A Signal Sequence Domain Essential for Processing, but Not Import, of Mitochondrial Pre-Ornithine Carbamyl Transferase

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Abstract. Studies using deletion mutagenesis indicate that a processing recognition site lies proximal to the normal cleavage position between gln32 and ser33 of pre-ornithine carbamyl transferase (pOCT). pOCT cDNA was manipulated to delete codons specifying amino acids 22–30 of the signal sequence. The mutant precursor, designated pOCTA22–30, was imported to the matrix compartment by purified mitochondria, but remained largely unprocessed; the low level of processing that was observed did not involve the normal cleavage site. Several manipulations performed downstream of the normal pOCT processing site (deletion, substitution, and hybrid protein constructions) affected neither import nor correct processing.

Our data suggest (a) that domains specifying import and processing site recognition may be functionally segregated within the signal peptide; (b) that processing is not requisite for import of pOCT; and (c) that a proximal region, not involving the normal signal peptide cleavage site, is required for processing site recognition.

Materials and Methods

General

Earlier studies describe the methods used for recombinant pSP64 transcription and subsequent translation in a rabbit reticulocyte lysate in the presence of [35S]methionine, isolation and purification of mitochondria from rat heart or liver, and analysis of total import products by SDS PAGE and fluorography (reference 25 and references cited therein). Further details are provided in the figure legends.

Mitochondrial Import Assay

After translation of recombinant pSP64 transcripts in a messenger-dependent rabbit reticulocyte lysate system for 20 min, an aliquot (46 μl) was removed, mixed with 4 μl of heart mitochondria (25 μg protein) suspended in 10 mM Hepes, pH 7.4, 0.25 M sucrose, 10 mM succinate, 2.5 mM K2HPO4, 0.15 mM ADP, and the incubation continued at 30°C for 60
Partial NH2-terminal Radiosequencing of Processed Products

After import, mitochondria were treated with proteinase K and processed products purified either by SDS PAGE and subsequent electrophoresis of excised bands (exactly as described in reference 7) or by immunoprecipitation with immobilized anti-OCT IgG (reference 1) followed by treatment with 2% SDS; in both cases, recovered samples were diluted with H2O and filtered to give a final concentration of SDS of <0.05% (wt/vol). Each polypeptide product, labeled with [3H]leucine, demonstrated a single radioactive band when re-analyzed by SDS PAGE. Samples (150,000-300,000 cpm) were subjected to automated Edman degradation using either a Beckman 890c spinning cup sequencer or an Applied Biosystems 470A gas-phase sequencer.

Results and Discussion

Rat liver pOCT (40 kD) is made as a larger precursor molecule containing at its amino terminus a transient signal sequence (Fig. 1) that functions to target pOCT into mitochondria (25); the signal has amphiphilic properties (6) and is characterized by a high net positive charge, containing eight basic residues distributed throughout its length. Sequence comparisons between full-length rat pOCT cDNA (17, 20, 25, 28) and mature enzyme protein (18) have revealed that normal processing of pOCT in vivo involves cleavage between gln32 and ser33 of the precursor polypeptide; we have confirmed by amino acid sequence analysis that this same cleavage site is used after import of pOCT into mitochondria in vitro (see Fig. 7A). Features of the precursor molecule that determine correct processing of pOCT, or indeed any other mitochondrial precursor protein, however, have yet to be elucidated, although the total lack of any consensus among amino acids at the cleavage site of a number of precursors to mammalian mitochondrial matrix proteins suggests that regions flanking the processing site may be important. Indeed, in the case of rat liver, pOCT is cleaved between gln12 and ser13, pre-carbamyl phosphate synthetase is cleaved between leu31 and leu32 (26), and pre-ornithine aminotransferase is cleaved between glu34 and gln35 (24). Though not yet proven, there likely exists a single matrix enzyme responsible for processing most, if not all, mitochondrial matrix precursors (4, 21, 30). To test the contribution of sequences lying proximal to the normal processing site of rat liver pOCT, we have carried out deletion mutagenesis.

Construction of pSPod2

The recombinant plasmid was derived from pSP019 (25) and was engineered to delete codons from pOCT cDNA that encode amino acids 22-30 of the signal sequence, by the protocol outlined in Fig. 2. After deletion of a 49-nucleotide Ncol-I-Pvu II fragment from pSP019, a synthetic oligonucleotide adaptor was used to introduce the desired modification, and in particular to reconstitute the normal processing site at amino acids 32 and 33. The derived plasmid, designated pSPod2, was sequenced to confirm the predicted construct. After in vitro transcription-translation of pSPod2, a polypeptide product was made whose mobility on SDS gels

![Diagram showing construction of pSPod2](image-url)

 specifying amino acids 21 and 31-36, and leaving a final deletion of codons encoding amino acids 22-30 of pOCT; the cDNA encoding pOCT was otherwise normal in all respects. Note that in native pOCT, val occupies positions 22 and 31; only the codon specifying val22 was deleted. The mutant plasmid, designated pSPod2, was linearized with Eco RI before transcription-translation. The correct processing site between gln32 and ser33 is indicated.
pOCTΔ22-30 Accumulates in Mitochondria

Results of import of pOCT and pOCTΔ22-30 in vitro are presented in Fig. 3; the primary translation products corresponding to the two precursors were derived by transcription-translation of their respective plasmids. As expected, pOCT (40 kD) was imported and processed to mature form (36 kD) by heart mitochondria in vitro. A 37-kD proteolytic fragment of pOCT was also recovered inside mitochondria, but its appearance is variable. The 36-kD polypeptide represents the input translation (see Fig. 6, lane 1), it is not observed but usually accounted for 10-30% of imported (protease-resistant) material. That such processing, albeit inefficient, resulted from the action of the normal matrix-processing enzyme, however, is suggested by the fact that O-phenanthroline, a chelator which inhibits the Zn++-dependent mitochondrial signal peptidase of rat liver (4), also inhibited pOCTΔ22-30 processing (Fig. 4). This finding further shows that processed Δ22-30 is not simply the mutant equivalent to the 37-kD proteolytic fragment of pOCT, because appearance of the latter is not sensitive to O-phenanthroline (Fig. 4, lanes 2 and 3). The same results were obtained using mitochondrial matrix extracts in place of the intact mitochondria used for Fig. 4; chelation of Zn++ by either O-phenanthroline or EDTA prevented processing (not shown).

Several attempts were made to sequence processed pOCTΔ22-30 labeled with [3H]leucine (containing up to 300,000 cpm) but, in striking contrast to correctly processed pOCT (see Fig. 7), only background radioactivity was recovered, strongly suggesting that processed Δ22-30 had a blocked NH2-terminus (data not shown). This fact, together with the finding that processed pOCTΔ22-30 did not co-migrate with processed pOCT (Fig. 3 and 4), suggests that processing of pOCTΔ22-30 must have occurred at a site other than between gln32 and ser33, although in the absence of sequencing data, such a conclusion cannot be made with certainty.

Location of Imported pOCTΔ22-30

Figs. 3 and 4 demonstrate that a protease-resistant pool of pOCTΔ22-30 accumulated within mitochondria after import.
Effects of Downstream Sequence Alterations on pOCT Import and Processing

Fig. 5 describes manipulations to pOCT cDNA that remove or substitute codons specifying basic residues and/or helix breakers (gly, pro) in the region immediately downstream of the normal pOCT processing site. Such alterations were chosen because basic residues and helix breakers are a feature of this region in a number of matrix precursor proteins. The mutants, pOCTΔ37-47E and pOCTΔ38-40, and the hybrid protein, pOCT-AS, were all capable of being imported and processed by purified mitochondria in vitro (Fig. 6). Radiosequencing of the processed products (Fig. 7) showed that in all cases processing took place in the correct position between gln32 and ser33.

Conclusions

We have demonstrated that deletion of about one-third of the pOCT signal sequence just proximal to the precursor processing site, while having no obvious effect on pOCT import, resulted in a product that accumulated within mitochondria where it was inefficiently and incorrectly processed. The results from mutagenesis, therefore, are consistent with findings in Neurospora (32) and yeast (30) that transmembrane import of matrix proteins is not obligately linked to precursor processing, although it should be emphasized that processing may affect the rate of such import (references 16 and 29). Moreover, our results suggest that distinct functional domains may exist within the pOCT signal sequence, conferring import capabilities on the one hand and processing recognition on the other. An examination of various mitochondrial signal sequences reveals that helix breakers (gly, pro) and basic residues (lys, arg) are often represented in the region preceding the cleavage site; recent studies of human pOCT, however, indicate that positive charges in positions 23 and 26 are not required (11). Residues 22–30, however, may very well contribute to a specific conformation within the signal sequence that specifies recognition by the mitochondrial processing enzyme. It may also serve to position pOCT and help orient the gln32-ser33 peptide bond toward the metal cofactor (Zn++) and catalytic residue within the peptidase’s active site. Such positioning may be critical for selecting which peptide bond is cleaved, and could explain how specificity is achieved for precursors exhibiting diverse amino acid compositions at their cleavage site. Moreover, alterations to the conformation of the processing recog-
Figure 7. Radiosequencing of $[^3H]leu$-labeled products after import and processing of pOCT, pOCTΔ37–47E, pOCTΔ38–40, and pOCT-AS. After import into mitochondria and digestion with proteinase K (Fig. 6), processed products of pOCT, pOCTΔ37–47E, and pOCTΔ38–40 were purified by an immunofinity binding procedure, exactly as described in reference 2; pOCT-AS processed product was purified by electroelution from gel slices after SDS PAGE, as described (7). In each case, purified processed product showed a single radioactive species after re-analysis by SDS PAGE and fluorography. Sequence positioning of $[^3H]leu$ was performed as described in Materials and Methods. The positions of leucine residues in each sequence is denoted by an asterisk; correspondence between the positions of leucine and peaks of radioactivity at a sequencing cycle were used to position the site of processing for each precursor (shown in Fig. 5). (A) pOCT; (B) pOCTΔ37–47E; (C) pOCTΔ38–40; (D) pOCT-AS.

nition site within the signal sequence may in some cases lead to the wrong peptide bond being positioned in the catalytic site, and thereby explain how incorrect processing might arise.

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