminal peptides diminished during prolonged incubation of the sample at 20 °C. Both the mitochondrial presequence and NH²-terminal peptides diminished significantly the fraction of pmAspAT immunoprecipitated with hsp70 antibodies (Fig. 5). The inhibition of protein synthesis caused by the mitochondrial synthetic peptides made the analysis of the binding of hsp70 to the newly synthesized protein under these conditions difficult, which initially was our main objective. The almost undetectable levels of pmAspAT synthesized in the presence of the presequence peptide precluded any further analysis of the binding of hsp70 to the newly synthesized protein under these conditions.

To rule out the possibility that the decrease in protein synthesis caused by this peptide was due to the presence of RNase activity in the peptide solution, we checked the stability in the cytosolic enzyme did not interfere with the synthesis of either pmAspAT or cAspAT (Fig. 4, A and B). In contrast, the 1–23 NH²-terminal peptide from the mitochondrial isozyme caused a partial decrease in the yield of protein synthesis. This inhibitory effect was not specific for the mitochondrial enzyme as the synthesis of cAspAT was almost equally affected. But the most dramatic effect was observed with the presequence peptide. At comparable concentrations, it almost completely abolished protein synthesis of the two AspAT isozymes and of the bromo mosaic virus protein used as control. These findings clearly indicate that the presence of an excess of the presequence peptide interferes with the normal functioning of the protein synthesis machinery of RRL.

The inhibition of protein synthesis caused by the mitochondrial synthetic peptides made the analysis of the binding of hsp70 to the newly synthesized protein under these conditions difficult, which initially was our main objective. The almost undetectable levels of pmAspAT synthesized in the presence of the presequence peptide precluded any further analysis of the translation product. The mitochondrial NH²-terminal peptide caused a slight decrease in the fraction of protein immunoprecipitating with hsp70 early during translation (data not shown), but the low quantities of protein synthesized hindered the accuracy of the measurements. As expected, the cytosolic NH²-terminal peptide did not have any effect on the level and time course of the immunoprecipitation of pmAspAT during protein synthesis (data not shown). We also examined whether any of these synthetic peptides was able to prevent the more stable binding of hsp70 to the folded, trypsin-resistant pmAspAT. The peptides were added after stopping the synthesis of pmAspAT with cycloheximide. Then the folding of the translation product and its association with hsp70 were followed during prolonged incubation of the sample at 20 °C. Both the mitochondrial presequence and NH²-terminal peptides diminished significantly the fraction of pmAspAT immunoprecipitated with hsp70 antibodies (Fig. 5). This inhibition was dependent on the concentration of peptide present and at –150 μM prevented almost completely the binding to hsp70. This effect was much less pronounced for the cytosolic NH²-terminal peptide (Fig. 5) which caused a partial decrease in immunoprecipitation only at the highest concentration used. These findings corroborate that hsp70 shows a clear preference for binding of the presequence and NH²-terminal peptides of the mitochondrial isozyme.

Binding of hsp70 to the presequence is not necessary for proper folding of the pmAspAT translation product in RRL. Neither the presequence nor the cytosolic NH²-terminal peptide affect the folding of pmAspAT in RRL (Fig. 6). However, the mitochondrial NH²-terminal peptide causes a marked decrease in the yield of protease-resistant pmAspAT. This effect is most likely due to a direct interference of this peptide with folding of the polypeptide chain rather than to depletion of the chaperone in the lysate. As indicated before, this region of the molecule appears to be critical for proper folding of the mitochondrial enzyme. During in vitro refolding studies of the acid-
It is now widely accepted that protein folding and assembly in the cell are mediated by molecular chaperones (Gething and Sambrook, 1992; Hendrick and Hartl, 1993). However, very little is known regarding the mechanism of action of these chaperones in vivo, mainly due to the scarcity of suitable analytical procedures and the complexities of the experimental systems under study. Several questions still remain open: are there any differences in the control of the folding processes of cytosolic and membrane translocated proteins?; and which features of a newly synthesized polypeptide sequence in a precursor protein binds hsp70

In this work we address some of these questions using the mitochondrial and cytosolic isozymes of malate dehydrogenase (Stanford et al., 1994). For this enzyme, a correlation was proposed between the overall hydrophobicity of the isozyme and the strength of its interaction with groEL. Mitochondrial malate dehydrogenase, which binds more tightly to groEL, has a higher hydrophobic potential than the cytosolic isozyme (Stanford et al., 1994). However, no such correlation exists for mAspAT and cAspAT. Both enzymes contain about equal amounts of hydrophobic amino acids (Mattingly et al., 1995) very similarly distributed along their primary structure (Mattingly et al., 1993b). Thus, this feature alone cannot explain the different affinity of these enzymes’ folding intermediates for chaperones. Perhaps the slightly greater spontaneous refolding rate of cAspAT is responsible for those differences. The rapid collapse of the unfolded protein to a compact structure could prevent a strong interaction with hsp70 which is known to favor binding of highly unstructured or extended regions of polypeptide chains (Flynn et al., 1991; Hartl et al., 1994; Palleros et al., 1994). Other chaperones, such as groEL, may recognize instead certain conformational features of intermediates in the folding pathway of the protein.

The percentage of total labeled pmAspAT present (aliquot not treated with trypsin) has been determined by monitoring acquisition of trypsin resistance. Data are expressed as the percentage of total labeled pmAspAT present (aliquot treated with trypsin) that has become trypsin-resistant (aliquot treated with trypsin). Symbols are: control in the absence of peptides; mitochondrial NH2-terminal peptide; or, cytosolic NH2-terminal peptide; or, presequence peptide.

Fig. 6. Effect of post-translational addition of presequence and NH2-terminal peptides on the folding of pmAspAT after synthesis in RRL. pmAspAT was synthesized in RRL as described under “Experimental Procedures.” After stopping protein synthesis, approximately 86 μL of the synthetic peptides, or an equivalent volume of DEPC water, were added to the translation mixtures. Samples were incubated at 20 °C, and folding of the translation product was followed by monitoring acquisition of trypsin resistance. Data are expressed as the percentage of total labeled pmAspAT present (aliquot not treated with trypsin) that has become trypsin-resistant (aliquot treated with trypsin). Symbols are: control in the absence of peptides; mitochondrial NH2-terminal peptide; or, cytosolic NH2-terminal peptide; or, presequence peptide.

In this work we address some of these questions using the mitochondrial and cytosolic isozymes of malate dehydrogenase (Pfanner et al., 1994; Verner, 1993), efficient uptake of mAspAT by mitochondria requires the presence of the NH2-terminal presequence peptide as well as an incompletely folded conformation (Mattingly et al., 1993a). From the latter requirement it follows that the folding of the newly synthesized protein must be slow enough to allow it to engage the import machinery in a competent conformation. Indeed, as we reported earlier, folding of the mitochondrial precursor synthesized in cell-free extracts proceeds at a slow rate (τ1/2 > 1 h at 15 °C) (Mattingly et al., 1993a). If mitochondria are introduced into the system immediately after finishing translation, the protein is imported into the organelle before it has a chance to fold into a protease-resistant conformation. In contrast, the cytosolic isozyme folds markedly faster under the same conditions (Mattingly et al., 1993b). How these two isozymes evolve to display such different folding rates in cytosolic-like environments is not known, but their distinct behavior may relate to their separate locations in the cell.

What determines the different folding rates of these two proteins after synthesis in cell-free extracts? Since the rates of refolding in buffer of the chemically denatured purified proteins are very similar (Mattingly et al., 1995), selective interaction with molecular chaperones is an obvious possibility. The findings presented in this work support this hypothesis, at least regarding hsp70. This chaperone could be detected associated with the mitochondrial translation product (Lain et al., 1994), whereas no such complex was found with the cytosolic form. From these data one can tentatively infer that perhaps cytosolic proteins do not need the collaboration of molecular chaperones for the regulation of their intracellular folding, because translocation through a membrane system is not part of their maturation process. In fact, mAspAT and cAspAT also show very different behavior with regard to their interaction with chaperones during in vitro refolding of the chemically denatured proteins. hsp70 arrests folding of acid-unfolded mAspAT but not cAspAT. Furthermore, the Escherichia coli chaperonin groEL completely stops the refolding of mAspAT at low temperatures, but only slows down the reaction for cAspAT (Mattingly et al., 1995). Interestingly, the cytosolic enzyme is able to refold from its guanidine HCl unfolded state at physiological temperature in the absence of chaperones. By contrast, mAspAT, perhaps because of a higher tendency of some of its folding intermediates to aggregate, requires the assistance of groEL/groES to refold at 37 °C after chemical denaturation (Mattingly et al., 1995).

Similar findings have been reported for the cytosolic and mitochondrial isozymes of malate dehydrogenase (Stanford et al., 1994). For this enzyme, a correlation was proposed between the overall hydrophobicity of the isozyme and the strength of its interaction with groEL. Mitochondrial malate dehydrogenase, which binds more tightly to groEL, has a higher hydrophobic potential than the cytosolic isozyme (Stanford et al., 1994). However, no such correlation exists for mAspAT and cAspAT. Both enzymes contain about equal amounts of hydrophobic amino acids (Mattingly et al., 1995) very similarly distributed along their primary structure (Mattingly et al., 1993b). Thus, this feature alone cannot explain the different affinity of these enzymes’ folding intermediates for chaperones. Perhaps the slightly greater spontaneous refolding rate of cAspAT is responsible for those differences. The rapid collapse of the unfolded protein to a compact structure could prevent a strong interaction with hsp70 which is known to favor binding of highly unstructured or extended regions of polypeptide chains (Flynn et al., 1991; Hartl et al., 1994; Palleros et al., 1994). Other chaperones, such as groEL, may recognize instead certain conformational features of intermediates in the folding pathway of the protein.

Are there linear sequence motifs that are particularly important for recognition and binding to hsp70? Previous results with the precursor and mature forms of mAspAT suggested that the presequence peptide may be one of the structural elements that are involved in this interaction (Lain et al., 1994). In addition, recombinant chicken pmAspAT expressed in

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E. coli copurifies with hsp70 (DnaK) tightly attached to its presequence peptide (Schmid et al., 1992). Our results with the fusion protein pcAspAT agree with these observations. Since cAspAT does not bind hsp70, the interaction of the chaperone with trypsin-resistant pcAspAT must involve the presequence peptide fully accessible in the folded protein. Several pieces of indirect evidence indicate that the presequence peptide lies exposed on the surface of native pmAspAT or pcAspAT precursors. For example, the peptide is extremely susceptible to trypsin hydrolysis (Martinez-Carrion et al., 1990). Furthermore, the presence of the presequence does not affect either the cataytic activity or the folding of the mature moiety of the proteins. Hence, although we do not know the exact arrangement of this peptide in the three-dimensional structure of the precursors, we envision it as an appendix at the NH₂-terminal end with only minimum, if any, contact with the surface of the protein dimer. Consequently, binding of hsp70 to the presequence peptide does not prevent the folding of the protein either.

However, if the presequence peptide in the folded proteins represents a linear targeting signal for hsp70, why were we unable to detect the transient complex of hsp70 with presequence-containing cAspAT forms during translation? One possibility is that the presequence is not available for recognition by hsp70 when the protein is emerging from the ribosome. More likely, the complex may form, but due to the rapid folding of the elongating cAspAT chain, the chaperone may dissociate during the dead time of our method of analysis. Additional investigation of the folding state of the cytosolic enzyme during translation would be required to clarify this point.

Since mature mAspAT, lacking the presequence peptide, also commumunoprecipitates with hsp70 antibodies early during translation, it is evident that the mature part of the mitochondrial protein contains additional recognition signals for the chaperone. We focused our attention initially on the NH₂-terminal region of the mature protein for several reasons. First, since protein synthesis proceeds from the amino- to the carboxyl-terminal end of the chain, this region would be the first to emerge from the ribosome immediately after the presequence peptide. Second, the low degree of sequence homology on this region of the two isozymes makes it a likely target for selective recognition of mAspAT by hsp70. Finally, appropriate restriction sites exist in the area which facilitates the manipulation of the parent cDNA in the preparation of chimeras.

Despite these predictions, the results from our studies on this sequence region are inconclusive. On the one hand, the deletion of this segment or its substitution with its equivalent from cAspAT eliminates the initial immunoprecipitation of the translation product with hsp70 antibodies during protein synthesis. The results obtained from competition studies using synthetic peptides corroborate that hsp70 shows a clear preference for binding to the mitochondrial NH₂-terminal (and presequence) peptides relative to the corresponding cytosolic sequence. However, its incorporation into the NH₂-terminal region of the cytosolic enzyme fails to convert this isozyme into a target for hsp70. It is likely, therefore, that the association of mAspAT with hsp70 involves other regions of the protein structure besides the NH₂-terminal peptide, probably with the participation of several chaperone molecules binding to different recognition signals along the polypeptide chain.

On the other hand, folding of mAspAT is drastically affected by deletion or replacement of its NH₂-terminal arm. Furthermore, only the mitochondrial NH₂-terminal peptide interferes with folding of pmAspAT, even when added posttranslationally. These results suggest that proper fitting of the first 10 residues at the NH₂-terminal end of one subunit into the hydrophobic pocket on the surface of its neighboring monomer is essential for efficient folding and/or assembly of the mAspAT dimer. The synthetic peptide probably competes for the same pocket on the surface of the protein that usually accepts the first 10 residues of the NH₂-terminal segment of the chain.

This anchoring contributes to the stabilization of the quaternary structure of the enzyme (McPhalen, 1992) and is also essential for the open-closed conformational transitions that accompany catalysis in both isozymes (Iriarte et al., 1984b). However, similar alterations in the corresponding region of cAspAT only decreases slightly the yield of folding in RRL (Fig. 3A), suggesting that the subunit interaction involving the NH₂-terminal arm is not as critical for dimerization of this isozyme. These observations correlate with our earlier reports showing that after limited trypsin cleavage, the NH₂-terminal peptide is easily removed from cAspAT (Iriarte et al., 1984a) but remains tightly associated with the protein core in mAspAT, suggesting a much stronger interaction at this subunit contact area in the mitochondrial protein. The significance of this difference in association energy between the two isozymes at this region of the subunit interface is not clear.

Perhaps the most surprising observation reported in this work is the dramatic effect of the mitochondrial peptides, particularly the presequence, on the synthesis of the mitochondrial and cytosolic AspAT as well as of the unrelated brome mosaic virus protein. The cytosolic peptide, however, is completely innocuous. Apparently, the mitochondrial peptides are competing with the nascent polypeptide chains for binding to some component of the protein synthesis machinery of RRL essential for completion of translation. Although the information available is insufficient to identify this factor(s), we can speculate that perhaps hsp70, which can bind the presequence and the mitochondrial NH₂-terminal peptides, is at least one of them. The interaction of this chaperone with a variety of nascent proteins while still attached to the ribosome is well documented (Beckmann et al., 1990; Hansen et al., 1994). It has been suggested that hsp70 may even have an active role in translation by aiding in the movement of the elongating chain through the ribosome channel, analogous to its role in protein translocation across membranes (Nelson et al., 1992). Consequently, depletion of free hsp70 by introduction of an excess of a suitable ligand, such as the presequence peptide of pmAspAT, would inhibit efficient protein synthesis.

In any case, this study suggests that hsp70 may not be directly involved in the slow posttranslational folding observed for mAspAT in RRL (Mattingly et al., 1993a). It may instead play a more essential role in the successful completion of the translation process or in the degree of cotranslational folding that the polypeptide chain achieves. The affinity of hsp70 for certain binding sites in the nascent chain, perhaps in combination with the intrinsic folding rate of the chain, could determine the stability and hence longevity of the initial complex. The two phenomena might be actually connected. It is possible that low affinity binding of hsp70 to the emerging cAspAT chain occurs and is sufficient to aid in the elongation of the chain, but not to slow down the intrinsically fast (cotranslational) folding of the translation product. This in turn would prevent binding of additional cytosolic factors, and the protein could finish folding quickly without assistance from other proteins. In contrast, a stronger association of hsp70 with the NH₂-terminal region of the nascent pmAspAT might slow down the initial acquisition of structure, thus allowing the subsequent binding of additional hsp70 molecules and/or other cytosolic proteins.

[4] Y.-H. Fu, A. Iriarte, and M. Martinez-Carrion, unpublished observations.
soluble components to recognition signals or hydrophobic regions that become transiently exposed in the elongating chain. Folding of the completed translation product after its release from the ribosome would then require its dissociation from this initial complex with chaperone(s), perhaps with the participation of other soluble cytosolic components (Lain et al., 1994; Mattingly et al., 1993a).

In summary, the interaction of molecular chaperones with nascent proteins is not solely by specific linear sequence elements. To transform proteins from low affinity or nonbinding to strong binding types, it is not just enough to incorporate into the structure those sequence elements suspected to be responsible for recognition and interaction of chaperones with their natural ligands. Presumably, additional information intrinsic to the conformation of folding intermediates, or even the folding of the protein itself, is needed. The specific interaction of only the translocated mitochondrial member of the AspAT isozyme system may have evolved to slow down the folding of the protein and hence give it the opportunity to be delivered to the mitochondrial import machinery in a suitable conformational state. By contrast, the cytosolic form, which remains in the compartment where it is synthesized, can skip such interactions and quickly acquire its native conformation. Further investigation is needed to explore the relative rate of cotranslational folding of the two isozymes and the nature of the complexes assembled around the mitochondrial AspAT nascent chain as it is being synthesized on the ribosome.

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