The Role of Arginine Residues in the Rat Mitochondrial Malate Dehydrogenase Transit Peptide*

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Arginine residues in the transit peptides of mitochondrial precursors are proposed to be important for uptake into mitochondria. To study this further, we have used cassette mutagenesis to create site-specific amino acid replacements within the transit peptide of rat mitochondrial malate dehydrogenase. Plasmids containing mutant sequences were expressed in vitro and tested in a mitochondrial uptake system utilizing isolated rat liver mitochondria. Substitution for arginine at position 14 with asparagine, glutamine, or alanine decreased the relative import level by 20–30% compared to the wild-type sequence when assayed in 1-h uptake experiments. Although lysine substitution did not alter import, substitution with glutamic acid decreased import by 40%. Alanine substitution for arginines at both positions 14 and 15 also dramatically decreased import. Uptake was partially restored in this mutant when positive charge was inserted at a new location within the transit peptide. Time course experiments showed that the initial rates of import were decreased in these mutants, as were the relative amounts of incorporated protein. These results were best explained by the loss of positive charge following amino acid substitutions for the arginine residues and suggest that the role of the charge is to enhance the efficiency of membrane translocation.

Most mitochondrial proteins are made as larger precursors in the cytoplasm and are post-translationally incorporated into mitochondria. The increased sizes of precursor molecules are due to NH₂-terminal extensions, or transit peptides, which are necessary to direct proteins into mitochondria (for reviews see Zimmerman, 1986; Colman and Robinson, 1986). Identification of structural features which enable transit peptides to target proteins into mitochondria is the focus of many current investigations. Transit peptides share some qualitative similarities, one being the abundance of basic amino acids, especially arginine, and the nearly complete absence of acidic amino acid residues. Site-specific mutagenesis of the human ornithine transcarbamylase transit peptide has shown that the arginine residues in this peptide are important for transport into the mitochondria as well as for proteolytic processing to the mature form (Horwich et al., 1985). One reason for this could be the contribution of positive charge. However, Arg⁹ of this peptide was found to be especially crucial and could be replaced by uncharged amino acids which preserved α-helical potential (Horwich et al., 1986). Other investigators have also suggested that transit peptides target proteins to mitochondria by virtue of amphiphilic, α-helical properties, and that a sequence which can assume a positively charged helix structure may be capable of functioning as a targeting signal (Roise et al., 1986; von Heijne, 1986). Whether charged residues must participate in a particular secondary structure remains an open question.

We have shown that several of the known rat transit peptides, including that of rat mitochondrial malate dehydrogenase, exhibit conservation of some amino acids (Grant et al., 1986). This region includes the pentapeptide Ala-Ala-Leu-Arg-Arg, which accounts for 2 of the 3 positively charged residues of the mitochondrial malate dehydrogenase transit peptide. This was, therefore, our choice for studying the roles of such residues. Experiments were designed to further define the role of positive charge in transit peptide function. To do this, a series of mutations in the mitochondrial malate dehydrogenase transit peptide was created. This paper reports the effects of amino acid substitutions for the arginine residues at positions 14 and 15 of mitochondrial malate dehydrogenase. Although all of these mutants were competent for uptake, marked differences in the extent and rate of import suggest that the net positive charge within transit peptides affects the initial rate of transport of precursor molecules into mitochondria.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized on an Applied Biosystems Model 370 A/B DNA synthesizer at the Protein Chemistry Facility at Washington University School of Medicine. Klenow fragment of DNA polymerase I was a gift from John Majors (Washington University School of Medicine). Other DNAs, chemicals, and enzymes were obtained from commercial suppliers.

Oligonucleotide-directed Mutagenesis—A series of restriction sites was constructed in the cDNA clone encoding mitochondrial malate dehydrogenase (Fig. 1). These were NdeI, HindIII, and BglII, which were chosen because they were unique to the cDNA sequence and because they did not result in any changes in amino acid sequence. The sites were inserted using oligonucleotide-directed mutagenesis. Fifteen pmol of a mutagenic oligonucleotide was annealed to 1 pmol of a single-stranded DNA template (M13mp18) containing the wild-type mitochondrial malate dehydrogenase sequence. Second-strand synthesis was performed using Klenow fragment and deoxyribonucleotides (Zoller and Smith, 1983), and was closed with T₄ DNA ligase. This material was used to transform Escherichia coli JM105 using a simple transformation protocol described by Hanahan (1985). Replica filter lifts were probed with the mutagenic oligonucleotide which had been labeled with ³²P using T₄ polynucleotide kinase. Positive plaques were picked and used to prepare single-stranded DNA templates (Barnes et al., 1983) for dyeoxy sequencing (Sanger et al., 1977). Replicative form double-stranded DNA was prepared

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polymerase promoter; inserted into a mitochondrial malate dehydrogenase (mMDH) cDNA. The EcoRI fragment containing the modified mitochondrial malate dehydrogenase was re-cloned into an altered pGEM 2 vector from which had been removed the HindIII site and three NarI sites. Malate dehydrogenase was re-cloned into an altered pGEM 2 vector from which had been removed the HindIII site and three NarI sites. Sp6 RNA polymerase promoter; T7, T7 RNA polymerase promoter; Amp', ampicillin resistance gene. Details of enzymatic steps are described in the text. B, immunoprecipitation of mitochondrial malate dehydrogenase. Plasmid pGMDH was linearized with DraI and transcribed in the text. B, immunoprecipitation of mitochondrial malate dehydrogenase. Isolated mitochondria were incubated with mitochondri malate dehydrogenase immune serum separated on an SDS-10% acrylamide gel. In lane 2, the total translation products were loaded onto the gel. The gel was fixed, dried, and autoradiographed for 16 h. The slight difference in migration of the major bands in lanes 1 and 2 may be due to the presence of large amounts of immunoglobulin in lane 1.

Plasmid pGMDH was linearized with DraI and transcribed with SP6 RNA polymerase. The RNA was translated in a reticulocyte lysate system. Lane 1 represents products immunoprecipitated with mitochondrial malate dehydrogenase immune serum separated on an SDS-10% acrylamide gel. In lane 2, the total translation products and the major product (lane 2, Fig. 1B) had an apparent product mass of 35,000. Following translation, mitochondrial malate dehydrogenase assay was performed as described by Folz and Gordon (1986) except that the RNA was recovered by ethanol precipitation. Where indicated, mitochondrial malate dehydrogenase was immunoprecipitated from the primary translation mixture (Grant et al., 1986) and analyzed by SDS-polyacrylamide gels. Labeled proteins were detected by autoradiography.

Removal of Restriction Sites from pGEM—The insertion of new restriction sites from pGEM—Excision of restriction sites from pGEM—Restriction sites were removed by cutting with the restriction enzyme, filing 5' "sticky-ends" with Klenow, and religating with T4 DNA ligase. Modified DNAs were used to transform competent E. coli strain DH-1. Bacterial colonies surviving the expected size for the mitochondrial malate dehydrogenase. Plasmid pGMDH was linearized with DraI and transcribed with SP6 RNA polymerase. The RNA was translated in a reticulocyte lysate system. Lane 1 represents products immunoprecipitated with mitochondrial malate dehydrogenase immune serum separated on an SDS-10% acrylamide gel. In lane 2, the total translation products were loaded onto the gel. The gel was fixed, dried, and autoradiographed for 16 h. The slight difference in migration of the major bands in lanes 1 and 2 may be due to the presence of large amounts of immunoglobulin in lane 1. The EcoRI fragment containing the modified mitochondrial malate dehydrogenase was re-cloned into an altered pGEM 2 vector from which had been removed the HindIII site and three NarI sites. Sp6 RNA polymerase promoter; T7, T7 RNA polymerase promoter; Amp', ampicillin resistance gene. Details of enzymatic steps are described in the text. B, immunoprecipitation of mitochondrial malate dehydrogenase. Plasmid pGMDH was linearized with DraI and transcribed in the text. B, immunoprecipitation of mitochondrial malate dehydrogenase. Isolated mitochondria were incubated with mitochondri malate dehydrogenase immune serum separated on an SDS-10% acrylamide gel. In lane 2, the total translation products were loaded onto the gel. The gel was fixed, dried, and autoradiographed for 16 h. The slight difference in migration of the major bands in lanes 1 and 2 may be due to the presence of large amounts of immunoglobulin in lane 1.

(Appliatis et al., 1982) and used in later cloning steps.

FIG. 1. A, construction of pGMDH. Restriction enzyme sites were inserted into a mitochondrial malate dehydrogenase (mMDH) cDNA (open arrow) by oligonucleotide mutagenesis using single-stranded DNA. The EcoRI fragment containing the modified mitochondrial malate dehydrogenase was re-cloned into an altered pGEM 2 vector from which had been removed the HindIII site and three NarI sites. RI, EcoRI; H, HindIII; Bn, BanI; Bg, BglII; Nd, NdeI; Sp6, SP6 RNA polymerase promoter; T7, T7 RNA polymerase promoter; Amp', ampicillin resistance gene. Details of enzymatic steps are described in the text. B, immunoprecipitation of mitochondrial malate dehydrogenase. Plasmid pGMDH was linearized with DraI and transcribed with SP6 RNA polymerase. The RNA was translated in a reticulocyte lysate system. Lane 1 represents products immunoprecipitated with mitochondrial malate dehydrogenase immune serum separated on an SDS-10% acrylamide gel. In lane 2, the total translation products were loaded onto the gel. The gel was fixed, dried, and autoradiographed for 16 h. The slight difference in migration of the major bands in lanes 1 and 2 may be due to the presence of large amounts of immunoglobulin in lane 1.

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Plasmid pGMDII was linearized with DraI and used as a template for SP6 RNA polymerase as described by Folz and Gordon (1986) except that the RNA was recovered by ethanol precipitation rather than Sephadex G-50 chromatography. Typically, 5-10 μg of RNA could be obtained from 1 μg of linearized plasmid. Four hundred to 800 ng of newly transcribed RNA were used to program a reticulocyte lysate in vitro translation system (Promega Biotec) containing 35S methionine. The major product (lane 2, Fig. 1B) had an apparent mass of 35,000, the expected size for the mitochondrial malate dehydrogenase precursor. Following translation, mitochondrial malate dehydrogenase antisera specifically precipitated the M, = 35,000 product (lane 1), indicating that this band was pre-mitochondrial malate dehydrogenase. Aliquots were mixed with mitochondria (see below) and recentrifuged at 10,000 × g. The final pellet was resuspended in homogenization buffer at a protein concentration of 10 mg/ml.

In Vivo Mitochondrial Import Assay—A 10-μl aliquot of a translation reaction was mixed with an equal volume of the mitochondrial suspension. Uptake was allowed to occur at 30 °C for 1 h, after which the mitochondria were pelleted by centrifugation and the supernatant and pellet were mixed with SDS gel buffer (0.125 M Tris, pH 6.8, 2% SDS, 10% glycerol, 0.7 M β-mercaptoethanol) and analyzed by SDS-PAGE. The pellet (Fig. 2, lane 3) contained two major labeled products, the larger of which co-migrated with the primary translation.
product. The other product migrated as an M, = 33,000 protein. In the supernatant, the major product was pre-mitochondrial malate dehydrogenase (the 35,000 product). The band at M, = 43,500 in the supernatant is encoded by an mRNA endogenous to the reticulocyte lysate because this material was present even if mitochondrial malate dehydrogenase RNA was omitted (data not shown). Alternatively, the incubation mixture was subjected to digestion with 0.1 mg/ml proteinase K at 4 °C for 15 min with or without prior treatment with 1% Triton and 1% deoxycholate. This treatment (lanes 4–7, Fig. 2) demonstrates that the smaller product (M, = 33,000) was internalized pre-mitochondrial malate dehydrogenase, whereas the larger product was pre-mitochondrial malate dehydrogenase residing outside the mitochondria. In these experiments, the smaller band in the mitochondrial pellet was resistant to degradation by proteinase K in the absence of detergent (lanes 4 and 5), but was digested after disruption of mitochondrial membranes by addition of Triton X-100 and sodium deoxycholate (lanes 6 and 7). Addition of 0.1 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP) to the mitochondria prior to uptake abolished formation of the processed product seen in the pellet (lanes 8 and 9). This compound dissipates the proton gradient across the inner mitochondrial membrane which is necessary for import of precursor proteins into mitochondria (Schleyer et al., 1982). This experiment provides further evidence that conversion to the mature form reflects mitochondrial import.

**Time Course of Uptake**—For time course assays, 135 µl of the translation reaction was mixed with an equal volume of the mitochondrial suspension. Forty-µl aliquots were removed at 2, 5, 10, 15, 20, and 50 min and 0.1 mM CCCP was added to arrest import. The aliquots were divided into two portions. The first portion (20 µl) was analyzed directly by SDS-PAGE and was used to quantitate change in percent of precursor and mature forms of mitochondrial malate dehydrogenase. Mitochondria were re-isolated from the second portion by centrifugation before and after digestion with proteinase K and were analyzed by SDS-PAGE. The material appearing in these fractions was used to determine the amount of mitochondrial malate dehydrogenase imported into mitochondria.

**Quantitation of Uptake**—Bands corresponding to precursor and mature forms of mitochondrial malate dehydrogenase were quantified from autoradiograms using a scanning laser densitometer. These signals fell within the linear range of film sensitivity (Demmer et al., 1986). Initially the areas of bands giving rise to major bands were also excised, dissolved by boiling in 30% H2O2, mixed with scintillation fluid, and counted in a liquid scintillation counter. These methods gave equivalent results, and thereafter laser densitometry was used to quantitate most uptake experiments.

**RESULTS**

**Construction and Expression of Mutants**—Having demonstrated the feasibility of in vitro expression and import of wild-type mitochondrial malate dehydrogenase, we proceeded to construct mutant forms of this protein. In order to effect changes in the amino acid sequence of the mitochondrial malate dehydrogenase transit peptide, the BanI to HindIII fragment was removed from the wild-type mitochondrial malate dehydrogenase cDNA and replaced with oligonucleotide cassettes designed to replace the arginine residue at position 14. These mutants were expressed in vitro in the same manner as ArgI4 as AlaI4, ArgI4 as LysI4, ArgI4 as AsnI4, ArgI4 as GluI4, ArgI4 as HisI4, ArgI4 as GluI4, ArgI4 as ArgI5 as AlaI4, and III-OTC. The mutant DNA was transfected into the mitochondria and uptake was allowed to occur. Fig. 3 shows examples of mitochondrial malate dehydrogenase precursor uptake experiments after 1 h of incubation. The entire reaction was loaded onto an SDS-PAGE lane in order to minimize losses during re-isolation of mitochondria. We found that the absolute amount of labeled mitochondrial malate dehydrogenase precursor which was converted to mature form varied with the particular preparation of mitochondria (Fig. 3, compare wild-type in A, B, and C). Nonetheless, there was a decrease in the ability of some mutants to import relative to the wild-type which was reproducible. To normalize the results between experiments, we expressed the level of import of mutants relative to the wild-type import in the same experiment as:

\[
\text{Import level} = \frac{\text{amount of mutant mature}}{\text{amount of mutant precursor and mature}}
\]

\[
\text{(amount of wild-type mature)/(amount of wild-type precursor and mature)}
\]

The results are compiled in Table I from a series of uptake experiments. All mutants were competent for uptake, but varied in the extent of import. The relative order was dependent on the charge of the side chain of the substituted amino acid, as ArgI4 as LysI4 was imported as well as the wild-type (1.0), whereas ArgI4 as GluI4 showed the lowest level of import (0.6). The neutral amino acid substitutions gave intermediate levels of import (0.7–0.8).

**Construction and Expression of Mutant ArgI4–ArgI5**

\[
\text{AlaI4–AlaI5} \rightarrow \text{AlaI4–AlaI5}
\]

A cassette of complementary oligonucleotides was designed to replace the BanI-HindIII fragment and change the 2 arginine residues in this segment to alanines (ArgI4–ArgI5 → AlaI4–AlaI5). The mutant DNA was transcribed in vitro, the RNA translated as for the wild-type protein, and the product tested in mitochondrial uptake experiments (Fig. 3, A and C). The average level of import of this mutant relative to the wild-type protein in a 1-h uptake experiment was 0.4 (Table I). This mutant demonstrated more severe impairment of import than the single-site mutations,
reflecting a greater reduction in net positive charge for this mutant.

Replacement with Ornithine Transcarbamylase Sequences—
To test the hypothesis that reduction of positive charge was responsible for decreased import of Arg^{14}-Arg^{15} → Ala^{14}-Ala^{15}, a new arginine residue was introduced into the mutant at a different site. The HindIII to BglII fragment was removed from the cDNA encoding the Arg^{14}-Arg^{15} → Ala^{14}-Ala^{15} mutant and was replaced with an oligonucleotide cassette which encoded amino acids 18–23 of the rat ornithine transcarbamylase transit peptide. Comparison of the predicted amino acid sequence of the mutant transit peptide, designated III-OTC, and the wild-type is as follows:

Wild-type MLASALARPVGAALRSFSSTSAQNN

III-OTC MLASALARPVGAALASFHSTSMYRN

Arginine at position 23 was necessary for import of the human ornithine transcarbamylase (Horwich et al., 1986). Partial import activity was regained by III-OTC compared to Arg^{14}-Arg^{15} → Ala^{14}-Ala^{15} (Fig. 3, Table I), suggesting that this sequence can restore uptake to the mitochondrial malate dehydrogenase precursor. A small amount of an intermediate-sized protein (M, = 33,500) was present in the primary translation product which may be due to an alternative start site at Met^{31}.

Time Course of Uptake—The different levels of uptake exhibited by these mutants could reflect different rates of import or different levels of total import occurring with the same initial rate. To examine the import of both wild-type and mutant proteins over time, uptake reactions were performed for 2, 5, 10, 15, and 50 min. Import was arrested by addition of 0.1 mM CCCP. The reactions were analyzed as before by SDS-PAGE of the total uptake reaction and separation of the mitochondrial pellet and the mitochondrial pellet after proteinase K digestion (Fig. 4). The results were expressed as percent precursor remaining and normalized amounts of mature form appearing as a function of time (Fig. 5). More than half of the wild-type protein was imported by 5 min, and there was little change in the amount of precursor material after 20 min. In this assay, 80% of the wild-type precursor was converted to mature size by 50 min.

This represented imported protein as shown by quantitative recovery of the mature-sized, protease-protected material in the mitochondrial pellet (Fig. 4). The intermediate band at 34,000 appeared at 2 min, and was present over the time course of the experiment. This intermediate product was most abundant in the total uptake reaction, with considerably less in the mitochondrial pellet and the proteinase K-protected material. The precursor form was associated with the mitochondrial pellet at early time points, but was accessible to protease. The precursor diminished over time as the mature form increased in intensity, suggesting that this externally bound precursor material was subsequently incorporated into mitochondria.

Import of Arg^{14}-Arg^{15} → Ala^{14}-Ala^{15} occurred more slowly than that of the wild-type (Fig. 5, A and B). After 50 min, only 50% of the mutant had been imported and processed. Moreover, a dramatic difference in rate of uptake compared to wild-type occurred over the first 5 min. During this time, <20% of the mutant precursor was imported, whereas over 50% of the wild-type was taken up. In contrast to the wild-type, the intermediate-sized product was not observed for the Arg^{14}-Arg^{15} → Ala^{14}-Ala^{15} mutant.

The rate of uptake for the III-OTC mutant was intermediate between those of the wild-type and the Arg^{14}-Arg^{15} → Ala^{14}-Ala^{15} mutant (Fig. 5B). At 15 min, 50% of the precursor had been processed and imported, whereas 66% was imported after 50 min. Again, the greatest difference in uptake rate occurred over the first 5 min, during which there was 30% import of III-OTC. A significant amount of an intermediate III-OTC form was observed in the primary translation product, and this was subtracted from the remaining time points to calculate the appearance of the mature form. In contrast to the wild-type, an intermediate form of III-OTC was also observed in association with the mitochondrial pellet and was protected from protease. Two possibilities to explain this are that processing of III-OTC may involve two proteolytic steps (Rosenberg et al., 1987), both of which occur within mitochondria, or alternatively, the intermediate form observed in the primary translation reaction may gain access to the mitochondria.

The Arg^{14} → Gln^{14} mutant was imported at an initial rate

![Fig. 4. Time course of uptake. Aliquots from import experiments were removed at 2, 5, 10, 15, 20, and 50 min. Half of the sample was analyzed directly on SDS-PAGE (Total Uptake). The remaining portion was divided and mitochondria were re-isolated directly from one fraction (Mito Pellet) and the other was treated with 0.1 mg/ml proteinase K before the mitochondria were re-isolated (+ Proteinase K). The amounts of mature-sized material in the protease-treated and untreated fractions were equivalent and accounted for the mature material seen in the total import reaction when corrected for sample size.](image-url)
DISCUSSION

To investigate the role of specific sequences in the function of mammalian transit peptides, we have created a series of mutations in a rat heart mitochondrial malate dehydrogenase cDNA. The first step in these studies involved expression of the wild-type cDNA in an in vitro system. The primary translation product had an apparent Mr of 35,000, which was recognized by anti-mitochondrial malate dehydrogenase antisera, and was imported and processed by isolated rat liver mitochondria to an Mr of 33,000 species. In a typical experiment, 70% of the incubated wild-type protein was imported into isolated mitochondria and processed to the mature form.

Arg14 was chosen as a target for mutagenesis because of its presence in the pentapeptide Ala-Ala-Leu-Arg-Arg, which is conserved in other rat transit peptides (Grant et al., 1986), and because other reports have suggested the importance for arginines in other transit peptides (Horwich et al., 1985; Horwich et al., 1986). In the experiments described here, a number of amino acids were substituted at this position. The mutations were chosen as part of a systematic approach to try to understand the effect of positive charge in the transit peptide on the uptake of mitochondrial precursor proteins. The results were quantified to allow comparisons between mutants of both the rate of import and the extent of uptake.

There was a marked difference in the relative extent of import among the mutants. The order of amino acids which resulted in the best import was Arg = Lys > Ala ≥ Asn = His = Gln > Glu. This result was striking in that substitution for a single amino acid residue could influence import in a manner which depended upon the charge of that residue.

When arginine residues at both positions 14 and 15 were replaced by alanine, the resulting protein had an average import level of only 0.4 relative to the wild-type, which was worse than any of the single substitution mutants. The import of the double mutant was partially restored when amino acids 18–23 from the rat ornithine transcarbamylase were inserted in place of amino acids 18–22 in Arg14-Arg15 → Ala14-Ala15, suggesting that functional domains of transit peptides are interchangeable to some degree. The alteration resulted in addition of 2 charged residues, histidine at position 18 and arginine at position 23. In this case, restoration of import could be due to increased positive charge, or could also depend on a proper, although not unique, location.

Arginine is remarkably abundant in transit peptides, and one possibility is that arginine is specifically required for import to occur. However, it is apparent that some import occurred in all substitution mutants during a 1-h uptake experiment. Also, the fact that lysine can be substituted for Arg14 in mitochondrial malate dehydrogenase with no apparent loss of function argues against an essential role for these residues per se. These findings suggest that positive charge, rather than the identity or location of the amino acid side chain bearing the charge, is the important feature contributed to transit peptides by arginine.

Our results from the fixed time point assays had shown moderate to severe impairment of import in some of the mutants. To study this further, we performed time course experiments with several mutants. The mutants were chosen to focus on the effects of charge differences in the transit peptide region. For example, Arg14 → Glu14 and Arg14 → Gln14 differ by amidation of the carboxylic acid, and III-OTC and Arg14 → Ala14-Ala15 differ by addition of arginine and histidine in the inserted ornithine transcarbamylase sequences. The results confirm the relative import levels seen in the 1-h experiments. They also indicate that much of the difference in the 1-h import level can be attributed to changes in the initial rate of import. Greater positive charge on the transit peptide accentuates the initial import rate (Fig. 5, B and C).

Two possibilities to explain the effects of reduced charge are (i) that binding of the transit peptide to a putative receptor is weakened, or (ii) that the initial translocation of the transit peptide into the matrix is slowed. Although the experiments described here cannot distinguish between these, we propose that the second explanation is more likely. The results of Schleyer and Neupert (1985) in Neurospora suggest that the first domain of a mitochondrial precursor protein which enters
the mitochondrion is the transit peptide and that this reaction is dependent on the membrane potential. Removal of positive charge may slow this process, resulting in a decreased initial rate of import seen in our time course assays. It may be that other residues in transit peptides serve other functions (Allison and Schatz, 1986), such as facilitating binding to mitochondria. Our results, in which eliminating 2 of 3 positively charged residues in the pre-mitochondrial malate dehydrogenase transit peptide did not abolish import, emphasize the need to define the roles of the uncharged amino acids. Our current studies suggest that other residues in the conserved pentapeptide Ala-Ala-Leu-Arg-Arg are crucial for the import of mitochondrial malate dehydrogenase.

It is unclear whether the intermediate band observed in the mitochondrial uptake experiments represents a true intermediate in the processing of premitochondrial malate dehydrogenase. Several examples of two-step processing have been described for some intermembranous space proteins (Gasser et al., 1982; Ohashi et al., 1982), and an intriguing possibility is that this is also true for matrix proteins. Time course experiments for the wild-type protein showed that an intermediate-sized species appears early in the uptake reaction, and plateaus over the 50-min time course. Although such behavior is consistent with a processing intermediate, this species was much less apparent in the re-isolated mitochondrial fraction and the protease-protected material. An intermediate form was not detected for Arg\textsuperscript{14}-Arg\textsuperscript{15} → Ala\textsuperscript{14}-Ala\textsuperscript{15} or Arg\textsuperscript{14} → Glu\textsuperscript{14}, suggesting that NH\textsubscript{2}-terminal processing may be responsible for the intermediate, but also that its formation may not be obligatory for import.

In summary, our results demonstrate that the redundancy of arginines in the mitochondrial malate dehydrogenase transit peptide is important because of positive charge. We have quantified import of transit peptide mutants to show that the charge of the transit peptide influences the uptake process by affecting the initial rate of import. We suggest that the positive charge in transit peptides aids translocation of this domain across the mitochondrial membranes.

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