Synthetic Transit Peptides Inhibit Import and Processing of Mitochondrial Precursor Proteins*

(Received for publication. January 10, 1989)

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We have demonstrated that a synthetic peptide corresponding to the rat mitochondrial malate dehydrogenase (mMDH) transit peptide (TP-28) inhibits the binding of pre-mMDH to isolated mitochondria. Synthetic peptides derived from chloroplast transit peptide sequences, which have a similar net charge, did not inhibit import. In addition, this peptide (TP-28) inhibits import of ornithine transcarbamylase, another mitochondrial matrix protein, thus suggesting that common import pathways exist for both mMDH and ornithine transcarbamylase. A smaller synthetic peptide corresponding to residues 1–20 of the mMDH transit peptide (TP-20) also inhibits binding. However, several substitutions for leucine-13 in the smaller peptide relieve import inhibition, thus providing evidence that this neutral residue plays a crucial role in transit peptide binding to the mitochondrial surface. Proteolytic processing of pre-mMDH by a mitochondrial matrix fraction to both the mature and intermediate forms of mMDH was also inhibited by TP-28. The ability of synthetic peptides to inhibit distinct steps in the import of mitochondrial precursor proteins corresponds precisely to their ability to interact with the same components used by transit peptides on intact precursors. Furthermore, inhibition at multiple points along the import pathway reflects the functions of several independent structures contained within transit peptides.

The majority of mitochondrial proteins are encoded by the nuclear genome and synthesized on cytoplasmic ribosomes. From the cytoplasm, these precursors of mitochondrial proteins are targeted to several mitochondrial subcompartments: the outer membrane, the inner membrane, the intermembranous space, and the matrix.

Mitochondrial protein import involves several steps: 1) binding of the precursor to the mitochondrial import apparatus; 2) membrane insertion of the precursor into the mitochondrial membrane; 3) proteolytic processing of precursors, and 4) assembly with other mitochondrial proteins. We have studied transit peptide function using isolated mitochondria and mitochondrial precursor proteins generated in vitro from cDNAs cloned into expression plasmids. In conjunction with mutagenesis techniques which allow alteration of transit peptide sequences, this system has allowed definition of critical structural features of the mitochondrial malate dehydrogenase (mMDH) transit peptide.

Several approaches have been used to identify the interactions between mitochondrial precursor proteins and the import apparatus. We have studied transit peptide function using isolated mitochondria and mitochondrial precursor proteins generated in vitro from cDNAs cloned into expression plasmids. In conjunction with mutagenesis techniques which allow alteration of transit peptide sequences, this system has allowed definition of critical structural features of the mitochondrial malate dehydrogenase (mMDH) transit peptide. Our previous results indicated a role of positive charge in membrane translocation, but not in binding (Chu et al., 1987a). We further defined structural features conferred upon the mMDH transit peptide by certain neutral residues (e.g. leucine-13) which promote binding to mitochondria (Chu et al., 1987b).

In the work reported herein, synthetic peptides corresponding to portions of the mMDH transit peptide were employed as inhibitors of import and processing of mitochondrial precursor proteins. These experiments, in agreement with previous findings, suggest that distinct structures within transit peptides are responsible for binding, translocation, and processing.

EXPERIMENTAL PROCEDURES

Preparation of Mitochondria—Mitochondria were prepared from livers of freshly killed, fasted rats by homogenization and differential centrifugation as has been previously described (Chu et al., 1987a). The mitochondrial pellet was resuspended in ice-cold homogenization buffer (5 mM Tris, pH 7.4, 220 mM mannitol, 70 mM sucrose) at a protein concentration of 5 mg/ml and used immediately for import or processing assays. The amount of precursor protein added to uptake is about 10⁻⁶ M.

Restoration of import to Energy-depleted Mitochondria—Mitochondrial import was inhibited with 20 or 100 μM CCCP, 8 μM oligomycin, or 2 μM antimycin. Retinoloyl lysate containing newly synthesized mMDH is added to these preparations at 10⁻⁵ M (Hurt et al., 1985; Emr et al., 1986). It has been suggested that these targeting signals interact with receptors located on the mitochondrial surface (Riezman et al., 1983; Gillespie, 1987).

Other work has demonstrated the necessity for energy in the translocation process, as both an electrochemical gradient (Schleyer et al., 1982) and ATP (Eilers et al., 1987; Chen and Douglas, 1987). Matrix proteins have been identified which are involved in the processing of precursor molecules (McAda and Douglas, 1982; Bohni et al., 1983; Miura et al., 1982; Kalousek et al., 1988), and, in some cases, multiple processing steps involving a single precursor have been demonstrated (Gasser et al., 1982; Hartl et al., 1986; Sztul et al., 1987, 1988).

The abbreviations used are: mMDH, mitochondrial malate dehydrogenase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; TP-28 and TP-20, synthetic transit peptides as delineated in Table I.

* This work was supported by Grant AM-20407 from the National Institutes of Health and by a grant from the Monsanto Co. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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RESULTS

Synthetic Peptides as Inhibitors of Mitochondrial Import—To study the function and specificity of transit peptides during mitochondrial protein import, chemically synthesized peptides were added as inhibitors in import assays. Because import of proteins into chloroplasts is mediated by transit peptides which are similar in composition and charge to those which direct import into mitochondria, peptides with sequences of chloroplast transit peptides for petunia 5-enolpyruvylshikimate-3-phosphate synthase (residues 1-24) plus 4 additional residues in parentheses, the first 2 of which are from the mature mMDH sequence. The arrows indicate normal processing sites in the mMDH and ornithine transcarbamylase precursors.

TABLE I
Sequences of wild-type and synthentic transit peptides

| Peptide            | 1  | 10  | 20  | 30 |
|--------------------|----|-----|-----|----|
| 5-enol-Pyruvylshikimate-3-phosphate synthase (210) | MAQ1NN | MAQ1NNMAQ | ↓ | ↓ |
| Ribulose-1,5-bisphosphate carboxylase | MASSMISSPAVTTVNRA | ↓ | ↓ |
| Tobacco F_{1}-ATPase (213) | MLSANLRLILLNAALKAHTSMNRVN | ↓ | ↓ |
| Ornithine transcarbamylase mMDH | FRYGKVYQ | ↓ | ↓ |
| TP-28 | SFSTS | ↓ | ↓ |
| TP-20 | SFSTS | ↓ | ↓ |
| TP-20N13 | SFSTS | ↓ | ↓ |
| TP-20P13 | SFSTS | ↓ | ↓ |
| TP-20E13 | SFSTS | ↓ | ↓ |

*TP-28 corresponds to the complete mMDH transit peptide (residues 1-24) plus 4 additional residues in parentheses, the first 2 of which are from the mature mMDH sequence. The arrows indicate normal processing sites in the mMDH and ornithine transcarbamylase precursors.

FIG. 1. Inhibition of in vitro import of wild-type mMDH precursor by synthetic transit peptides. Radiolabeled wild-type mMDH precursor generated by translation of mMDH mRNA transcribed from the plasmid containing the cDNA was incubated with freshly isolated rat liver mitochondria in the presence of the indicated concentrations of synthetic peptide. For processing inhibition, the entire reaction mixture was analyzed by SDS-PAGE and autoradiography. Only the relevant parts of the autoradiographs were photographed. The synthetic peptide sequences are given in Table I. 210 is MAQ1NN, and 211 is MAQ1NNMAQ, both derived from the chloroplast protein 5-enol-pyruvylshikimate-3-phosphate synthase. 213 is a MASSMISSPAVTTVNRA, derived from the transit peptide of the tobacco F_{1}-ATPase subunit. mMDH refers to TP-28, the entire wild-type mMDH transit peptide. In all gels, the upper band co-migrates with the mMDH precursor, the lower band co-migrates with mature mMDH, and, in some lanes, a faint intermediate band is present.

mitochondrial F_{1}-ATPase β-subunit (residues 1-17), and the rat mMDH transit peptide (residues 1-24 + 4) were prepared and tested for import inhibition (see Table I for comparison of peptide sequences). The peptides were added at concentrations of 0-100 μM in the mitochondrial import reaction with wild-type pre-mMDH synthesized in vitro.

At these concentrations, only the F_{1}-ATPase β-subunit and the mMDH transit peptides were inhibitory (Fig. 1). The F_{1}-ATPase β-subunit peptide was partially effective at 60 μM and completely inhibitory at 100 μM. In contrast, the mMDH peptide caused significant inhibition by 5 μM and completely inhibited uptake, as measured by pre-mMDH processing, at
concentrations above 10 μM. The results demonstrate that inhibition of import by synthetic peptides is specific for those sequences which normally target proteins to mitochondria. Despite the similarities in composition and net charge, peptides derived from chloroplast targeting sequences were ineffective at the concentrations employed in this experiment. In contrast, these chloroplast transit peptides did inhibit import of the 5-enol-pyruvylshikimate-3-phosphate synthase precursor into chloroplasts, and the intact mMDH precursor was not incorporated into chloroplasts. This discrimination reflects the mechanism by which chloroplast and mitochondrial proteins become segregated in plant cells in that the targeting information in the mMDH transit peptide is specific for mitochondria.

**Fig. 2. Mature and intermediate mMDH do not bind to mitochondria.** Wild-type 5-S-labeled mMDH precursor was incubated with mitochondria treated with 100 μM CCCP in the presence of 100 μg/ml mature mMDH (indicated by + mMDH) or intermediate mMDH (indicated by + imMDH). The imMDH was purified from E. coli expressing an mMDH encoding plasmid and, as characterized by NH2-terminal sequence analysis, contained the last 8 amino acids of the transit peptide and all of mature mMDH. The binding reactions were separated into a supernatant (S) and pellet (P) and analyzed by SDS-PAGE and fluorography as in the legend to Fig. 1. The major band co-migrates with mMDH precursor.

**Synthetic Targeting Signals Block Binding of Precursors to Mitochondria—**To determine the step at which synthetic peptides interfere with mitochondrial import, we tested the ability of synthetic mMDH transit peptide to block binding to CCCP-treated mitochondria. The results demonstrate that increasing concentrations of peptide, in the same range which inhibit import into untreated mitochondria, block binding of precursor (Fig. 4A). However, after precursor addition to CCCP-treated mitochondria in the absence of peptide, the import of prebound precursor following rescue with β-mercaptoethanol occurred even if peptide were added along with the β-mercaptoethanol (Fig. 4B). These results indicate that import inhibition by synthetic transit peptides occurs by prevention of precursor binding to mitochondria and not by damage to mitochondria.

**Import of Both Ornithine Transcarbamylase and mMDH Is Inhibited by Synthetic Peptides—**Import inhibition of other mitochondrial protein precursors by mMDH-derived synthetic peptides was tested using rat ornithine transcarbamylase in a parallel set of experiments. As with mMDH, import of ornithine transcarbamylase into isolated rat liver mitochondria was blocked by TP-28, the mMDH transit peptide (Fig. 5). The F1-ATPase β-subunit transit peptide from tobacco also inhibited pre-ornithine transcarbamylase import, but peptides corresponding to chloroplast transit peptide sequences did not (not shown). The concentration of TP-28 required for effective inhibition was similar for both pre-mMDH and pre-ornithine transcarbamylase, with half-max-

**Fig. 3. Prebound precursor mMDH can be subsequently imported into mitochondria.** Wild-type precursor mMDH was incubated with rat liver mitochondria poisoned with 20 μM (lanes 3–6) or 100 μM (lanes 7–10) CCCP or with antimycin or oligomycin (lanes 11–14). The mitochondrial pellet was recovered and divided into separate portions. The supernatants (S) and pellets (P) of one aliquot from each treatment were analyzed by SDS-PAGE, as shown (lanes 3, 4, 7, 8, 11, and 12). The remaining mitochondria were rescued by resuspension in fresh reticulocyte lysate containing either 7 mM β-mercaptoethanol (SME) (lanes 5, 6, 9, and 10) or 8 mM ascorbate and 2 mM TMPD (lanes 13 and 14), and the reactions were separated into supernatants (S) and pellets (P) for analysis. The wild-type mMDH precursor before (lane 1) and after (lane 2) incubation with untreated mitochondria is shown for comparison.

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2 G. della Ciopa, personal communication.

3 M. Montgomery and A. Strauss, unpublished data.
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FIG. 4. The synthetic peptides derived from the mMDH wild-type precursor inhibit binding to mitochondria. A, binding of radiolabeled wild-type precursor mMDH to CCCP-treated mitochondria was performed as described in the legend to Fig. 3, but in the presence of the indicated concentrations of TP-28, the synthetic peptide containing the entire wild-type mMDH transit peptide sequence. Following the incubations, the reactions were separated into supernatants (S) and pellets (P) and analyzed as in previous figures. Note the absence of bound precursor in the pellets of mitochondria incubated with the transit peptide, and the inhibition of binding by synthetic peptide to untreated mitochondria. B, mMDH precursor was incubated with CCCP-treated mitochondria as described in Fig. 3. After resolation of the mitochondrial pellet, rescue was performed with β-mercaptoethanol in the presence of the indicated concentrations of TP-28. Import of the prebound precursor was not inhibited.

FIG. 5. Inhibition of import of pre-ornithine transcarbamylase and pre-mMDH by a synthetic mMDH transit peptide occurs in parallel. Radiolabeled ornithine transcarbamylase and mMDH precursors were incubated separately with mitochondria without any additions or with the indicated amounts of TP-28, the complete synthetic wild-type mMDH transit peptide. Quantification of inhibition of import was by densitometry of the fluorographs. Note that pre-ornithine transcarbamylase import is less efficient than pre-mMDH, such that the abscissa scales are different.

The ability of synthetic peptides to inhibit the import process corresponds precisely with our previous findings with mutation of the mMDH transit peptide. That is, the wild-type mMDH precursor protein is readily incorporated into mitochondria, and the wild-type transit peptide, either as a 28- or 20-mer, is a strong competitive inhibitor of uptake. On the other hand, synthetic peptides containing mutations of leucine-13 which cause defective binding of the precursor mutant mMDHs to mitochondria are inactive in blocking uptake of pre-mMDH or pre-ornithine transcarbamylase. These results support the concept that inhibition of protein import into mitochondria by synthetic peptides reflects the usual mechanism by which transit peptides cause binding to the mitochondrial surface.

Two Regions of the mMDH Transit Peptide Are Important for Proteolytic Processing—Following the binding and translocation steps in mitochondrial protein import, transit peptides are removed by a proteolytic processing event. Recently, we have shown that the mMDH transit peptide is removed in two steps via formation of an intermediate (i-mMDH) resulting from cleavage following arginine-15 (Sztul et al., 1988, Kalousek et al., 1988). Mature mMDH is formed by processing after asparagine-24 (see Table I for sequences). To examine the role of transit peptide sequences in the processing reac-

| Test peptide | Concentration peptide added (μM) | % precursor |
|--------------|---------------------------------|-------------|
| TP-28        | 0                               | 100         |
| TP-20        | 0                               | 100         |
| TP-20N13     | 100                             | 100         |
| TP-20P13     | 42                              | 42          |
| TP-20E13     | 33                              | 33          |

Substitution for Leucine-13 Abolishes the Ability of Synthetic Peptides to Inhibit Import into Mitochondria—Our previous mutagenesis experiments had indicated that substitution with proline, asparagine, or glutamic acid for leucine-13 of the mMDH transit peptide markedly reduced binding of pre-mMDH to the mitochondrial surface (Chu et al., 1987b). To determine the function of this residue to the synthetic peptides, we constructed peptides with substitutions for leucine-13 and tested these in the import inhibition assay. As was demonstrated for the intact precursor, substitution with asparagine eliminated the ability of the synthetic peptide to block import, although the 20-mer peptide containing the wild-type sequence (Fig. 6) was highly effective. Similarly, substitutions with glutamic acid or proline for leucine-13 also relieve inhibition of mMDH import (Table II).
These results suggest that mammalian mitochondria, in at least one of their potential conformations, are capable of excluding proteins destined for chloroplasts, but not to mitochondria. Our results are consistent with those described by others (see Keegstra, 1989, for review) which demonstrate both in vitro and in vivo that chloroplast transit peptides direct precursors to chloroplasts, but not to mitochondria, and plant mitochondrial transit peptides direct precursors exclusively to mitochondria. This conclusion differs from that of Hurt and co-workers (Hurt et al., 1986) who demonstrated that the plant chloroplast transit peptide of the ribulose-1,5-bisphosphate carboxylase fused to mature dihydrofolate reductase could direct this fusion protein into yeast mitochondria, although less efficiently than with an authentic yeast mitochondrial transit peptide, and suggested that mitochondrial and chloroplast presequences perform their functions through a similar mechanism. In our results, the synthetic transit peptide from the tobacco mitochondrial F$_{1}$-ATPase was much less effective than the mMDH-derived synthetic transit peptides. This relative inactivity of a plant mitochondrial transit peptide in the mammalian uptake system suggests that there are differences among mitochondrial import systems in various phyla. Taken together, our data and those of others suggest that precursor protein uptake into mitochondria and chloroplasts shares some general features, but that the kinetics and extent of uptake are sufficiently different between organelles and among organisms, that organelar specificity for uptake exists.

The similar behavior of both the mMDH and ornithine transcarbamylase precursors in our experiments with mammalian mitochondria suggests that their import mechanisms are shared. The transit peptides for these proteins contain regions of sequence homology, especially the Ala-Ala-Leu-Pro-Arg/Lys peptide which we have shown is critical for binding to mitochondria (Chu et al., 1987, a and b), although divergence is present in other regions (Table I). These experiments indicate that the targeting information of transit peptides is unlikely to be dependent on a single common property, such as net charge or secondary structure, but instead may be a result of multiple functional features which can be optimized for interaction with the import machinery from a particular organelle or source of mitochondria.

Other workers have studied the effect of chemically synthesized peptides on mitochondrial protein import (Gillespie et al., 1985; Roise et al., 1986, 1988; Cote et al., 1988). Some have suggested that transit peptides destabilize membranes because they form amphipathic $\alpha$-helices and subsequently insert into membranes. Although our data cannot completely exclude this interaction because we have not, as yet, assessed uptake of mutant precursors in vivo after transfection into cells, we believe that this physical property alone is unlikely to explain our results. First, only peptides corresponding to the native mMDH sequence are effective as inhibitors of pre-mMDH and pre-ornithine transcarbamylase import into mitochondria, despite the similarities in overall composition of all peptides tested. Second, we have demonstrated that the order of addition of precursor and peptide affects the ability of the latter to inhibit import (Fig. 4). This finding suggests that the peptides act by blocking access to one or more components of the import apparatus (Gillespie, 1987), rather than by disrupting the membrane. Third, the wild-type peptides do, in fact, inhibit binding to pre-mMDH to the mitochondrial surface, but certain mutations of leucine-13 in the peptide sequence allow removal of import inhibition. We have previously reported that these mutations at position 13 abolish binding of the intact precursor to mitochondria (Chu et al., 1987b). The absolute and complete correlation of the effects of the wild type and mutant mMDH precursors and the corresponding synthetic peptides in these two different types of mitochondrial import assays strongly supports our contention that leucine-13 is essential for conferring upon the transit peptide a structure required for binding to the postulated mitochondrial receptor. Fourth, we have previously reported (Chu et al., 1987b) that mutations which scramble the Ala-
The Leu-Arg peptide sequence at positions 12–14 of the mMDH transit peptide do not affect uptake into mitochondria in vitro. These mutations would, of necessity, alter the amphipathic α-helical structure which has been predicted as essential for transit peptide function (Roise et al., 1986). Our results, therefore, seem incompatible with the amphipathic, α-helical model. Fifth, the wild type peptides TP-28 and TP-20 also inhibit processing of mitochondrial precursor proteins by a mitochondrial matrix fraction, indicating that interactions with membranes alone may not account for their activities. Therefore, we propose that at least one of the many potential conformations of the synthetic peptides, like the transit peptides from which their sequences are derived, functions as a result of specific interactions with the protein molecules which form the import machinery.

These results presented here, in agreement with our previous findings, suggest that distinct structural features of transit peptides mediate binding, translocation, and processing of mitochondrial precursor proteins. Mutagenesis of the mMDH transit peptide in intact precursors or in synthetic peptides has demonstrated a critical role for leucine-13 in binding of pre-mMDH to the mitochondrial surface, perhaps because it influences the secondary or tertiary structure of the protein in this region. The importance of this residue can be distinguished from that of positive charge which is important for membrane translocation (Chu et al., 1987a), but does not affect binding (Chu et al., 1987b). Other workers have found that substitution for leucine residues in the ornithine transcarbamylase transit peptide also diminishes import, possibly because such mutations interfere with binding to mitochondria or components of the import apparatus (Skerjanc et al., 1988).

However, leucine-13 does not affect processing. Mutants of TP-20 containing leucine-13 substitutions were as effective as native peptide in blockage of i-mMDH to mature mMDH conversion. In contrast, they differed markedly with respect to inhibition of pre-mMDH import into intact mitochondia, clearly indicating that different regions or secondary structures of the transit peptide are required for binding, as compared to processing. It is likely that regions other than the cleavage sites themselves with the transit peptide are needed to define the proper sites for cleavage. As might be predicted, TP-28 inhibits conversion of pre-mMDH to both i-mMDH and mature mMDH. However, TP-20 and its mutants inhibit conversion of i-mMDH to mature mMDH, but not of pre-mMDH to i-mMDH. Thus, this inhibition cannot be explained by a simple competitive mechanism in which an excess of processing sites is supplied by the peptides because TP-20, which lacks the cleavage site required for conversion of i-mMDH to mature mMDH, nonetheless, inhibits the second processing step. These results contrast with those obtained by others (Cote et al., 1988) in which a 19-mer synthetic peptide of the ornithine transcarbamylase transit peptide did not inhibit processing of pre-ornithine transcarbamylase by a matrix fraction. Comparison of the pattern of inhibition of our mMDH synthetic transit peptides upon processing of pre-ornithine transcarbamylase will be necessary to elucidate the reasons for these apparently different results. The surprising inhibition of the second processing step by TP-20 suggests that some structure or sequence within this peptide interacts with the second processing protease. Similar interactions may also occur between the ornithine transcarbamylase transit peptide and the second processing protease as suggested by experiments in which substitutions for Asn-24 or Arg-26 of ornithine transcarbamylase inhibit the second cleavage at Gln-32 (Horwich et al., 1987). The lack of inhibition by TP-20 of the first processing step of mMDH suggests that the substrate-protease interaction requires amino acids and/or structure in the last 8 residues of the mMDH transit peptide. Perhaps more importantly, this lack of inhibition of the first protease, with effective inhibition of the second, strongly supports the concept (Sztul et al., 1988; Kalousek et al., 1988) that two distinct enzymes exist.

In conclusion, we have employed synthetic peptides to examine the interactions between transit peptides and the import apparatus of mitochondria. These synthetic peptides retain several of the functional characteristics of native transit peptides, including those which mediate binding to the mitochondrial surface and proteolytic processing of precursor molecules. The results presented here suggest that these functions are conferred by distinct amino acid sequences within transit peptides which can be tailored to direct import to specific organelles. Recognition of the various functions of transit peptides mediated by distinct structural domains within them will allow a better understanding of the complexities of the mitochondrial import apparatus.

Acknowledgments.—We thank Dr. Gregory Grant and Mark Frazier from the Washington University Protein Chemistry Facility for their expert services in peptide synthesis and Dr. Gay della Ciopa of Monsanto Corp. for peptides derived from chloroplast sequences. Michael Montgomery purified iMDH expressed in Escherichia coli. We also thank Drs. Franta Kalousek, Joe Hendrick, and Leon Rosenberg of the Dept. of Human Genetics, Yale University School of Medicine, who provided the pre-ornithine transcarbamylase-containing plasmid and the crude mitochondrial matrix fraction used in some of the experiments. Dr. Steve Adams and Harold Sims stimulated helpful discussions.

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