An Amino-terminal Signal Sequence Abrogates the Intrinsic Membrane-targeting Information of Mitochondrial Uncoupling Protein*

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Mitochondrial uncoupling protein, a polytopic integral protein of the inner membrane, is initially made in the cytoplasm as a soluble polypeptide (307 amino acids) lacking a cleavable targeting (signal) peptide. Earlier studies (Liu, X., Bell, A. W., Freeman, K. B., and Shore, G. C. (1986) J. Cell Biol. 107, 503-509) identified internal regions of the molecule that are critical for targeting and membrane insertion. Here, we demonstrate that the ability of uncoupling protein to insert into the inner membrane is abrogated when the molecule is fused behind the matrix-targeting signal of preornithine carbamyltransferase; the hybrid protein was imported across the inner membrane and deposited in the matrix where it was processed. In this context, however, the processed product remained in the matrix and was incapable of inserting into the inner membrane.

Uncoupling protein (UCP)1 is an integral protein of the mitochondrial inner membrane in brown adipose tissue (1). It shares strong structural similarities with two other proteins of the inner membrane, ADP/ATP carrier (AAC) and phosphate carrier (2, 3). All three consist of a 3-fold repeat of two membrane-spanning segments connecting an ectodomain, which, in the case of AAC and UCP, is exposed to the matrix and was incapable of inserting into the inner membrane.

Experiments using the amino acid sequence of UCP were performed in accordance with 18 U.S.C. Section 1734 solely to indicate the amino-terminal third of the molecule. Whether insertion occurs during unidirectional translocation of UCP across the inner membrane or whether UCP follows a “conservative” sorting pathway in which the molecule is translocated first to the soluble matrix compartment and then back into the inner membrane (10) is not presently known.

Here, we have examined the fate of a hybrid molecule in which the entire 3-fold repeat structure of AAC, containing all of the requisite topogenic information for mitochondrial targeting and membrane insertion, is placed behind a matrix-targeting signal derived from the precursor to the matrix enzyme, ornithine carbamyltransferase. The hybrid protein was imported to the matrix compartment where the preornithine carbamyltransferase signal sequence was removed. The resulting UCP molecule remained in the matrix rather than inserting into the inner membrane.

**EXPERIMENTAL PROCEDURES**

**General**—Routine procedures for recombinant DNA manipulation, transcription in the pSP64 system, translation in a rabbit reticulocyte lysate in the presence of [35S]methionine, isolation of rat heart mitochondria, in vitro import into mitochondria, and analysis of import products by SDS-PAGE and fluorography were described previously (5, 11, 12). Further details are provided in the figure legends.

**Construction of pSPp-UCP, the Plasmid Encoding p0-UCP**—pSPUCP (5) was linearized with SphI and then partially digested with BglII to delete a fragment encoding the NH₂-terminal 16 amino acids of AAC. It was replaced with two fragments: an SphI-PvuII fragment from pUC19 (12) encoding the entire signal sequence of preornithine carbamyltransferase and the first 4 amino acids of the mature enzyme (UCP1) and a PvuII-BglII oligonucleotide adaptor, CTGCAACACCCACGGGTTCA, that restores the BglII site and amino acids 10–16 of UCP except for amino acid 13 (methionine); it also restores the PvuII site and the 5th amino acid of processed preornithine carbamyltransferase.

**Succinate Cytochrome c Oxidoreductase Activity—Mitochondria (50 μg of protein) were added to 1 ml of assay medium (exactly as described in Ref. 13) to initiate the reaction. After 2 min, 10 μl of 1 N HCl were added and the absorbance at 550 nm determined. Total enzyme activity was routinely observed to be ~100 nmol of cytochrome c reduced/mg of protein/min, which is in good agreement with published findings (13).**

**RESULTS AND DISCUSSION**

Transcription-translation of UCP cDNA in a pSP64 vector yields two products. Analysis of these products by SDS-PAGE is consistent with translation initiation occurring at UCP codons 1 and 13 (5). Both products, designated UCP and UCP12, respectively, are imported to the inner membrane of rat heart mitochondria in vitro, as judged by their acquisition of Δν-dependent resistance to both exogenous protease and extraction at pH 11.5 (5). UCP is not made as a larger
precursor molecule so that the requirement of an electrochemical potential for membrane insertion is necessary to distinguish between product inserted into the inner membrane (Δψ-dependent) and product adventitiously and perhaps cryptically associated with the surface of the organelle (Δψ-independent) (5). As illustrated previously, the deletion mutant UCPd112 is imported and inserted into the inner membrane indicating that UCP amino acids 1–12 are dispensable for both targeting and membrane anchoring.

Import of pO-UCP—cDNA encoding UCP was inserted into the pSP64 vector and was then modified to include a cDNA fragment encoding the first 37 amino acids of preornithine carbamyltransferase fused to UCP amino acids 10–307; as well, the ATG codon at UCP amino acid position 13 was deleted to avoid internal initiation of translation at this position. Thus, removal of the preornithine carbamyltransferase signal sequence (32 amino acids) from the hybrid protein would yield a “mature” product in which 5 amino acids of mature processed preornithine carbamyltransferase (SQVQVL) replace 9 amino acids (MYSSTTSEV) from the NH2-terminus of UCP. The intrinsic targeting information of UCP, however, exists downstream of amino acid 12 (5).

Synthesis of pO-UCP in a rabbit reticulocyte lysate in vitro yielded a single polypeptide product with an expected size of ~37 kDa (Fig. 1, lane 1). The hybrid precursor polypeptide was imported and processed by isolated mitochondria (Fig. 1, lane 4) in a manner that was dependent on an electrochemical potential across the inner membrane (Fig. 1, lanes 2 and 3); precursor on the surface of the organelle was sensitive to exogenous protease, whereas the processed product was protected (Fig. 1, lanes 3 and 5). The extent and characteristics of pO-UCP import into mitochondria were very similar to that observed for UCPd112, except of course that processing of UCPd112 to a smaller product did not take place (Fig. 1, lanes 6–10).

Because pO-UCP was processed to the expected size following import into mitochondria in vitro, its NH2-terminal preornithine carbamyltransferase signal sequence presumably gained access to the matrix compartment where the Zn2+-dependent preornithine carbamyltransferase processing enzyme is located (14). As expected, therefore, import of pO-UCP in the presence of o-phenanthroline, a Zn2+ chelator, resulted in partial inhibition of precursor processing, with the result that the accumulated precursor acquired resistance to exogenous trypsin (Fig. 2, lane 2); this is in contrast to import in the absence of chelator in which all of the full-size precursor that cosedimented with mitochondria was sensitive to the protease (Fig. 1, lanes 3 and 5).

Localization of Precursor and Processed pO-UCP—An initial examination of processed pO-UCP inside mitochondria revealed that it was not integrated into a lipid bilayer and remained completely extractable by 0.1 M Na2CO3, pH 11.5 (not shown). This analysis was extended to include the full-size precursor that was allowed to accumulate in a protease-resistant compartment following import in the presence of o-phenanthroline (Fig. 2, lane 2); it too was extracted by alkali (Fig. 2, lane 3). When the inhibition of processing was relieved by the addition of excess Zn2+, most of the precursor was processed (Fig. 2, lane 4), and the resulting processed product was released by alkaline treatment (Fig. 2, lane 5). This is in contrast to imported UCPd112, which was resistant to alkaline extraction (Fig. 2, lanes 7 and 8). Following import, mitochondria were treated with trypsin to remove UCP adhering to the surface of the organelle (Fig. 1) and were then extracted with 0.1 M Na2CO3, pH 11.5, and the membranes recovered by high speed centrifugation (Fig. 2). The percent recovery of imported UCPd112 after protease, then alkaline, treatment was similar to the recovery of an endogenous marker for integral proteins of the inner membrane of heart mitochondria, ADP/ATP carrier protein (not shown).

The fact that import of pO-UCP was dependent on an electrochemical potential (Fig. 1) provides additional support for the role of UCPs in the regulation of mitochondrial potential. 

![Figure 1](image1.png)

**Fig. 1.** pO-UCP is imported and processed by rat heart mitochondria in vitro. Plasmids encoding pO-UCP and UCPd112 were transcribed and translated in a rabbit reticulocyte lysate in the presence of [35S]methionine. Import was carried out for 30 min, as described in Ref. 5, in the presence (lanes 2, 3, 7, and 8) or absence (lanes 4, 5, 9, and 10) of 1 μM carbonyl cyanide m-chlorophenylhydrazine (CCCP). Samples were divided into two aliquots. One aliquot (lanes 3, 5, 8, and 10) was incubated with trypsin (TRY), final concentration 50 μg/ml at 4°C for 30 min at which time soybean trypsin inhibitor was added to 1 mg/ml. The other aliquot (lanes 2, 4, 7, 9) was incubated with both soybean trypsin inhibitor and trypsin (mock trypsin treatment). Mitochondria were recovered by centrifugation and analyzed by SDS-PAGE and fluorography. Lanes 1 and 6 represent 10% of input pO-UCP and UCPd112, respectively. p and m, precursor and mature forms of pO-UCP, respectively.

![Figure 2](image2.png)

**Fig. 2.** Precursor and processed pO-UCP are released by alkaline extraction following import into mitochondria. Lanes 2–5, import of pO-UCP was performed as described in Fig. 1 but in the presence of 1 mM o-phenanthroline (O-PHE) and 5 mM EDTA. After trypsin treatment (Fig. 1), mitochondria were collected by a brief centrifugation at 12,000 × g and resuspended in import medium (11) containing 500 μg/ml soybean trypsin inhibitor. Samples were divided into two aliquots: to one aliquot was added 0.5 mM each of o-phenanthroline and EDTA (lanes 2 and 3) to maintain inhibition, while the other aliquot (lanes 4 and 5) received ZnCl2 to a final concentration of 1.5 mM. Both aliquots were incubated at 30°C for 30 min. Excess Zn2+ (lanes 4 and 5) was chelated by the addition of 1 mM o-phenanthroline and 5 mM EDTA, followed by incubation at 30°C for 10 min. Samples were divided: mitochondria in one aliquot (lanes 3 and 5) were extracted with 0.1 M Na2CO3, pH 11.5, and the membranes recovered by high speed centrifugation (6); the other aliquot was untreated. Mitochondria or the derived alkali-insoluble membrane fraction were analyzed by SDS-PAGE and fluorography. Lane 1 represents 20% of input pO-UCP; lane 6, 20% of input UCPd112; lanes 7 and 8, import of UCPd112 followed by trypsin treatment as described in Fig. 1; lane 7, mitochondrial pellet; lane 8, membrane pellet following extraction with 0.1 M Na2CO3, pH 11.5.
The other aliquot was examined for succinate-cytochrome c oxidoreductase activity as described under "Experimental Procedures." Enzyme activity was measured relative to the activity recorded after mitochondrial protein (PRO), respectively. OCT, processed preornithine carbamyltransferase.

Mitochondria were resuspended in import medium (0.5 mg protein/ml) (11). The mitochondria were frozen in a dry ice-acetone bath and thawed. Trypsin was added to a final concentration of 25 µg/ml, and samples were incubated on ice for the indicated period of time. 100 µl were withdrawn at each time point, and soybean trypsin inhibitor was added (500 µg/ml) to inhibit further digestion. The mitochondria were recovered and analyzed by SDS-PAGE and fluorography. T1 and T2, tryptic fragments of UCPd1-12 (see text).

FIG. 3. Effect of digitonin on the accessibility of precursor (p) and mature (m) forms of pO-UCP to trypsin. Following import of preornithine carbamyltransferase and pUCPd1-12 into mitochondria, digitin (twice recrystallised from ethanol) was added to a final concentration as indicated. Incubation was at 4 °C for 15 min after which the mitochondria were collected by centrifugation. Mitochondria were resuspended in import medium (11) and the resuspended divided into two aliquots. One aliquot was treated with trypsin (100 µg/ml) and analyzed by SDS-PAGE and fluorography. The other aliquot was examined for succinate-cytochrome c oxidoreductase activity as described under "Experimental Procedures." Enzyme activity was measured relative to the activity recorded after mitochondria were incubated in 10 mM Tris-HCl, pH 8.0, and 25 mM sucrose, conditions which osmotically rupture the mitochondrial outer membrane. Numbers 1-6 on the bar graph correspond to treatments with 0, 0.1, 0.25, 0.5, 0.75, and 1.0 µg of digitonin (DIG)/mg of mitochondrial protein (PRO), respectively. OCT, processed preornithine carbamyltransferase.

FIG. 4. The processed form of pO-UCP lacks a trypsin-sensitive domain extending into the intermembrane space. Import of UCPd1-12 and pO-UCP was performed as in Fig. 1. After a brief digestion (5 min on ice) by trypsin (50 µg/ml) to remove precursor proteins on the surface of the mitochondria, the protease was inhibited by 1 µg/ml soybean trypsin inhibitor. Mitochondria were collected, washed to remove the trypsin inhibitor, and resuspended in import medium (0.5 mg protein/ml) (11). The mitochondria were frozen in a dry ice-acetone bath and thawed. Trypsin was added to a final concentration of 25 µg/ml, and samples were incubated on ice for the indicated period of time. 100 µl were withdrawn at each time point, and soybean trypsin inhibitor was added (500 µg/ml) to inhibit further digestion. The mitochondria were recovered and analyzed by SDS-PAGE and fluorography. T1 and T2, tryptic fragments of UCPd1-12 (see text).

reflected partial damage to the inner membrane; by 1.0 µg of digi-tonin, complete sensitivity to trypsin of the three polypeptides was observed as well as a net loss in total reductase activity (Fig. 3), indicating that the inner membrane was severely damaged, resulting in accessibility of the matrix compartment to the protease.

Finally, Ekerskorn and Klingenberg (15) have recently observed that native UCP in brown adipose mitochondria yields two fragments upon partial digestion by trypsin of mitochondria that were frozen and thawed to disrupt the outer membrane. The major fragment (T1) was ~30 kDa in size and was generated by removal of ~2 kDa from the COOH-terminus of UCP; a minor fragment (T2) of ~25 kDa arose with a time delay from T1 (15). As shown in Fig. 4, fragments similar to those demonstrated in Ref. 15 (T1 and T2) were obtained when rat heart mitochondria containing newly imported UCPd1-12 were treated under similar conditions; treatment of frozen-thawed mitochondria with trypsin for progressively longer times led to the disappearance of full-size UCPd1-12 and the appearance of T1 and T2 (Fig. 4, lanes 1–6). This is in contrast to the situation for processed pO-UCP which remained largely intact following trypsin treatment of frozen-thawed mitochondria (Fig. 4, lanes 7–12). These findings (Fig. 4) are consistent with the conclusion that UCPd1-12 was integrated into the inner membrane in a disposition similar to native UCP, while processed pO-UCP resided in the matrix. The minor band that appeared immediately below processed pO-UCP in mitochondria that had been treated with trypsin for 60–90 min (Fig. 4, lanes 11 and 12) was also evident in certain incubations that had not received trypsin treatment (e.g. see Fig. 1, lane 4) and, therefore, might result from the action of endogenous proteases over the extended period of these incubations.

CONCLUDING REMARKS

The polytopic disposition of UCP in the inner membrane of mitochondria is determined by multiple topogenic signals which are located within amino acids 12–105 as well as downstream of this position (6). Here, we have demonstrated that the membrane-anchoring function of UCP, which results in the assembly of 3 pairs of amphiphilic α-helices spanning the inner membrane (2, 3), is abrogated when UCP amino acids 10–307 are fused behind a matrix-targeting signal derived from preornithine carbamyltransferase. In this context, the fusion protein is translocated to the soluble matrix compart-
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...ment of mitochondria in vitro where it is processed by the Zn$^{2+}$-dependent matrix-processing enzyme. Presumably, the presence of the preornithine carbamyltransferase signal sequence confers a conformation to the rest of the molecule which is no longer compatible with insertion into the inner membrane, i.e. via paired amphiphilic helices led by internal matrix-targeting domains (5). It may also be that UCP and pO-UCP employ different receptors for import (10) and that only the UCP receptor is capable of presenting the protein to the inner membrane in a form competent for insertion. Interestingly, however, insertion into the inner membrane is abrogated even after the preornithine carbamyltransferase signal sequence has been removed in the matrix. Either an insertion-incompetent conformation is retained in the processed form of the protein or the machinery requisite for protein integration into the inner membrane is not available to the processed product when the protein is presented to the membrane from the matrix side.

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