# 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 $gln^{32}$ and $ser^{33}$ 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 pOCTΔ22–30, 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.

STUDIES using hybrid proteins (5, 12–14, 25) and synthetic signal peptides (9) have recently established that information for targeting and subsequent translocation of nuclear-coded precursor proteins into mitochondria resides exclusively within their amino-terminal leader sequence; though not yet proven, recognition of such targeting sequences by mitochondria is presumably mediated by an import receptor located on the surface of the organelle. After translocation into mitochondria, the transient leader (or signal) peptide is cleaved from the precursor molecule by a specific metalloprotease (mitochondrial signal peptidase) located within the matrix compartment (3, 4, 19, 22). A large number of mitochondrial signal peptides have now been sequenced and although little homology exists between them, they all share certain similarities, including a high net positive charge and an overall composition of predominantly polar residues interspersed with short stretches of 1–3 hydrophobic amino acids. Such characteristics confer amphiphilic properties to mitochondrial signal sequences (6, 27), but how this relates to the import process is not yet known.

With respect to correct processing of signal sequences, the specificity requirements for mitochondrial signal peptidase have yet to be elucidated; in particular, there appears to be little or no consensus for a preferred amino acid composition at the cleavage site. Recently, however, Horwich et al. (11) identified a midportion of the pre-ornithine carbamyl transferase (pOCT)[1] signal sequence as essential both for import of the precursor protein into mitochondria and for removal of its signal peptide, and Hurt et al. (13) have demonstrated that an amino-terminal fragment of a signal peptide (lacking the normal cleavage site) directs import of a hybrid protein without concommitant processing. In the present communication, we show that amino acids 22–30 of pOCT contribute to a critical processing recognition site, and we further demonstrate that despite the deletion of this site from pOCT, the precursor molecule is imported and accumulates within mitochondria. Manipulations that remove basic residues and helix breakers immediately downstream of the pOCT cleavage site affected neither import nor processing of the precursor.

1. *Abbreviation used in this paper*: pOCT, pre-ornithine carbamyl transferase.

## 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 [$^{35}$S]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 $K_2HPO_4$, 0.15 mM ADP, and the incubation continued at 30°C for 60


*Figure 1.* Primary structure of rat liver pOCT signal sequence. The one-letter amino acid code has been used to denote the primary sequence deduced from cDNA clones (4, 13–15). The solid arrowhead designates the normal processing site between amino acids 32 and 33 of pOCT; the space denotes the deletion of amino acids 22–30 in pOCTΔ22–30; the open arrowhead indicates where correct processing of pOCTΔ22–30 should take place.

min at which time the mitochondria were recovered by centrifugation and analyzed directly by SDS PAGE. (See reference 25).

### *Partial $NH_2$-terminal Radiosequencing of Processed Products*

After import, mitochondria were treated with proteinase K and processed products purified either by SDS PAGE and subsequent electroelution 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 $H_2O$ and filtered to give a final concentration of SDS of <0.05% (wt/vol). Each polypeptide product, labeled with [$^3$H]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 gln[32] and ser[33] 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. 7 *A*). 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 gln[32] and ser[33], pre–carbamyl phosphate synthetase is cleaved between leu[38] and leu[39] (26), and pre–ornithine aminotransferase is cleaved between glu[34] and gln[35] (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 pSPO19 (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 NcoI–Pvu II fragment from pSPO19, 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

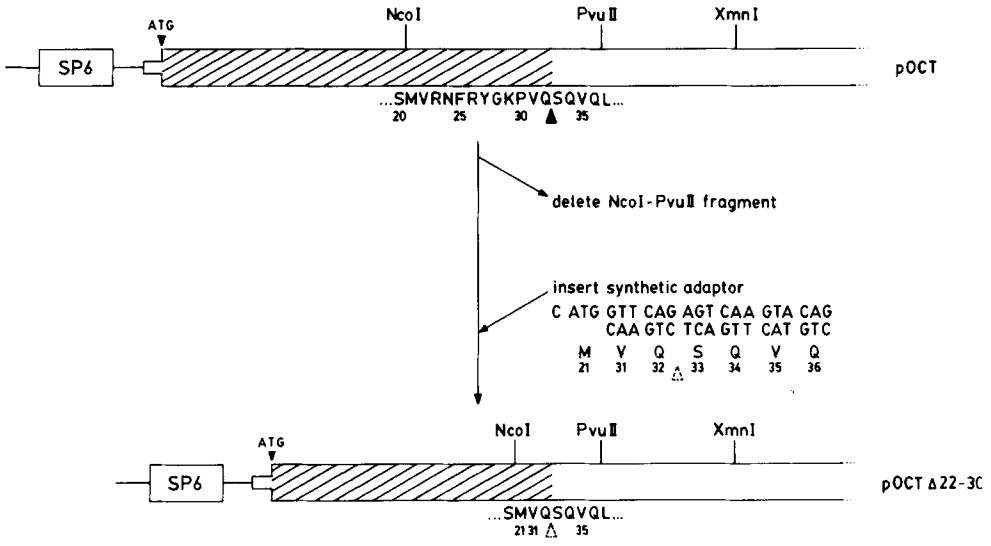


*Figure 2.* Construction of pSPOd2 encoding pOCTΔ22–30. pSPO19 is derived from pSP64 and contains a cDNA insert coding for a functional full-length copy of rat liver pOCT (25). pSPO19 was digested to completion with Nco I and partially digested with Pvu II to generate a 4.8-kb fragment. The latter represented pSPO19 lacking 49 nucleotides encoding amino acids 21–36 of pOCT. After purification by agarose gel electrophoresis, the 4.8-kb fragment was annealed with a double-stranded Nco I–Pvu II adaptor, synthesized on an ABS 380A DNA synthesizer, which reconstituted codons 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 val[22] was deleted. The mutant plasmid, designated pSPOd2, was linearized with Eco RI before transcription-translation. The correct processing site between gln[32] and ser[33] is indicated.

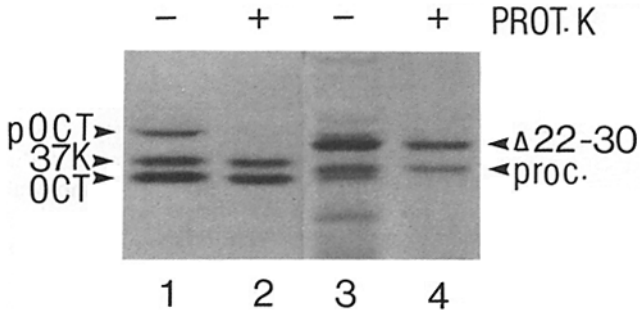


*Figure 3.* Import of pOCT and pOCTΔ22–30 by heart mitochondria in vitro. pSPO19 and pSPOd2 encoding native pOCT and pOCTΔ22–30, respectively, were linearized with Eco RI and transcribed to yield capped mRNA as described in reference 25. After translation in a rabbit reticulocyte lysate, import was performed as described in Materials and Methods, at which time import mixtures were placed on ice and treated with or without proteinase K (200 μg/ml) (±PROT. K) for 30 min at 4°C. After addition of phenylmethylsulfonyl fluoride (2 mM final), mitochondria were isolated by centrifugation at 12,000 *g* for 5 min, briefly rinsed, and dissolved in SDS sample buffer and loaded directly onto gels for analysis by SDS PAGE and fluorography. The positions of pOCT, processed ornithine carbamyl transferase (OCT), pOCTΔ22–30, and processed Δ22–30 (*proc.*) are indicated. 37K denotes a 37-kD proteolytic fragment of pOCT (see text). Lane *1*, pSPO19 translation products without protease digestion; lane *2*, as in lane *1* but with proteinase K digestion; lane *3*, pSPOd2 translation products without protease digestion; lane *4*, as in lane *3* but with proteinase K digestion.

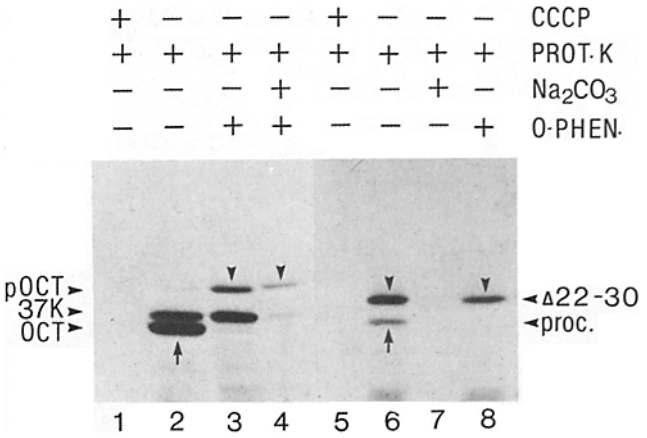


*Figure 4.* Fate of imported pOCTΔ22–30. Import of pOCT (lanes *1–4*) and pOCTΔ22–30 (lanes *5–8*) was performed as described in Fig. 3, in the presence or absence of 5 mM EDTA and 0.1 mM O-phenanthroline (±O-PHEN), or 1.0 μM carbonyl cyanide *m*-chlorophenyl hydrazone (±CCCP). After import, incubation mixtures were treated with or without proteinase K as described in Fig. 3 (±PROT. K), and mitochondria were isolated by centrifugation. Mitochondria were either dissolved directly for analysis by SDS PAGE (−$Na_2CO_3$) or they were suspended in 150 μl 0.1 M $Na_2CO_3$, pH 11.5 (0.2 μg protein/μl), sonicated, incubated on ice for 30 min, and membranes recovered by centrifugation for 10 min in an airfuge operating at 130,000 *g* (+$Na_2CO_3$). Arrows indicate processed products; arrowheads indicate precursors. Abbreviations are as in Fig. 3.

was consistent with the expected size differential resulting from removal of nine amino acids from pOCT (Fig. 3).

### *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 (2, 15, 21), it is not present in the input translation (see Fig. 6, lane *1*), it is not observed in some downstream mutants (see Fig. 6, lane *4*) or the human homologue of pOCT (11), and may result from nonspecific events (2). The 36-kD polypeptide represents bona fide processed product, as determined by sequence analysis of its amino terminus (Fig. 7 *A*).

After treatment of postimport mixtures with proteinase K, all residual pOCT was degraded whereas processed OCT remained unaffected (Fig. 3). The results are consistent with repeated observations that a measurable pool of unprocessed pOCT does not exist inside mitochondria; rather, once pOCT is imported, it is immediately processed to mature product. A very different observation, however, was made for pOCTΔ22–30. After import, pOCTΔ22–30 remained largely unprocessed and was largely resistant to external protease (Figs. 3 and 4). Relatively minor levels of a processed Δ22–30 product appeared during these import assays, but it did not co-migrate with mature OCT which, as demonstrated in Figs. 1 and 2, would have been the case had pOCTΔ22–30 been processed between $gln^{32}$ and $ser^{33}$. The amount of processed Δ22–30 varied from experiment to experiment, 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 [$^3$H]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 $NH_2$-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 $gln^{32}$ and $ser^{33}$, 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 im-

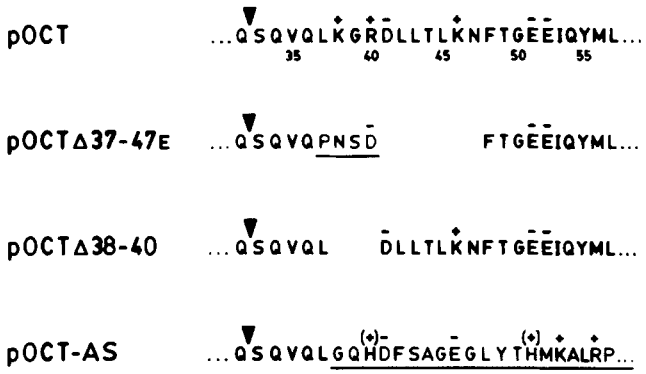


*Figure 5.* Derived amino acid sequences of plasmids encoding pOCT, pOCTΔ37–47E, pOCTΔ38–40, and pOCT-AS. pSP64 plasmids were constructed to encode the four polypeptides as follows: pOCT, see reference 25 and Fig. 2; pOCTΔ37–47E, the Pvu II–Xmnl fragment shown in Fig. 2 was deleted and replaced with a single copy of an Eco Rl linker; pOCTΔ38–40, oligonucleotide-directed loop-out deletion was performed after subcloning in M13, using the oligonucleotide GGTGAGGAGGTCCAGCTGTACTTG, by the technique of Zoller and Smith (31); pOCT-AS, reference 25 describes construction of the plasmid, pSPOA5, encoding a hybrid protein consisting of the first 36 amino acids of pOCT followed by the COOH-terminal 250 amino acids of the cytosolic enzyme of *Escherichia coli*, asparagine synthetase (AS). The one-letter amino acid code is shown for the region downstream of the normal processing site of pOCT, between $gln^{32}$ and $ser^{33}$. Deletions are represented by spaces and substitutions by underlined sequences. The predicted plasmid constructs and their corresponding polypeptide products were confirmed both by DNA sequencing and by amino acid sequencing of [$^3$H]leu-labeled processed polypeptide (Fig. 7). The arrowheads denote the processing site as determined in each case in Fig. 7.

port in vitro. Resistance to external protease was also observed after treatments (e.g., digitonin) that selectively rupture the outer membrane (10, 23) (not shown). However, when import was performed in the presence of carbonyl cyanide *m*-chlorophenyl hydrazone, an uncoupler which collapses the electrochemical potential across the mitochondrial inner membrane, pOCTΔ22–30 was entirely degraded by proteinase K (Fig. 4, lane *5*), suggesting that pOCTΔ22–30 is otherwise imported either into or across the inner membrane. Newly imported pOCTΔ22–30 was extractable with 0.1 M $Na_2CO_3$, pH 11.5 (Fig. 4, lane *7*), a procedure which releases mitochondrial content and membrane-peripheral proteins and converts the organelle to open membrane sheets (8); in this regard, the properties of pOCTΔ22–30 were similar to those of pOCT whose processing had been blocked by *O*-phenanthroline (Fig. 4, lanes *3* and *4*). Taken together, therefore, the results suggest that pOCTΔ22–30 is translocated to the matrix compartment, although it cannot be ruled out that it retains a pH-sensitive association with the matrix-aspect of the inner membrane.

### *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

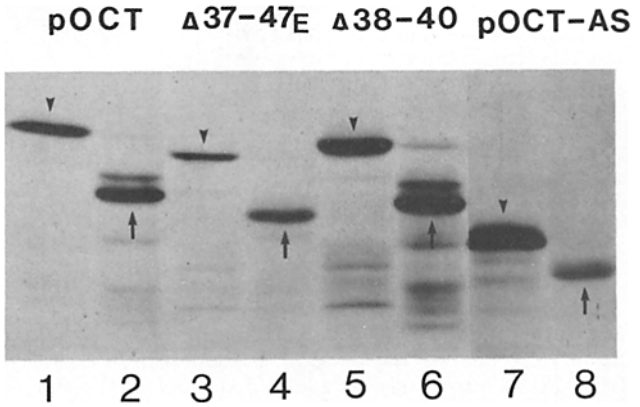


*Figure 6.* In vitro import and processing of pOCT, pOCTΔ37–47E, pOCTΔ38–40, and pOCT-AS. Transcription-translation of the pSP64 derived plasmids was performed as described in Materials and Methods and in Fig. 3; products were analyzed by SDS PAGE and fluorography either directly after translation (lanes *1*, *3*, *5*, and *7*) or after incubation with mitochondria and subsequent proteinase K treatment (lanes *2*, *4*, *6*, and *8*). Arrowheads indicate precursors; arrows indicate processed products.

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 $gln^{32}$ and $ser^{33}$.

## *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 preceeding 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 $gln^{32}$–$ser^{33}$ 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-

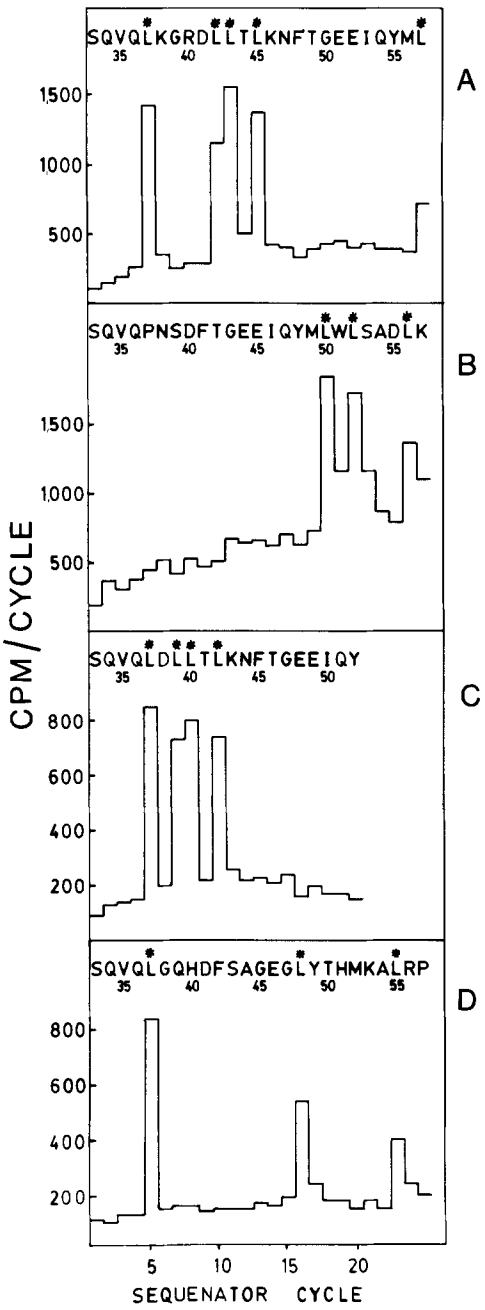


*Figure 7.* Radiosequencing of [$^3$H]leu-labeled products after import and processing of pOCT, pOCTΔ37–47E, pOCTΔ38–40, and pOCT-AS. After import into mitochondria and digestion with proteinase K (Fig. 6), processed products of pOCT, pOCTΔ37–47E, and pOCTΔ38–40 were purified by an immunoaffinity binding 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 [$^3$H]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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