

# The Ornithine Transcarbamylase Leader Peptide Directs Mitochondrial Import through Both Its Midportion Structure and Net Positive Charge

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**Abstract.** The cytoplasmically synthesized precursor of the mitochondrial matrix enzyme, ornithine transcarbamylase (OTC), is targeted to mitochondria by its NH<sub>2</sub>-terminal leader peptide. We previously established through mutational analysis that the midportion of the OTC leader peptide is functionally required. In this article, we report that study of additional OTC precursors, altered in either a site-directed or random manner, reveals that (a) the midportion, but not the

NH<sub>2</sub>-terminal half, is sufficient by itself to direct import, (b) the functional structure in the midportion is unlikely to be an amphiphilic  $\alpha$ -helix, (c) the four arginines in the leader peptide contribute collectively to import function by conferring net positive charge, and (d) surprisingly, proteolytic processing of the leader peptide does not require the presence of a specific primary structure at the site of cleavage, in order to produce the mature OTC subunit.

**M**OST mitochondrial proteins are encoded in the nucleus and translated in the cytosol as larger precursors containing NH<sub>2</sub>-terminal cleavable portions called leader peptides. Leader peptides comprise 20–60 amino acids and are both necessary and sufficient to direct posttranslational import of precursors by mitochondria (for reviews, see Colman and Robinson, 1986; Douglas et al., 1986). They must somehow be specifically recognized by the organelles, perhaps by receptors in the outer mitochondrial membrane (Hennig et al., 1983). Subsequently, they are translocated across the mitochondrial membranes, apparently at points of contact between inner and outer membranes (Schleyer and Neupert, 1985), by a mechanism requiring an intact electrochemical potential gradient across the inner membrane. Mature sequences follow their respective leader peptides into mitochondria in a manner that is not dependent upon the electrochemical gradient (Schleyer and Neupert, 1985), but that requires assumption of conformations different from those of the mature intramitochondrial forms (Eilers and Schatz, 1986). After entry into the mitochondria, the leader peptides are cleaved by one or more mitochondrial matrix proteases to produce the mature proteins.

What features of leader peptides are required for directing these events of recognition, translocation, and proteolytic processing? Comparison of those leader peptides analyzed to date reveals limited sharing of primary structure by a few peptides, but, overall, no amino acid sequence common to the peptides. A compositional feature is shared by virtually all of the peptides, however. They are strikingly basic in overall amino acid composition (Douglas et al., 1986). That is, they are essentially devoid of acidic residues while con-

taining average to above-average content of basic residues, mostly arginines. We recently explored the functional role of both various regions and basic residues in the leader peptide of the precursor of the human mitochondrial matrix enzyme ornithine transcarbamylase (OTC)<sup>1</sup> (Horwich et al., 1986). We found by deletion analysis that the midportion of the 32-residue OTC leader peptide is absolutely required to direct import of the precursor, whereas neither NH<sub>2</sub>-terminal nor penultimate COOH-terminal portions are essential. When single substitutions of glycine were made for each of the four arginines in the leader, import was completely blocked only with substitution for arg 23. Other substitutions at that position suggested that arg 23 may be part of a secondary structure constituting a required functional element in the midportion of the peptide.

Although one functional element of the OTC leader has thus been identified, a substantial number of questions concerning both this element and additional ones remain. For example, although the midportion is required for directing import, is it alone sufficient? Does the midportion function as an amphiphilic  $\alpha$ -helix? What is the role of the other three arginine residues? What regions or residues are required for proteolytic processing of the leader peptide by a mitochondrial matrix protease(s)? In this report, we have addressed these questions by carrying out both directed alteration and random, single-base substitution of the OTC leader coding sequence.

1. *Abbreviation used in this paper:* OTC, ornithine transcarbamylase.

## Materials and Methods

### Mutagenesis

For construction of deleted leader coding sequences, restriction endonucleases were used to excise appropriate portions from the previously described plasmid, pSPROTC (Horwich et al., 1986), and double-stranded synthetic DNA segments were then joined to construct circular plasmid derivatives containing the desired coding sequence (Fig. 1). For generation of point mutations, procedures of gap misrepair or of chemical treatment of single-stranded DNA were employed. Gap misrepair was carried out as described by Shortle et al. (1982) with several modifications. Steps of ligation and purification of gap reaction products were omitted because the predominant product of the nicking reaction was form II DNA, with no form I detected, and with only a small amount of form III present. After fill-in with three deoxyribonucleotide triphosphates and ligation, the products were digested with an excess of the restriction enzyme whose recognition site was predicted to be absent in the desired mutants. Despite this step, only 30% of the plasmids derived after transformation of HB101 were resistant to cleavage with the enzyme. The sequence of the leader region of 10 of the resistant mutants was analyzed (Maxam and Gilbert, 1977). Base changes reflecting misincorporation were detected in all cases, with changes occurring in the same direction from the original restriction site in every plasmid analyzed, probably reflecting a preferred strand for nicking. Nucleotide changes extended from a minimum of eight nucleotides from the predicted site of nicking to a maximum of 50 nucleotides. In two cases, a one-base-pair deletion was detected immediately 3' to the predicted site of the original nick. The leader coding region was excised from selected mutants and inserted into a nonmutagenized SPROTC backbone.

Chemical treatment of single-stranded DNA was carried out as described by Myers et al. (1985). A single-stranded circular molecule containing the leader coding sequence, L clamp (see Horwich et al., 1986), was treated in separate reactions with formic acid,  $\text{KMnO}_4$ , nitrous acid, and hydrazine. A 5'- $^{32}\text{P}$ -labeled primer was used in the subsequent extension reaction to enable monitoring by display of a portion of the products in a 6% acrylamide gel. Because of initial difficulty in achieving extension beyond 40–50 bases, gene 32 protein (Pharmacia Fine Chemicals, Piscataway, NJ), 8  $\mu\text{g}$  per reaction, was included both in a preincubation reaction, carried out with primer and template for 10 min at 37°C, and during the subsequent reverse transcription reaction. After this modification, extension was significantly lengthened, with at least 30% of the products reaching a length greater than 120 bases. The extension products were digested with restriction enzymes to release the double-stranded mutagenized leader coding segment and in all cases the 98 base pair fragment was readily detected in a 5% acrylamide gel by staining with ethidium-bromide. The fragments from the various chemical reactions were individually recovered by electroelution and used exactly as described by Myers et al. (1985). Ultimately, 24 individual plasmid molecules judged to be mutant by their mobility in Lerman gels were subjected to sequence analysis of the leader region. Seven molecules contained single-base changes that were silent, whereas 17 of the molecules contained single-base changes that directed an amino acid alteration. Seven of the amino acid changes were represented twice; one additional change had been previously produced by oligonucleotide-directed mutagenesis.

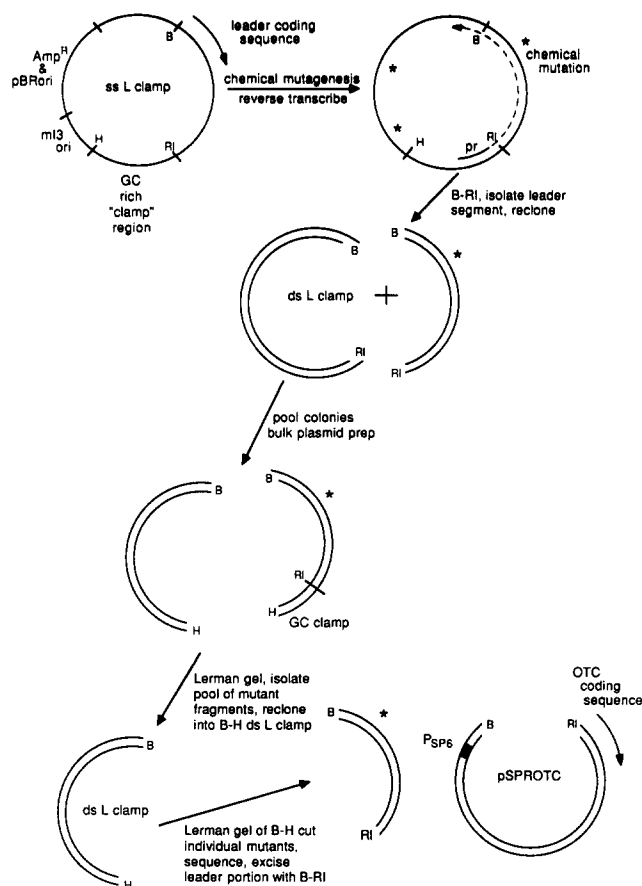
### In Vitro Protein Synthesis and Incubation

In vitro transcription and translation, and incubation of [ $^{35}\text{S}$ ]methionine-radiolabeled products with isolated mitochondria were carried out as previously described (Horwich et al., 1986). Fluorograms of the anti-OTC-precipitable incubation products were scanned with a Beckman DU-8 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA). Peaks corresponding to precipitable OTC species were integrated and summed. Extent of conversion of altered precursors was expressed in relation to wild type, assigning the latter as 100%, by dividing in each case the percentage of mature form recovered from the altered precursor by the percentage of mature form recovered from the wild-type precursor in the same experimental series.

## Results

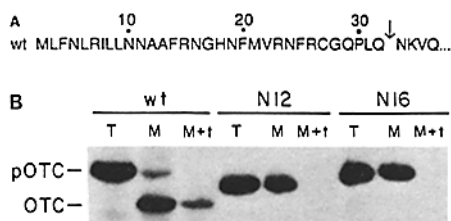
### Neither 12 nor 16 $\text{NH}_2$ -Terminal Residues of the OTC Leader Peptide Are Sufficient to Direct Import

Although deletion analysis had indicated previously that the



**Figure 1.** Strategy of chemical mutagenesis of OTC leader coding sequence and identification of mutants. The single-stranded DNA plasmid L-clamp carrying the leader coding strand between BamHI and EcoRI recognition sequences (see Horwich et al., 1986) was subjected to chemical mutagenesis as described in Materials and Methods. A 20-nucleotide primer was used to direct reverse transcription of the leader region, producing a double-stranded segment that could be excised with BamHI and EcoRI. The excised fragments were cloned into the BamHI-EcoRI-digested clamp plasmid backbone and the population of plasmids digested with Hind III and BamHI to release fragments containing both the clamp and leader coding region. The collection of fragments migrating differently in the Lerman gel system from wild type was recovered from the gel and cloned into Hind III-BamHI-digested clamp plasmid backbone, and single clones were then similarly studied using Lerman gels. Plasmids with Hind III-BamHI fragments migrating differently from wild type were subjected to sequence analysis of the leader region, and mutant segments of interest were transferred as BamHI-EcoRI segments to the BamHI-EcoRI-digested expression plasmid, pSPROTC. Circle with single line corresponds to single-stranded (ss) DNA; double line corresponds to double-stranded (ds) DNA; arrow parallel to circle indicates normal direction of transcription of coding region. Dashed arrow parallel to circle indicates direction of reverse transcription. Asterisk indicates site of chemical mutation. ( $P_{SP6}$ ) *Salmonella* phage promoter region.

midportion of the 32-residue OTC leader peptide is necessary for both import and proteolytic processing of the OTC precursor, it did not establish which regions of the peptide are sufficient by themselves to supply these functions. Therefore, additional altered OTC precursors have been programmed. The first two, called N12 and N16, contain only  $\text{NH}_2$ -terminal portions of the peptide (see Fig. 2 A for wild-



**Figure 2.** Incubation with mitochondria of OTC precursors comprising NH<sub>2</sub>-terminal leader portions joined with the mature subunit. (A) NH<sub>2</sub>-terminal amino acid sequence of wild-type human OTC precursor. Arrow designates normal site of leader peptide cleavage. (B) SP6-derived plasmids with the designated number of NH<sub>2</sub>-terminal leader codons were transcribed in vitro and the products used to direct reticulocyte lysate translation reactions containing [<sup>35</sup>S]methionine. After translation, an aliquot of each mixture was immunoprecipitated with anti-OTC antiserum. A second aliquot was incubated for 1 h at 27°C with isolated rat liver mitochondria, after which the reaction mixture was detergent-solubilized and immunoprecipitated. A third aliquot of the mixture was incubated with mitochondria, after which the entire mixture was incubated with trypsin (1:1,000, trypsin:total protein) for 15 min at 27°C. The mixture was then detergent-solubilized and immunoprecipitated. Products of immunoprecipitation were electrophoresed through an 8% SDS-polyacrylamide gel and the gel was fluorographed. (T) Translation products; (M) products after mitochondrial incubation; (M+t) products after trypsin treatment of the mitochondrial incubation mixture; (pOTC) preornithine transcarbamylase; (OTC) mature subunit.

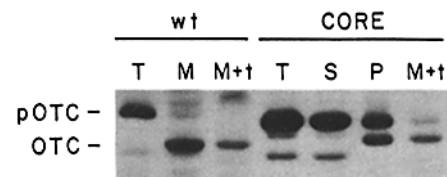
type sequence), either 12 residues or 16 residues, respectively, joined with the mature subunit sequence at residue 32. Because the mature human OTC subunit contains the residues asparagine-lysine at its amino terminus (residues 33 and 34), contributing a basic residue, these were changed to ser-gln, the amino terminus of rat OTC. In control experiments the human OTC leader has been shown to be able to direct the mature subunit of rat OTC to mitochondria (Horwich, unpublished observation). The precursors were programmed, as described in Materials and Methods, by manipulation of the cloned OTC cDNA sequence present in the previously described plasmid, pSPROTC, which joins a human OTC cDNA sequence with the *Salmonella* phage SP6 promoter.

Plasmids encoding either wild-type or altered precursors were transcribed in vitro using SP6 polymerase, and the resulting RNAs were translated in rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine. A portion of each translation mixture was solubilized and immunoprecipitated with rabbit anti-rat OTC antiserum. As shown in Fig. 2 B, in the lanes designated T, the wild-type OTC precursor migrates in an SDS-polyacrylamide gel as a 40-kD protein, whereas the precursor proteins of N12 and N16 migrate slightly more rapidly, consistent with their smaller molecular sizes. A second portion of each translation mixture was incubated with intact rat liver mitochondria for 1 h at 30°C, and the products recovered by solubilization and immunoprecipitation (lanes designated M). After incubation of the wild-type precursor with mitochondria, the major precipitable product was a 36-kD species, corresponding in size to the mature OTC subunit. After incubation of N12 or N16 translation mixtures with mitochondria, the only forms detected corresponded in size to the primary translation products. To determine the lo-

calization of these products, a portion of each mitochondrial incubation mixture was treated with trypsin, and the mixtures were then solubilized and immunoprecipitated (lanes designated M+t). In the case of the wild-type protein, complete proteolytic degradation of the precursor was observed, consistent with its localization outside the organelles, although a substantial fraction of the mature OTC subunit failed to be proteolytically degraded, consistent with its localization within a protected compartment (established from other studies to be the mitochondrial matrix space). When mitochondrial mixtures containing the N12 and N16 proteins were incubated with trypsin, the products were completely degraded, indicating that they had failed to reach the matrix space.

### *An 18-Residue CORE Portion of the OTC Leader Can Direct Import of the Precursor*

To address whether the midportion of the leader peptide might be sufficient to direct import, a third altered precursor, called CORE, was programmed, containing an NH<sub>2</sub>-terminal methionine residue, leader residues 8–25, glutamine 32, and the mature subunit. The coding sequence for this protein was produced by joining in an SP6 plasmid the NH<sub>2</sub>-terminal leader coding portion of the previously studied deletion mutant d2–7 with the COOH-terminal coding portion of the mutant d26–31. As compared with the N12 and N16 precursors, which contain glutamine at the second position in their mature portion, we note that the CORE precursor contains the usual lysine as the second residue in the mature subunit portion. When CORE transcripts were translated, as shown in Fig. 3, lane T, three discrete species precipitable by anti-OTC antiserum were observed: a major species of ~39 kD; and two minor species of ~37 and 34 kD. The major species corresponds in size to that predicted for the primary CORE translation product. The minor species approximate in size the products that would result if translation initiated at the second and third in-frame AUG codons in the transcript. The translation mixture programmed to synthesize CORE product was incubated for 1 h with mitochondria, and the mixture was then separated into supernatant and mitochondrial pellet fractions which were solubilized and immu-



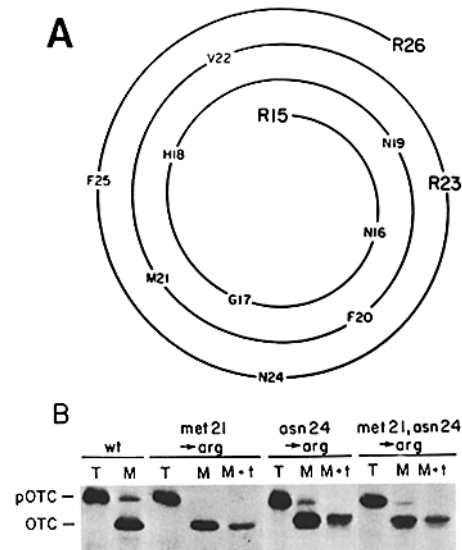
**Figure 3.** Incubation of CORE OTC precursor with mitochondria. A pSPROTC-derived plasmid encoding the NH<sub>2</sub>-terminal methionine, residues 8–25, gln 32, and the mature OTC subunit was transcribed in vitro and the products used to direct translation in reticulocyte lysate. An aliquot of each mixture was immunoprecipitated with anti-OTC antiserum. A second aliquot was incubated with isolated rat liver mitochondria. After 1 h at 27°C, the wild-type mixture was detergent-solubilized and immunoprecipitated. The CORE mixture was centrifuged at 10,000 g for 10 min and the supernatant and pellet fractions were solubilized and immunoprecipitated separately. (T) Translation products; (M) products of mitochondrial incubation; (S) supernatant fraction of mitochondrial incubation mixture; (P) pellet fraction; (M+t) products of trypsin treatment of mitochondrial incubation mixture.

noprecipitated. In the supernatant fraction (lane *S*), three proteins were detected corresponding precisely to the CORE translation products. The 39-kD protein remained as the major species. In the pellet fraction (lane *P*), however, two major species were observed. One corresponded to the 39-kD translation product and the other exhibited an apparent size of  $\sim 36$  kD—similar to that for the wild-type mature subunit (see *wt*, lane *M*). The amount of the 36-kD species detected was severalfold greater than the total amount of the 37-kD translation product added to the mitochondrial incubation mixture (lane *T*), suggesting that the 36-kD species was almost certainly the product of proteolytic processing of the 39-kD translation product. To establish intramitochondrial localization of the 36-kD CORE product, a trypsin-protection experiment was carried out. After the incubation of the translation products with mitochondria, an aliquot of the mixture was treated with a small amount of trypsin, then solubilized and immunoprecipitated. When wild-type OTC was examined in this fashion, only mature OTC was recovered (lane *wt*, *M+t*). When the CORE mixture was examined with exactly the same amount of trypsin, the 36-kD species was recovered, indicating that it was localized within the mitochondria. In addition, a small amount of the 39-kD species was also detected, suggesting that a portion of this precursor species may also localize within the organelles.

#### Insertion of Additional Arginine Residues in the Region of Arginine 23 Does Not Interfere with Import

In a previous study, analysis of substitutions at position 23 suggested that the arginine normally present participates in a functional secondary structure, possibly an  $\alpha$ -helix. The position of residues in a helical region can be approximated using a helical wheel nomogram, as shown in Fig. 4 *A*, which predicts that the arginine residues at positions 15, 23, and 26 occupy the same aspect of an  $\alpha$ -helix, whereas the opposite aspect of the helix is occupied by noncharged and hydrophobic residues. The putative helix thus appears amphiphilic in character, bearing charge on one aspect and hydrophobic character on the opposite. To address a possible functional requirement for this putative amphiphilic helix, we examined three altered precursors in which additional positively charged arginine residues were introduced into the leader peptide at positions near residue 23, which would be predicted to reside in the noncharged aspect of the putative  $\alpha$ -helix.

As shown in Fig. 4 *B*, when arginine was substituted for met 21, there was no effect upon import and proteolytic processing of the substituted precursor. When arginine was introduced in place of asparagine at position 24, there was no apparent effect upon import of the substituted precursor as judged by protection of the cleavage products from exogenously added trypsin, but the major proteolytic cleavage product of this precursor migrated more slowly than the mature OTC subunit, which was also detected in very small amounts. The arginine 24 protein is apparently cleaved at an unusual position in the COOH-terminal region of the leader peptide by a mitochondria-associated protease. Finally, a doubly substituted mutant, placing arginines at both positions 21 and 24, was also examined. Here, the same result as that obtained with *arg* 24 was observed: an unusual cleavage product predominated. Once again, the unusual cleavage



**Figure 4.** Incubation with mitochondria of OTC precursors substituting arginines for methionine 21 and asparagine 24. (*A*) Helical wheel plot of OTC leader residues 15–26. An angle of  $110^\circ$  is assigned between residues. The three arginine residues in the displayed sequence are indicated with large single-letter designations. (*B*) Plasmids with the designated codon substitutions were transcribed *in vitro* and the products used to direct translation reactions. Aliquots of the translation products were directly analyzed, incubated with mitochondria, or incubated with mitochondria followed by trypsin treatment. Products were immunoprecipitated, electrophoresed through an 8% SDS-polyacrylamide gel and the gel was fluorographed. (*T*) Translation products; (*M*) products of mitochondrial incubation; (*M+t*) products of trypsin treatment of the mitochondrial mixture.

product was sequestered by the mitochondria in a trypsin-insensitive location (lanes *M+t*).

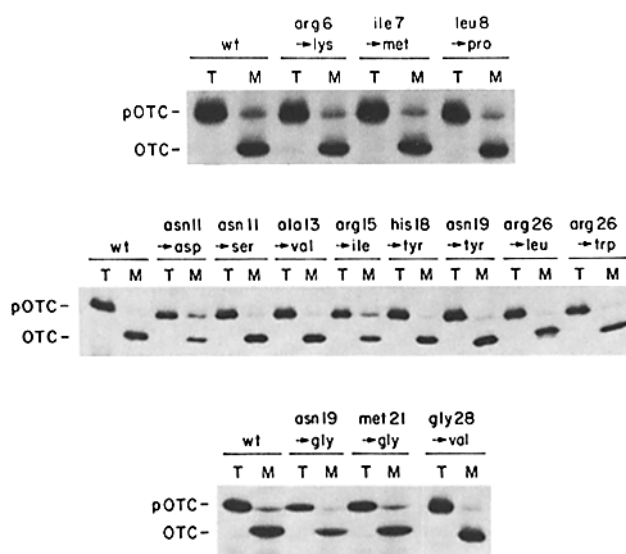
#### Analysis of Single Residue Substitutions in the Leader: Import Is Affected When Arg 15 Is Changed to Ile or When Asn 11 Is Changed to Asp

To identify particular residues in the leader peptide that are critical to function, both within and outside the midportion region, a series of precursors containing single residue substitutions was examined. Coding sequence alterations programming these precursors were produced using three different methods. Mutations affecting codons 6, 7, and 8 were produced by the procedure of misincorporation mutagenesis (Shortle et al., 1982), taking advantage of the presence of a unique BamHI site at this location. To produce mutations distributed along the length of the sequence between codons 8 and 37, the procedure developed by Myers and colleagues involving chemical mutagenesis of single-stranded DNA and identification of mutants in denaturing gels, was employed (Myers et al., 1985). The details of these manipulations as applied to the OTC leader coding sequence are presented in Fig. 1 and in Materials and Methods. DNAs identified as containing mutations directing single-codon changes were used to produce altered precursors. Nine such proteins were examined. The third method of mutagenesis, used to produce glycine substitutions at positions 19 and 21, involved replacement of a restriction fragment encoding a portion of the wild-

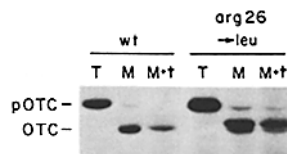
type leader with a synthetic double-stranded DNA fragment containing the desired codon change.

Each of the 14 single substitution products was incubated with isolated rat liver mitochondria, and the products were displayed as shown in Fig. 5. Of the mutants 11 were translocated and processed in a manner indistinguishable from that observed with wild-type OTC precursor. In all of these cases the mature subunit was found to be protected from digestion by exogenously added trypsin (data not shown). Only three of the mutants produced a different result. Two of the mutant precursors, asp 11 and ile 15, were converted to mature-sized OTC to an extent less than wild type, as measured densitometrically: 68% in both cases, compared with 100% for wild type. These mature forms were also protected from trypsin digestion, indicating their localization inside the organelles. Interestingly, the amino acid changes in both of these mutant precursors reduced the net positive charge of the leader peptide, by replacing either an uncharged asparagine with a negatively charged aspartate at position 11 or a positively charged arginine with an uncharged isoleucine at position 15.

The third unusual mutant, leu 26, produced another kind of result. When this precursor was incubated with mitochondria, most of it was converted to a product whose apparent size was intermediate between that of precursor and mature forms. To address whether this product localized within the mitochondria, a trypsin protection experiment was carried out as shown in Fig. 6. The intermediate-sized product detected after mitochondrial incubation was resistant to proteolysis, as was a product indistinguishable in size from ma-



**Figure 5.** Incubation with mitochondria of OTC precursors containing single-residue substitutions in the leader peptide. Plasmids with the designated codon substitutions were transcribed and the products used to direct reticulocyte lysate translation reactions. After translation, an aliquot of each mixture was immunoprecipitated with anti-OTC antiserum. A second aliquot was incubated for 60 min at 27°C with isolated rat liver mitochondria, after which the entire mixture was detergent-solubilized and immunoprecipitated. Products were electrophoresed through an SDS-polyacrylamide gel and the gel was fluorographed. (T) Translation products; (M) products of mitochondrial incubation.



**Figure 6.** Analysis of unusual cleavage product observed after mitochondrial incubation of leu 26 precursor. Equal amounts of in vitro-synthesized precursors were directly analyzed, incubated with mitochondria, or incubated with mitochondria followed by trypsin treatment as in Fig. 2. Products were immunoprecipitated and analyzed in an SDS-polyacrylamide gel.

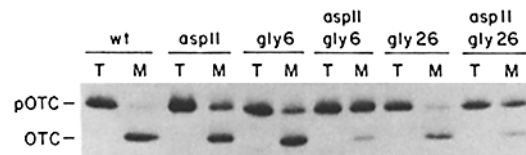
ture OTC. These results are similar to those observed with the arg 24 substitution mutant (Fig. 4 B).

### Import of Doubly Substituted Precursors Gly 6, Asp 11 and Asp 11, Gly 26 is Less Extensive than Import of Three Single-substitution Precursors Gly 6, Asp 11, and Gly 26

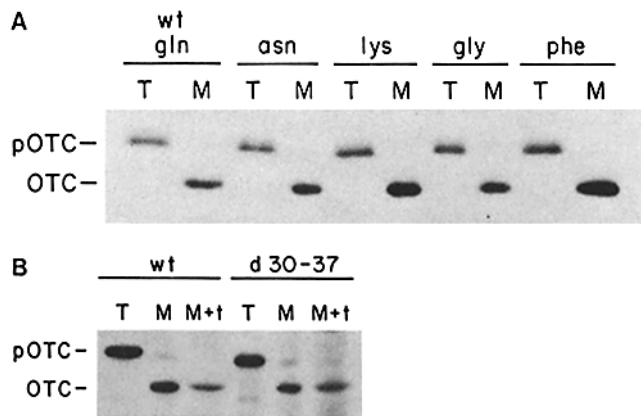
Given the observations in Fig. 5 that the only two leader substitution mutants whose import was impaired had either lost a basic residue or acquired an acidic one, we examined the hypothesis that a decrease of net positive charge of the leader peptide is associated with decreased extent of import. This hypothesis would predict that double mutants producing a loss of net positive charge at two positions should be impaired in import to a greater degree than the parental single-substitution products. Indeed, in a previous study (Horwich et al., 1986), the double-mutant gly 15,26, replacing arginines at both positions, was found to be imported substantially less extensively than either of the parental mutants gly 15 or gly 26. Fig. 7 shows the results of analysis of two additional double-substitution products, gly 6, asp 11 and asp 11, gly 26. In the first case (gly 6, asp 11), the extent of conversion to mature OTC was 8%, as compared with 80% and 68% for the respective gly 6 and asp 11 parental single mutants; in the second case (asp 11, gly 26), conversion to mature OTC was 28%, as compared with 68% and 95%, respectively, for the parental asp 11 and gly 26 mutants. Thus, as net positive charge was reduced in three different double-substitution products, there was a corresponding loss of import function.

### Neither Substitution for the Terminal Leader Residue Gln 32, nor Deletion of Residues 30-37 Interferes with Proteolytic Processing

In a previous study we observed that certain mutations affect-

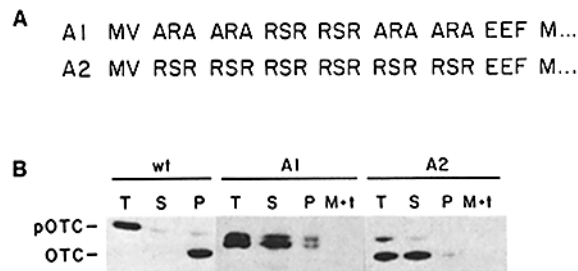


**Figure 7.** Incubation with mitochondria of OTC precursors containing single or double substitutions that reduce the net positive charge of the leader peptide. Plasmids with the designated codon substitutions were transcribed and the products used to direct in vitro translation. An aliquot of the translation mixture was immunoprecipitated with anti-OTC antiserum. A second aliquot was incubated with rat liver mitochondria, after which the entire mixture was detergent-solubilized and immunoprecipitated. Anti-OTC precipitable products were analyzed in an SDS-polyacrylamide gel. (T) Translation products; (M) products of mitochondrial incubation.



**Figure 8.** Incubation with mitochondria of OTC precursors altered at the site of leader peptide cleavage. (A) Plasmids with the designated substitutions for codon 32 were transcribed in vitro and the products used to direct in vitro translation reactions. An aliquot of the translation products was immunoprecipitated. A second aliquot was incubated with mitochondria and the incubation mixture was then detergent-solubilized and immunoprecipitated. The precipitable products were displayed in an SDS-polyacrylamide gel. (B) Plasmids encoding either the wild-type OTC precursor or a protein deleted of residues 30–37 were employed as described in A, except that an additional aliquot of each translation mixture was incubated with mitochondria and then treated with trypsin. (T) Translation products; (M) mitochondrial incubation products; (M+t) products of trypsin treatment of mitochondrial incubation mixture.

ing the midportion of the leader peptide prevented proteolytic cleavage, and speculated that secondary structure in this region might be required for cleavage (Horwich et al., 1986). To determine whether there is also a requirement for primary structure near the site of cleavage, we have now examined the effect of a number of amino acid substitutions at the residue preceding the cleavage, replacing gln 32 with asparagine, lysine, glycine, or phenylalanine. As shown in Fig. 8 A, these substituted precursors were all cleaved as extensively as the wild-type precursor, and the products exhibited a mobility identical to that of the mature OTC subunit. Trypsin protection analysis (not shown) indicated that in all cases the proteolytic products localized within the mitochondria. Because there is no apparent requirement for a specific residue at position 32, and because the deletion mutant d26–31 is cleaved as extensively as wild-type precursor, we were left to conclude that there is no primary structural requirement for proteolytic processing at the NH<sub>2</sub>-terminal side of the cleavage site. To determine whether there is a primary structure requirement at the COOH-terminal side of the cleavage site, residues 30–37 were deleted. This removed the terminal leader codons pro, leu, and gln, all four of the NH<sub>2</sub>-terminal mature subunit codons (asn, lys, val, gln) that precede the EcoRI linker present in the “wild-type” SPROTC plasmid construct, and the linker codon his. As shown in Fig. 8 B, lane M, when the d30–37 precursor was incubated with mitochondria, it was nearly completely cleaved to a form that migrated identically to the wild-type mature subunit. As shown in the lane designated M+t, this product was protected from proteolysis by exogenously added trypsin, indicating its localization within the organelles. Thus, even in the complete absence of the cleavage site that produces mature



**Figure 9.** Incubation with mitochondria of OTC precursors containing artificial leader peptides. (A) NH<sub>2</sub>-terminal amino acid sequence of two precursors designated A1 and A2, as predicted from DNA sequence analysis of the corresponding plasmids. The last three residues displayed, EFM, correspond to residues 38–40 in the SPROTC-encoded wild-type precursor. (B) Plasmids pA1 and pA2 were transcribed in vitro and the products were translated in reticulocyte lysate. An aliquot of each of the in vitro translation reactions was immunoprecipitated with anti-OTC antiserum. A second aliquot was incubated with rat liver mitochondria, then separated by centrifugation into supernatant and pellet fractions which were solubilized and immunoprecipitated separately. A third aliquot of the A1 and A2 translation mixtures was first incubated with mitochondria after which the entire mixture was treated with trypsin. Abbreviations as in Fig. 2.

OTC and its surrounding region, the precursor was proteolytically processed at a nearby site, most likely by the same mitochondrial matrix enzyme that normally cleaves the wild-type leader peptide.

#### Artificial Leader Peptides Containing Multiple Arginine Residues and Predicted to Assume an $\alpha$ -Helical Conformation Fail to Direct Import

To address whether the two elements, positive charge and  $\alpha$ -helical structure, are alone sufficient to direct mitochondrial import, we constructed, using synthetic oligonucleotides, several sequences that could encode such artificial leader peptides. The artificial leader coding sequences were derived from double-stranded synthetic 18mer DNA segments, which encoded on one strand ala-arg-ala-ala-arg-ala and on the opposite strand arg-ser-arg-arg-ser-arg. The blocks were joined using T4 ligase and segments of varying length were recovered from an acrylamide gel. Fragments were then joined with a plasmid vector containing an SP6 promoter and the mature OTC coding sequence. Two of the derived plasmids, designated pA1 and pA2, were subjected to DNA sequence analysis of the leader coding region. The predicted NH<sub>2</sub>-terminal amino acid sequences are shown in Fig. 9 A. The leader peptide of A1 up to the first glutamate residue is 20 residues in length and corresponds to that predicted from joining three consecutive oligonucleotide segments in a manner that places the outside segments in the same orientation and the internal segment in an inverted orientation. The programmed A1 leader region thus contains eight arginine residues and is composed entirely of residues with high  $\alpha$ -helix potential (Chou and Fasman, 1978). The predicted leader of A2 up to the first glutamate residue is also 20 residues in length and corresponds to joining of three consecutive oligonucleotide segments in the same orientation. The A2 leader region contains 12 arginines and, like A1, contains only residues with high  $\alpha$ -helix potential.

Plasmids pA1 and pA2 were transcribed *in vitro* and the RNAs were then translated. Fig. 9 B shows, in the lanes designated *T*, that two products were detected for both plasmids, in one case (*A1*) a 38-kD species and a 37-kD species, and in the other (*A2*) a 38-kD species and a 35-kD species. In both cases, the larger size species corresponds to the primary translation product predicted from the sequence. The origin of the smaller species is unknown although they might represent products of proteolysis of the larger species.

When the *A1* and *A2* translation products were incubated with mitochondria, no new species were detected, indicating that the products had not been cleaved by a mitochondrial protease. When the mitochondrial incubation mixtures were separated into supernatant (*S*) and pellet (*P*) fractions, only a small portion of the translation products was associated with the mitochondrial pellets. When the mitochondrial mixtures were treated following incubation with a small amount of trypsin, the associated species were completely degraded (lanes *M+t*), indicating that the *A1* and *A2* translation products had failed to be taken up into the mitochondria.

## Discussion

In order to define the features of the OTC leader peptide responsible for mitochondrial localization, a set of alterations affecting the peptide was programmed. Some of these alterations were specifically programmed to address particular hypotheses concerning leader function. In addition, a set of random single-residue substitutions directed by chemical mutagenesis of the leader coding sequence was designed to identify, in a nonselective manner, additional critical residues. Import of the altered OTC precursors was quantitated as the percentage of OTC species recovered, relative to wild type, that had been converted to intermediate or mature size. Such a measurement is valid because, when trypsin-protection experiments were carried out, the cleavage products were found to be protected from degradation, indicating their presence within the organelles, whereas the precursor forms were not. The results of the studies carried out here, and results from previous studies, are summarized in Table I.

### Functional Regions of the OTC Leader Peptide

The effects of removal of various portions of the leader peptide as shown in the first part of Table I, establishes that its midportion, between residues 8 and 25, is both necessary and sufficient to direct mitochondrial import. In contrast, the NH<sub>2</sub>-terminal portion is neither necessary nor sufficient to direct import. Additionally, the COOH terminus appears not to be necessary, because both a penultimate deletion product, d26-31, and a cleavage site deletion product, d30-37, were imported as extensively as wild type. These observations are noteworthy in two respects. First, studies of two leader peptides from *Saccharomyces cerevisiae* revealed that NH<sub>2</sub>-terminal portions containing either 9 or 12 residues were sufficient to direct mitochondrial localization of the respective precursor protein (Keng et al., 1986; Hurt et al., 1985). In clear contrast, the corresponding functionally sufficient domain in the OTC leader peptide lies in its midportion. It remains to be seen whether the midportion of other leader peptides directing mammalian precursors to the matrix will be generally critical. The other surprising obser-

vation produced by regional analysis is that both import and proteolytic cleavage proceed in the complete absence of the site of proteolysis that leads to the formation of mature OTC. Apparently, the matrix protease that catalyzes this process-

Table I. Percent Conversion of Altered OTC Precursors to Mature Subunit

Leader construct	Percent conversion to mature form
<i>wt</i>	100
Deletions, fusions	
d2-3	93
d2-7	50
d2-12	21
d8-22	0
N12	0
N16	0
CORE*	21
d26-31	94
d30-37	94
Single substitutions	
arg 6→gly	80
arg 6→lys	100
ile 7→met	100
leu 8→pro	98
asn 11→ser	100
asn 11→asp	68
ala 13→val	97
arg 15→ile	68
arg 15→gly	45
his 18→tyr	96
asn 19→gly	86
met 21→gly	84
asn 19→tyr	97
met 21→arg	87
arg 23→gly	0
arg 23→ala	51
arg 23→asn	22
arg 23→lys	79
asn 24→arg	88 i
	12 m
arg 26→gly	95
arg 26→leu	82 i
	13 m
arg 26→trp	84
gly 28→val	98
gln 32→asn	94
gln 32→gly	98
gln 32→lys	100
gln 32→phe	100
Double substitutions	
arg 21, arg 24	74 i
	16 m
gly 15, gly 26	17
asp 11, gly 6	8
asp 11, gly 26	28
Triple substitutions	
gly 15, gly 23, gly 26	0

The conversion of altered OTC precursors to mature subunits after mitochondrial incubation was determined as described in Materials and Methods. i, intermediate-sized form; m, mature-sized form.

\* Percent conversion of this precursor was determined essentially as for the others except that the sum of the products recovered in the supernatant and pellet fractions was the denominator.

ing does not require a specific primary structure at the site of cleavage. This lack of specificity differentiates this matrix protease from analogous processing proteases such as signal peptidase, which mainly recognizes small, uncharged residues preceding the site of cleavage (von Heijne, 1983), and prohormone-cleaving enzymes, which require one or two basic residues at this position (Steiner et al., 1980).

### ***Function of the Midportion Region in Import***

In a previous study, we observed that a number of substitutions for arg 23 interfered with import of the OTC precursor (Table I). The results were consistent with the idea that residue 23 contributes to a secondary structure which might be an  $\alpha$ -helix. In this regard, it has recently been hypothesized that leader peptides direct import via amphiphilic  $\alpha$ -helices containing positively charged residues on one aspect and hydrophobic residues on the opposite aspect (von Heijne, 1986). In order to test this hypothesis, we have examined the effects of a number of additional amino acid substitutions in the vicinity of arg 23. Two types of substitution seemed particularly likely to disrupt import. The first involved replacement of noncharged residues that occupy positions opposite arg 23 in a putative  $\alpha$ -helix with arginines. If a crucial amphiphilic  $\alpha$ -helix were present in the OTC leader in the region of arg 23, then simultaneous substitution of arginines at both positions 21 and 24 should have impaired import by disrupting the hydrophobic face of the helix. No such effect was observed—the mutant was imported in the same fashion as wild type, although it was cleaved at an unusual position. We thus conclude that, if an  $\alpha$ -helical structure exists in the region of arg 23, its amphiphilicity is not essential.

A second type of substitution directed at the functional role of a putative  $\alpha$ -helix in the midportion involved the placement of glycine residues at positions 21 and 19. We expected that substitution at these positions, only 2 and 4 residues away from arg 23, might recapitulate the loss of function observed with the gly 23 substitution, particularly if an  $\alpha$ -helix extended through this region. No effect was observed, however; the mutants were imported and cleaved exactly as wild type. In the case of gly 19, this result was not so surprising because the residue replaced at this position is asparagine, one that already exhibits low  $\alpha$ -helix forming potential (Chou and Fasman, 1978). In the case of gly 21, however, the residue replaced is methionine, which has a very high  $\alpha$ -helix potential. We conclude from these findings that either the functional secondary structure in the region of arg 23 is not an  $\alpha$ -helix or, if an  $\alpha$ -helix is present, its structure remains stable to the alterations programmed here. The latter possibility is supported by the previous observation that insertion into the deletion, d8–22, of residues with high  $\alpha$ -helix potential, ala-ala-ala-ile, could restore a measure of import function (Horwich et al., 1986). It is evident that direct structural analysis of the OTC leader peptide will be required in order to identify the features that make arg 23 stand out from its neighbors as a functionally preeminent residue.

### ***Function of the Midportion in Proteolytic Processing***

We had previously postulated a critical role for the midportion in directing proteolytic processing of the leader peptide because we observed that proteolytic cleavage was completely blocked by deletion of the midportion region in the

mutant d8–22 or by substitution of glycine at position 23. In the present study, two single amino acid substitutions in the region of arg 23 further established a role for this region. Substitution of either arginine for asn 24 or leucine for arg 26 produced cleavage at an unusual site lying within the COOH-terminal portion of the leader. The position 26 substitution argues for involvement of secondary structure because the unusual cleavage was observed only with substitution of leucine at this position and not with substitutions of tryptophan or glycine (Fig. 5, 7). An additional interpretation is worth mentioning. Incubation of the OTC precursor from rat and mouse with intact mitochondria has repeatedly revealed the presence of an OTC species intermediate in size between precursor and mature OTC (Mori et al., 1982; Conboy et al., 1982; Rosenberg et al., 1983). If mature OTC was, in fact, formed by two sequential cleavage events, then mutations blocking the second cleavage step could lead to accumulation of such an intermediate-sized species.

### ***Role of Positive Charge in Import***

In a previous study, we substituted glycine for each of the four arginines in the leader peptide and observed a range of effect on import, from virtually 100% import of gly 26, to 80% import of gly 6 and 45% of gly 15, to no import of gly 23. Additional substitutions clearly established a major role for structure at position 23, but charge is also likely to play a role at this position because the most extensive import with a noncharged residue at this position, ala 23, is substantially less than with the positively charged residues arg 23 or lys 23. At position 6, a similar preference for charge is noted, with lys 6 imported to an extent identical to wild-type while gly 6 is imported less extensively. Similarly, at position 15, import of either ile 15 or gly 15 was about 50% as extensive as wild type. Because ile has strong  $\alpha$ -helix potential, a functional role of  $\alpha$ -helical secondary structure at this position (which might be argued to have been impaired by substitution of glycine) is extremely unlikely. At position 26, single substitutions of tryptophan and leucine recapitulated the result with glycine; i.e., import was unaffected. This result is consistent with the previous observation that residues 26–31 can be deleted without any effect on import. Based on the effect of these various substitutions for the arginine residues, we conclude that the positive charge of the first three arginines in the leader plays a critical role in directing import.

Examination of two kinds of additional amino acid substitution leads to the more general conclusion that the net positive charge of the leader peptide, conferred by the basic residues, is required to direct import. One informative substitution involved replacement of a charge-neutral residue, asn 11, with a negatively charged acidic residue, aspartate. This led to impairment of import. In contrast, substitution at the same position with serine had no effect upon import. It has been previously noted that leader peptides are, in nearly all cases, devoid of acidic residues (Hay et al., 1984). The observation here that import is impaired with introduction of an acidic substitution at a normally charge-neutral position supports the notion that the presence of a negatively charged side chain within the leader peptide is detrimental to import function. A second type of informative substitution involved double replacements that reduced the net positive charge of the leader peptide, either by subtracting two positive charges or by simultaneously adding one negative charge



and subtracting one positive charge. Both types of double substitution led to a degree of impairment of import substantially greater than that observed with either corresponding parental single substitution. Interestingly, this observation extended to arginine residue 26, for which single charge-neutral substitutions were without effect. Specifically, double substitutions that involved residue 26 were imported to an extent substantially less than that of the other parental single substitution. This suggests that the positive charge at position 26 may be recruited to assist import function once net positive charge is reduced by a single substitution at another position.

How might net positive charge of the leader peptide play a role in import of the precursor? Several possibilities deserve mention. One possibility is that leader peptides interact electrostatically with mitochondria. The potential gradient across the mitochondrial inner membrane is oriented with relative negative charge at the inside, making it conceivable that the positively charged leader peptide is electrophoresed across the membrane. Strongly supporting this possibility is the recent observation that the electrochemical gradient was required only for translocation of leader peptides and not for translocation of adjoining mature sequences (Schleyer and Neupert, 1985). It is also possible, however, that the positively charged peptide may interact with negatively charged phospholipid head groups in one or both of the mitochondrial membranes, as suggested by the model of von Heijne (1986). An additional possibility is that the positively charged peptide interacts with a negatively charged protein, perhaps an outer membrane receptor molecule, or with a protein that comprises a channel.

Whatever the mechanism by which net positive charge plays a role in directing mitochondrial import, this feature alone is not sufficient. The artificial leader peptides examined here, devoid of acidic residues and rich both in arginine and in residues with high  $\alpha$ -helix potential, failed to direct import. This contrasts with the recent results of Allison and Schatz (1986) who found that a stretch of 11 residues comprised of arginine, leucine, and serine, and predicted to assume an  $\alpha$ -helical confirmation, could direct mitochondrial localization in *S. cerevisiae* of the mature portion of cytochrome oxidase subunit IV. Conceivably, the specificity of recognition of precursor proteins by mitochondria of mammals is greater than that of yeast although this remains to be directly addressed. We suspect that the artificial leaders tested here meet the requirement for net positive charge, but that they fail to meet particular structural requirements. It seems unlikely that simply the length of the artificial leader segments was responsible for their failure, that is, that they were not long enough to be functional, because we observed that the CORE leader, which is precisely the same length, conferred import. Rather, the inability of the artificial sequences to assume the functionally required secondary structure present in the midportion of the OTC leader peptide seems to us much more likely to be the shortcoming of the artificial peptides. Clearly, direct structural analysis of the OTC leader peptide and additional modeling studies with

artificial segments will be required to define the structural elements in the OTC leader peptide that, in concert with its net positive charge, direct the OTC precursor to its matrix destination.

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