Topogenesis of Mitochondrial Inner Membrane Uncoupling Protein. Rerouting Transmembrane Segments to the Soluble Matrix Compartment

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Abstract. Brown adipose tissue uncoupling protein (UCP), an integral polytopic protein of the mitochondrial inner membrane, is composed of at least six transmembrane segments whose net hydrophobic character derives from paired amphiphilic helices. The protein is synthesized in the cytoplasm as a polypeptide (307 amino acids) lacking a cleavable targeting (signal) peptide. Deletion mutagenesis and fusion protein constructions revealed the existence of at least two import signals: one lying between UCP precursor amino acids 13-105 and the other downstream of position 101. The former resulted in both targeting and membrane insertion of a fusion protein, whereas the latter targeted UCP 102-307 into the organelle but failed to result in membrane insertion. When a strong matrix-targeting signal derived from precarbamoyl phosphate synthetase was fused to UCP amino acids 169-307 or 52-307 (containing three and five transmembrane domains, respectively), the fusion proteins were efficiently imported to the soluble matrix compartment where correct signal cleavage took place. We suggest that assembly of UCP into the inner membrane follows a coordinate insertion pathway for integration and may use more than one signal sequence to achieve this. In this respect, it might share certain mechanistic features with the insertion of polytopic proteins into the endoplasmic reticulum. The data also suggest, however, that integration of the amino-terminal third of UCP into the inner membrane may be required to help or enhance insertion of the remaining UCP transmembrane domains.

MITOCHONDRIAL inner membrane uncoupling protein (UCP)[1] is responsible for thermogenesis in brown adipose tissue by functioning to return protons that have been expelled by the respiratory chain, thus circumventing ATP synthase. The uncoupling of respiration from ATP synthesis results in production of heat (for a review. see reference 19).

UCP shows strong structural similarities to two other inner membrane proteins, the ADP/ATP carrier (2, 28) and phosphate carrier (28). All three contain a similar threefold repeat of ~100 amino acids. In the case of UCP, physical–chemical analyses (16) and computer modeling (2, 28) predict a protein that is largely buried in the lipid bilayer; the amphiphilic character of the transmembrane domains, however, suggest that they are stabilized in the membrane by paired helical structures (2). Runswick et al. (28) predict six transmembrane segments, whereas Aquila et al. (2) suggest a similar arrangement, but with an additional β-strand spanning the bilayer.

The three proteins are the products of nuclear genes; they are synthesized in the cytoplasm and subsequently imported into mitochondria by a posttranslational mechanism (8, 28, 29). UCP and the ADP/ATP carrier are made without a transient (targeting) signal sequence whereas the precursor to the phosphate carrier protein contains an NH$_2$-terminal extension of 49 amino acids (28). In the case of yeast ADP/ATP carrier protein, topogenic information facilitating import has been shown to reside within the amino-terminal one-third (115 amino acids) of the protein (1), and more recently (26) a distal targeting function was identified in the carboxyl-terminal two-thirds of the *Neurospora crassa* homologue.

Although the mechanism of protein insertion into mitochondrial membranes is not well understood, it may share a number of similarities to the analogous process in the endoplasmic reticulum (ER) (5). At least in the case of simple bitopic transmembrane proteins, it has been proposed that two functionally distinct topogenic domains are used: an NH$_2$-terminal matrix-targeting signal and a distal stop-transfer sequence (11, 20, 23). Precursor proteins destined for the matrix lack the stop-transfer domain and, therefore, follow a default pathway analogous to protein sorting in the secretory apparatus (15); the position of the stop-transfer domain relative to the matrix-targeting signal has been found to influence sorting to the outer versus the inner membrane during import into mitochondria in vitro (23).

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1. *Abbreviations used in this paper*: CCCP, carbonyl cyanide m-chlorophenylhydrazone; ER, endoplasmic reticulum; pCPS and CPS, precursor and mature carbamyl phosphate synthetase, respectively; pOCT and OCT, precursor and mature carbamyl transferase, respectively; UCP, uncoupling protein.
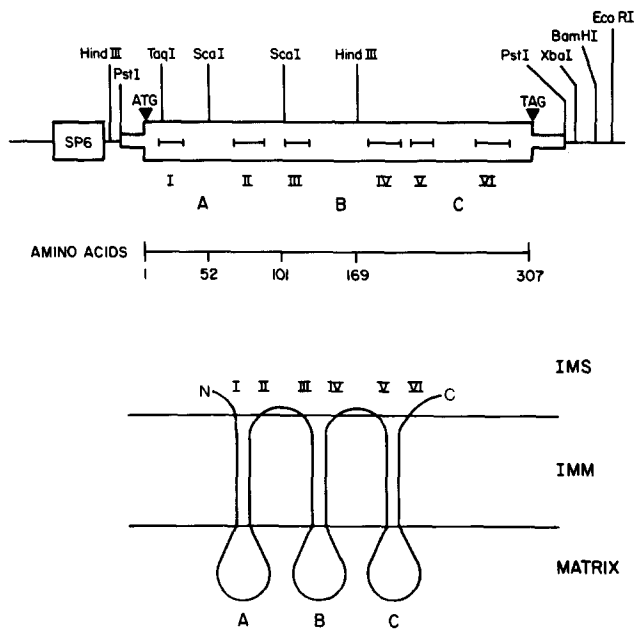
Figure 1. Schematic representation of UCP cDNA and topology of the corresponding polypeptide in the mitochondrial inner membrane. The transmembrane segments of UCP, designated I-VI, connect three relatively hydrophilic domains (*A, B, C*) located at the matrix side of the inner membrane (*IMM*) with short domains in the intermembrane space (*IMS*) (after references 2, 28). The positions of these regions in the linear coding sequence of UCP cDNA are indicated in the upper panel, while the lower panel presents a simplified scheme for their disposition in the inner membrane (2, 28). The middle panel shows the position of certain amino acids of the UCP precursor which has an amino-terminal methionine (amino acid 1) lacking in the mature protein. Recombinant pSP64 constructs. (*Upper panel*) *pSPUCP* encodes full-length UCP polypeptide. UCP cDNA was excised from pUCPrat15 (27) with Pst I and further digested with Taq I to remove the 5'-untranslated region; the Taq I–Pst I fragment was purified and inserted into the Pst I site of the SP64 polylinker via an oligonucleotide adaptor (GACCATGGTGAGTT), which also restored the correct UCP coding sequence. *pSPUCPdl-12* encodes UCP lacking amino acids 1–12 and was constructed as above except that the adaptor, GGTGAGTT, was used for ligation into pSP64. *pSPUCP13-51-OCT* and *pSPU-CP13-105-OCT* encode UCP amino acids 13–51 and 13–105, respectively, attached to 209 carboxyl-terminal amino acids from OCT (21). A Pst I–Bgl II fragment, encoding the amino-terminal 146 amino acids of pOCT, was deleted from pSP019 (21), and the deleted fragment replaced with either of two fragments purified from pSPUCPdl-12 after digestion to completion with Pst I and partial cutting with Sca I; for *pSPUCP13-51-OCT,* the shorter Pst I–Sca I fragment (encoding UCP amino acids 13–51) was inserted directly after blunting of the Bgl II end of the cut plasmid with Klenow enzyme. For *pSPUCP13-105-OCT,* the longer fragment (encoding UCP amino acids 13–101) was inserted via the oligonucleotide linker, ACTTCTCTTCA, which creates a cohesive end for Bgl II and restores UCP up to amino acid 105. *pSPUCP102-307* codes for a carboxyl-terminal fragment of UCP. A Sca I–Pst I fragment coding for UCP amino acids 102–307 was inserted between the Hind III and Pst I sites in the pSP64 polylinker, using an oligonucleotide adaptor (AGCTTGGGCTGCAGACCATGGGGT) which creates a Hind III end (UCP 101-307 begins with met-gly). *pSPCPS-UCP169-307* and *pSPCPS-UCP52-307* encode hybrid proteins containing the amino-terminal 96 amino acids from pCPS (21) fused to the carboxyl-terminal 169–307 or 52–307 amino acids of UCP. They were constructed by purifying a 596-bp Pst I fragment from pHN291 (24) corresponding to the 5'-end of pCPS cDNA, inserting it into

Compared with bitopic transmembrane proteins, UCP presents a different set of problems: first, it is a polytopic protein spanning the membrane at least six times and, second, unlike bitopic proteins anchored by a single, uniformly hydrophobic transmembrane segment, the individual transbilayer segments of UCP are amphiphilic and, therefore, acquire a net hydrophobic character via pairing with another segment (2). In the case of polytopic polypeptides assembled into the ER, the current evidence supports the idea that individual domains are inserted via separate signal sequences (4, 9). In view of the fact that we demonstrate here the existence of at least two targeting signals in UCP, a similar mechanism may apply to polytopic proteins of the mitochondrial inner membrane as well. However, amphiphilic transmembrane domains of UCP lose the ability to insert into the inner membrane when placed in the context of an amino-terminal nonUCP polypeptide. We provide evidence suggesting that insertion of the amino-terminal one-third of UCP into the inner membrane may be required to facilitate insertion of the remainder of the polypeptide.

Materials and Methods

General

Routine procedures for recombinant DNA manipulations, transcription in the pSP64 system, translation in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine, isolation and purification of rat heart mitochondria, and analysis of total import products by SDS-PAGE are described in previous articles (3, 21). Details are provided in the figure legends.
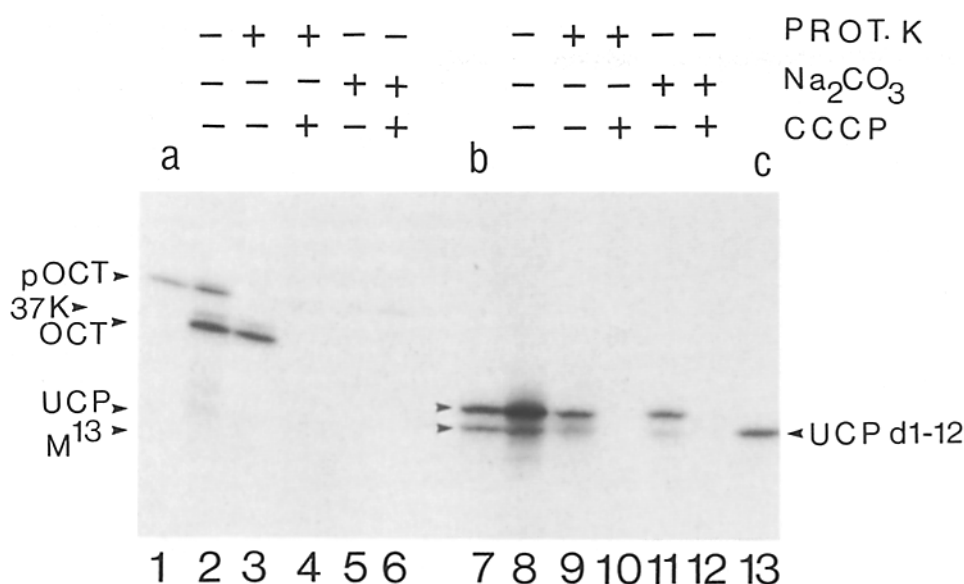
Mitochondrial Import

Recombinant pSP64 transcripts were translated in a messenger-dependent rabbit reticulocyte lysate system containing 1 mCi/ml of [^{35}S]methionine (1,000 Ci/mmol) for 30 min at 30°C. Aliquots were diluted to 75 μl with KMH (10 mM Hepes, pH 7.5, 80 mM KCl, and 2 mM magnesium acetate) and added to 75 μl of freshly purified mitochondria from rat heart uniformly suspended in MRM-succinate (10 mM Hepes, pH 7.5, 250 mM sucrose, 1.0 mM dithiothreitol, 1.0 mM ATP, 0.08 mM ADP, 2.0 mM K$_2$HPO$_4$, pH 7.5, and 5 mM sodium succinate), to yield a final concentration of 0.5 mg/ml mitochondrial protein in the import assay. The mixtures were incubated for 30 min at 30°C, the mitochondria recovered by centrifugation in a microfuge operating for 5 min at 12,000 *g*, and dissolved in hot SDS sample buffer for analysis by SDS-PAGE. Additional details are provided in the figure legends.

Results

The various plasmid constructs used in this study are described in the legend to Fig. 1. They include all or parts of rat UCP cDNA (27) cloned in the pSP64 in vitro expression vector system. We found, however, that removal of the 5'-un-

the Pst I site in the polylinker of pSP64, linearizing at an internal Nco I site, filling in the cohesive ends with Klenow enzyme, and inserting the appropriate UCP cDNA fragment: for *pSPCPS-UCP-169-307,* a Hind III fragment containing the 3' half of UCP cDNA was isolated from pSPUCP, blunt-ended with Klenow, and inserted into the blunt-ended Nco I site of the pCPS plasmid; for *pSPCPS-UCP52-307,* a Sca I–Pst I fragment (blunted with T4 DNA polymerase) encoding UCP amino acids 52–307 was isolated from pSPUCP and inserted into the blunt-ended Nco I site of the pCPS plasmid. For in vitro transcription, the recombinant pSP64 plasmids were linearized with Eco RI.

Figure 2. Import of pOCT and UCP by heart mitochondria in vitro. pSPO19, pSPUCP, and pSPUCPdl-12 were transcribed and translated in a reticulocyte lysate in the presence of [^{35}S]methionine, after which import was carried out as described in Materials and Methods; total products were analyzed by SDS-PAGE and fluorography. (*a*) pOCT; (*b*) UCP; (*c*) UCPdl-12. Lanes *1* and *7*, 20% of input pOCT and 33% of input UCP, respectively; lanes *2* and *8*, mitochondrial pellets after import; lanes *3* and *9*, before isolating mitochondria after import, mixtures were incubated at 4°C for 30 min with 100 μg/ml proteinase K (*PROT.K*), at which time phenylmethylsulfonyl fluoride (final concentration, 2 mM) was added and the mixture incubated for an additional 10 min; lanes *4* and *10*, as in lanes *3* and *9* except that import was performed in the presence of 1.0 μM CCCP; lanes *5* and *11*, after import, mitochondria were recovered, suspended (0.5 μg protein/μl) in 0.1 M Na$_2$CO$_3$, pH 11.5, sonicated vigorously, incubated on ice for 30 min, and the membranes recovered after centrifugation in an airfuge operating at 30 psi for 10 min; lanes *6* and *12*, as in lanes *5* and *11* except that import was performed in the presence of CCCP. Lane *13*, UCPdl-12. The arrowheads denote pOCT, mature OCT, 37K (an intermediate fragment routinely observed during pOCT import and processing in vitro), UCP, UCPdl-12, and M^{13} (resulting from internal initiation of polypeptide synthesis at UCP methionine-13).

translated sequence (or at least the poly GC portion) from UCP cDNA was required for efficient translation to take place.

Fig. 1 indicates the positions of the transmembrane segments (*I–VI*) and extramembrane matrix domains (*A–C*) in the linear cDNA and polypeptide sequences (upper panel), as well as showing a simplified schematic illustration (lower panel) of the disposition of these regions in the mitochondrial inner membrane (2, 28); the extramembrane domains may associate peripherally with the surface of the membrane on the matrix side rather than extend into the matrix space as illustrated (2).

Import of UCP and UCPdl-12

After transcription–translation of UCP cDNA lacking the majority of its 5′-untranslated sequence, two polypeptide products were observed: full-length UCP, with an apparent molecular mass of 32 kD on SDS gels, and a slightly smaller product migrating with a size of ∼30 kD (Fig. 2, lanes *7–12*). Because the latter comigrated with a mutant of UCP in which amino acids 1–12 had been deleted (Fig. 2, lane *13*), the smaller translation product likely derived from polypeptide initiation at an internal AUG coding for methionine at position 13 of the UCP polypeptide sequence (6, 27).

Import and insertion of UCP and UCPdl-12 into the inner membrane of isolated heart mitochondria is demonstrated by the data presented in Figs. 2 and 3; for comparison, import of pOCT, a well-characterized precursor to a matrix protein (13, 22), was also documented. To distinguish between the large amount of high input levels of UCP and UCPdl-12 which sedimented with mitochondria after import incubations (Fig. 2, lane *8*) and that fraction that entered the organelle and assembled into the inner membrane, two criteria were used: acquisition of Δψ-dependent resistance to exogenous proteinase K and Δψ-dependent insolubility in 0.1 M Na$_2$CO$_3$, pH 11.5. Earlier studies (12, 25) have established that a mitochondrial electrochemical potential (Δψ) is required for protein import into or across the inner membrane; thus, incubation in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP), an uncoupler which collapses the electrochemical gradient across the inner membrane, should render input UCP and UCPdl-12 entirely sensitive to exoge-
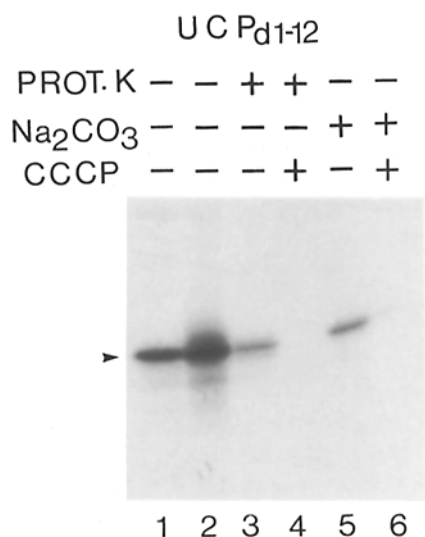
Figure 3. Import of UCPdl-12. Conditions and treatments were the same as in Fig. 2. Lane *1*, one-third of input; lanes *2–6*, mitochondrial pellets. Arrowhead denotes UCPdl-12.
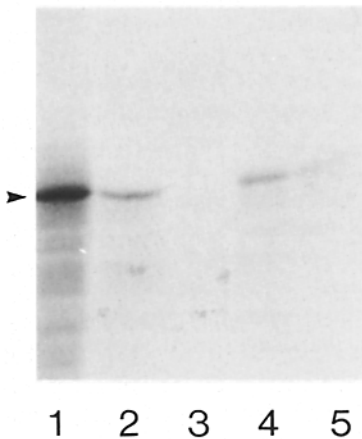
UC P13-51¯OCT

$$- \quad - \quad + \quad - \quad \text{PROT. K}$$
$$- \quad - \quad - \quad + \quad \text{Na}_2\text{CO}_3$$

1 2 3 4
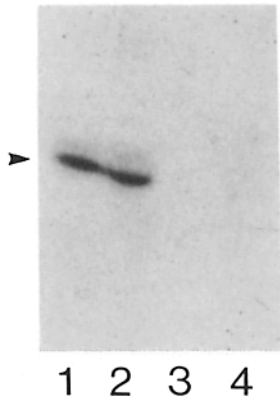
Figure 4. UCP amino acids 13–51 fail to deliver a reporter protein into mitochondria. UCP13-51-OCT was produced after transcription–translation of pSPUCP13-51-OCT (Fig. 1); conditions and treatments were the same as described in Fig. 2. Lane *1*, one-third of input; lanes *2–4*, mitochondrial pellets. Arrowhead denotes UCP13-51-OCT.

UC P13-105¯OCT

$$- \quad + \quad + \quad - \quad - \quad \text{PROT. K}$$
$$- \quad - \quad - \quad + \quad + \quad \text{Na}_2\text{CO}_3$$
$$- \quad - \quad + \quad - \quad + \quad \text{CCCP}$$

1 2 3 4 5

Figure 5. Import of UCP13-105-OCT. The polypeptide was produced by transcription–translation of pSPUCP13-105-OCT (Fig. 1). Conditions and treatments of import assays are described in Fig. 2. Lane *1*, one-third of input; lanes *2–5*, mitochondrial pellets. Arrowhead denotes UCP13-105-OCT.

nous protease. That this was the case is demonstrated in Fig. 2, lanes *9* and *10* (see also Fig. 3). Acquisition of protease resistance, therefore, was due to protein transport into or beyond the inner membrane rather than resulting from UCP gaining access to cryptic sites in the outer membrane. Furthermore, CCCP rendered the input polypeptide completely extractable by alkaline Na_2CO_3 (Fig. 2, compare lanes *11* and *12*). Sonication of mitochondria in 0.1 M Na_2CO_3, pH 11.5, releases the mitochondrial-soluble content (e.g., mature ornithine carbamyl transferase (OCT), Fig. 2, lane *5*) and membrane peripheral proteins and converts the organelle to open membrane sheets composed of a phospholipid bilayer with retained integral proteins (10); the fact that UCP was entirely released by this procedure after incubation in the presence of CCCP (Fig. 2, lane *12*) suggests that imported UCP otherwise acquires resistance to alkaline extraction as a consequence of a strong interaction with the inner membrane phospholipid bilayer.

As illustrated in Figs. 2 and 3, UCPdl-12 (M^{13} in Fig. 2) appeared to import with somewhat less efficiency than full-length UCP; in other experiments, however, such a difference was not observed. Thus, to avoid polypeptide initiation at two sites in UCP mRNA, hybrid proteins were constructed using the UCPdl-12 derivative.

Topogenesis Conferred by UCP Amino Acids 13–105

To assay the presence of topogenic information located toward the amino terminus of UCP, two hybrid proteins were constructed in which UCP amino acids 13–51 or 13–105 (see Fig. 1) were fused to a reporter polypeptide. For the latter, we used a COOH-terminal 209–amino acid fragment from OCT; this fragment does not itself carry targeting information but is imported when fused behind a heterologous mitochondrial targeting signal (20). UCP13-105-OCT (Fig. 5), but not UCP13-51-OCT (Fig. 4), was imported by mitochondria in vitro, though not with the same efficiency as UCP or UCPdl-12 (Figs. 2 and 3). Furthermore, by the criterion of $\Delta\psi$-dependent acquisition of resistance to alkaline extraction (Fig. 5, lanes *4* and *5*), UCP13-105-OCT appeared to be delivered and inserted into the inner membrane; the low amount of carbonate-resistant product seen in Fig. 5, lane *5*, may reflect nonspecific binding to membranes. In the case of UCP13-51-OCT, a large fraction of input polypeptide sedimented with mitochondria after import but it remained sensitive to external protease (Fig. 4). Although the data show that UCP amino acids 13–51 provide insufficient information to direct import, they do not rule out the possibility that this region contributes to the overall process. Indeed, deletion of this region (d2-51) was found to severely retard UCP import (data not shown).

Large Carboxyl-terminal Fragment of UCP Is Inefficiently Imported

To extend the findings that UCP amino acids 13–105 are sufficient to direct both import and membrane insertion of a chimeric protein (Fig. 5), we examined the possibility that a second set of topogenic sequences may reside downstream of this region in UCP. A deletion in UCP cDNA was performed (Fig. 1) in which codons specifying UCP amino acids 1–101 were removed and replaced with codons specifying met-gly; transcription–translation of the mutant cDNA re-
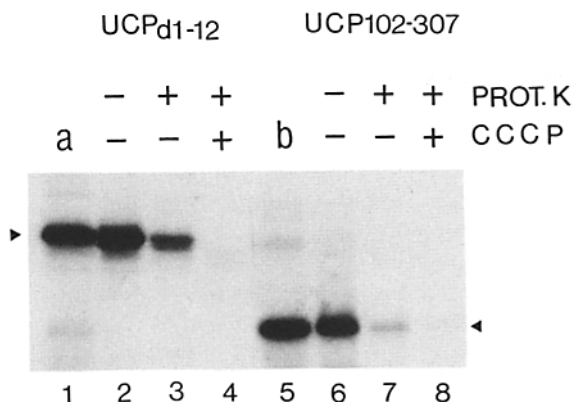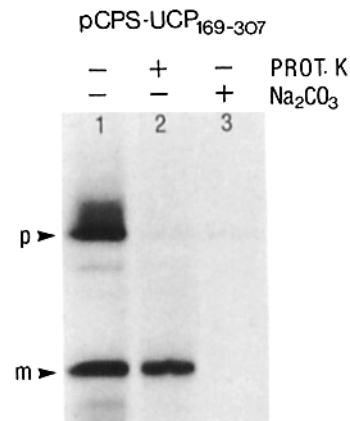
Figure 6. Import of UCPd1-12 and UCP102-307. The two polypeptides were produced by transcription-translation of (a) pSPUCPd1-12 and (b) pSPUCP102-307. Conditions and treatments of import assays are given in Fig. 2. Lanes 1 and 5, one-third of input; lanes 2–4 and 6–8, mitochondrial pellets. Arrowheads denote UCPd1-12 and UCP102-307.

Figure 8. Radiosequence analysis of [³H]leucine-labeled product after import and processing of pCPS-UCP169-307. The import described in Fig. 7 was repeated except that [³H]leucine replaced [³⁵S]methionine in the translation mixture; after import and proteinase K treatment, the product designated m in Fig. 7 was electroeluted and subjected to automated Edman degradation on a gas-phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA), as previously described (22). Correspondence between peaks of radioactivity at cycles 1 and 12 with positions of leucine in the amino acid sequence of mature CPS indicates correct processing between leu[38] and leu[39] (24) of the precursor polypeptide.
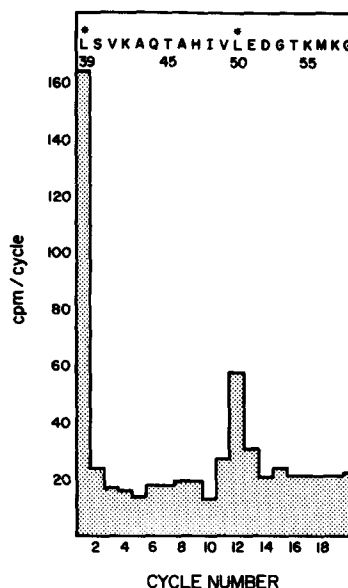
sulted in the synthesis of a polypeptide beginning with met-gly followed by amino acids 102–307 of UCP (designated UCP102-307). The fragment was efficiently synthesized in vitro (Fig. 6 b). After addition of mitochondria, a small portion was imported and rendered inaccessible to exogenous protease in a Δψ-dependent manner (Fig. 6, compare lanes 7 and 8). Compared with import of UCPd1-12 under identical conditions, however, the extent of import of UCP102-307 was relatively modest, attaining levels of only about one-fifth of those observed for the product of UCPd1-12 import (Fig. 6, compare lanes 3 and 7), as determined by densitometric analysis. Furthermore, in a number of separate experiments, we routinely found that UCP102-307 after import was not resistant to extraction with alkaline Na₂CO₃ (not shown), indicating its lack of integration into the mitochondrial inner membrane bilayer.

Strong Matrix-targeting Signal Translocates UCP Transmembrane Domains to the Soluble Matrix Compartment

In view of the fact that UCP102-307 was imported into mitochondria but did not integrate into the inner membrane, the possibility arises that such integration cannot occur without
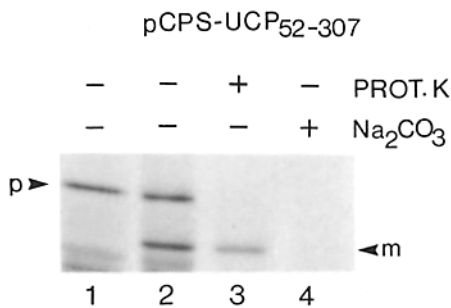

Figure 7. Import and processing of pCPS-UCP169-307. The precursor was produced by transcription–translation of pSPCPS-UCP169-307 (Fig. 1). Conditions and treatments of import assays are described in Fig. 2. Lane 1, mitochondrial pellet; lane 2, treatment with proteinase K; lane 3, treatment with 0.1 M Na₂CO₃, pH 11.5. p, precursor; m, processed product.

prior insertion of the amino-terminal one-third of UCP. To examine this idea further, and to ensure that transport into mitochondria follows a standard import pathway, carboxyl-terminal fragments of UCP comprising either three or five transmembrane segments (Fig. 1) were fused behind a strong matrix-targeting signal derived from pCPS (pCPS and CPS are precursor and mature carbamoyl phosphate synthetase, respectively); the hybrid proteins were designated pCPS-UCP169-307 and pCPS-UCP52-307, respectively (Fig. 1). The contribution from pCPS corresponded to its amino-terminal 96 amino acids, the first 38 of which comprise the signal sequence (21, 24).

As shown in Fig. 7, pCPS-UCP169-307 was imported into purified heart mitochondria in vitro and processed to mature product (Fig. 7, lane 1); the processed product but not the precursor was resistant to exogenous proteinase K (Fig. 7, lane 2), import was prevented by CCCP (not shown) indicating that translocation was otherwise into or across the inner membrane, and imported mature product was not retained with membrane after treatment with alkaline Na₂CO₃ (Fig. 7, lane 3). Radiosequencing analysis (Fig. 8) revealed that pCPS-UCP169-307 had been cleaved at the normal pCPS processing site between leu[38] and leu[39] of pCPS (24), indicating that processing took place in the matrix where mitochondrial signal peptidase is located (7, 18). The sequencing data, together with the CCCP and Na₂CO₃ observation, demonstrate that pCPS-UCP169-307 was imported across the inner membrane and deposited in the soluble matrix

pCPS-UCP$_{52-307}$

	−	−	+	−	PROT. K
	−	−	−	+	Na$_2$CO$_3$

p▶

◀m

1 2 3 4

Figure 9. Import and processing of pCPS-UCP-52-307. The precursor polypeptide was produced by transcription–translation of pSPCPS-UCP52-307 (Fig. 1). Conditions and treatments of import assays are described in Fig. 2. Lane *1*, one-third of input; lanes *2-4*, mitochondrial pellets. (*p* and *m*) Precursor and processed pCPS-UCP52-307, respectively.

compartment where it was correctly processed to remove the pCPS targeting sequence. Similar results were obtained for pCPS-UCP52-307 (Fig. 9).

Finally, the fate of UCP13-307 (i.e., UCPdl-12) which otherwise is imported and inserted into the mitochondrial inner membrane (Figs. 2, 3, and 6) was also examined when fused behind either the pCPS or pOCT signal sequence. The hybrid proteins were very inefficiently imported and processed, suggesting that the primary translation product largely assumed an import incompetent conformation; nevertheless, the small component that was imported was also freed from membrane after alkaline extraction (not shown). It would appear, therefore, that the ability of UCP amino acids 13-101 to signal membrane insertion (Fig. 5) was abrogated by the presence of a strong matrix-targeting signal located upstream of this region.

Discussion

The aims of this study were twofold: (*a*) to identify regions in the UCP primary translation product that contain topogenic information for import and insertion into the mitochondrial inner membrane, and (*b*) to determine if fragments of UCP containing multiple transmembrane segments can insert into the inner membrane when fused behind a strong matrix-targeting signal derived from the amino terminus of a heterologous protein, pCPS.

Our results indicate that the amino-terminal one-third of UCP is essential for both import and membrane insertion of UCP. Upon deletion of this region, however, a second import signal located within the carboxyl-terminal two-thirds of the molecule was detected, but the downstream import signal supported only inefficient uptake into mitochondria and did not mediate membrane insertion. Whether or not this latter signal functions in the intact polypeptide is not known, but its presence is interesting in view of the fact that multiple signal sequences appear to play an important role in assembling polytopic integral proteins into the ER (4, 9). Our findings, however, suggest that insertion of the amino-terminal third of UCP (i.e., the first repeat domain, I-A-II, Fig. 1) into the mitochondrial inner membrane may be required to facilitate integration of the remainder of the polypeptide, perhaps by inducing an appropriate conformation in the carboxyl-ter-

minal fragment so that the second signal sequence can help direct insertion. Because UCP is composed of a threefold repeat structure (2, 28), we are currently investigating the possibility that downstream signals reside within each of the last two (III-B-IV and V-C-VI, see Fig. 1).

To date, studies on the mechanism of insertion of proteins into mitochondrial membranes have concentrated on bitopic polypeptide precursors containing a single hydrophobic transmembrane domain located downstream of an amino-terminal matrix-targeting signal. The evidence suggests that in such situations the membrane anchor can function as a stop-transfer sequence, causing arrest of the polypeptide en route to the matrix and consequent insertion into either the outer or inner membrane phospholipid bilayer (11, 23). In this respect, the mechanism of sorting and membrane insertion of mitochondrial proteins may be analogous to that of the ER (5); indeed, viral ER stop-transfer sequences have been shown to function as membrane anchors in mitochondrial membranes (20, 23), though apparently not in chloroplasts (17).

A variation on this model might be necessary to explain the mechanism of membrane insertion for proteins such as UCP and the ADP/ATP carrier in which the targeting sequence is not at the extreme amino terminus and which lack uniformly hydrophobic transmembrane segments. The amphiphilic transmembrane segments of UCP would presumably acquire a hydrophobic character sufficiently compatible with a lipid environment as a result of interactions with other segments in the protein; insertion into the membrane of these interacting segments could occur during import led by internal targeting signals (perhaps localized in the ectodomains A, B, and C, see Fig. 1). It might be expected, therefore, that alterations to the import-competent conformation of UCP might disrupt such interactions and prevent polypeptide arrest and assembly into the inner membrane. This would explain our findings that pCPS-UCP169-307, pCPS-UCP52-307, and perhaps UCP102-307 were all imported but failed to insert into the inner membrane, despite the fact that they contain multiple segments which otherwise span the bilayer.

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