IMPORT OF PROTEINS INTO MITOCHONDRIA: BIOGENESIS OF THE UNCOUPLING PROTEIN AND IDENTIFICATION OF A MITOCHONDRIAL SIGNAL PEPTIDE BINDING PROTEIN

by

XINGQUAN LIU

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Department of Biochemistry

McGill University

Montréal, Québec

ABSTRACT

The inner membrane uncoupling protein (UCP) of rat brown fat mitochondria has been imported into rat heart mitochondria *in vitro*. Two import signals have been detected in UCP. The intrinsic membrane insertion information of UCP has been abrogated by a signal sequence fused in front of UCP, resulting in the rerouting of UCP into the matrix. Following removal of the signal sequence from the hybrid protein, the UCP moiety remained in the soluble matrix space indicating an incompatibility of UCP insertion into the inner membrane from the matrix side.

An integral mitochondrial membrane protein (p30) that binds a mitochondrial signal peptide in intact mitochondria *in vitro* has been purified by an affinity approach. The protein has been identified as a member of the ADP/ATP carrier (AAC) family based on both immunoblotting and peptide mapping. The irreversible association of the signal peptide with AAC in intact mitochondria has been correlated with inhibition of protein import into the organelle.

RESUME

La protéine de découplage (PDC ou termogénine), présente dans la membrane interne des mitochondries de la graisse brune de rat, a été importée *in vitro* dans des mitochondries de rat. Deux signaux importants pour l'importation de PDC ont été démontrés. L'information intrinsèque nécessaire pour l'insertion membranaire de PDC a été abragée par une séquence signale d'importation attachée en avant de la partie codante de PDC, résultant en une relocalisation de PDC dans la matrice mitochondriale. Suivant le clivage de la séquence signale de la protéine hybride, PDC est resté dans la partie soluble de la matrice mitochondriale, indiquant une incompatibilité d'insertion de PDC à partir de la face matricielle de la membrane interne.

Une protéine intégrale de la membrane mitochodriale (p30), qui attache un peptide signal mitochondrial *in vitro* en présence de mitochondries intactes, a été purifiée en utilisant une approche d'affinité. La protéine a été identifiée comme étant un membre de la famille des transporteurs d'ADP/ATP (TAA) en se basant sur des essaies immunologiques et de la cartographie de peptides. L'association irréversible du peptide signal avec TAA en présence de mitochondries intactes a montré être en corrélation avec l'inhibition de l'importation de protéines de l'organelle.

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PREFACE

In accordance with the regulations described in item 7 of the 'Guidelines Concerning Thesis Preparation' of McGill University Faculty of Graduate Studies and Research, as cited in full below, and as approved by the Department of Biochemistry, three manuscripts published or to be published have been incorporated into this thesis.

'The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.
It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary material is almost always necessary.
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Chapter 2. Liu, X., Bell, A.W., Freeman, K.B., and Shore, G.C. 1988. J. Cell Biol. 107:503-509.

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Except for figure 6A in Chapter 4 which was performed by Dr. Laura Gillespie, and the radiosequence analysis described in figure 8 of Chapter 2 which was performed by Dr. Alex Bell, the work described in all three manuscripts is my own. Dr. Karl Freeman provided the full length UCP cDNA

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ASGP	asialoglycoprotein
AAC	ADP/ATP carrier protein
ATP	adenosine triphosphate
BPTI	bovine pancreas trypsin inhibitor
BS^3	bis(sulfosuccinimidyl)suberate
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cDNA	complementary deoxyribonucleic acid
COXIV	cytochrome oxidase subunit IV
DHFR	dihydrofolate reductase
ER	endoplasmic reticulum
$F_1\beta$	F_1 ATPase β subunit
GIP	general insertion protein
hsp60 and hsp70	heat shock proteins of 60 kDa and 70 kDa, respectively
kB	kilobase pairs
kDa	kilodaltons
MBP	maltose binding protein
MPP	matrix processing protein
OCT	ornithine carbamyl transferase
OmpA	outer membrane protein A (*E. coli*)
p30	a 30 kDa mitochondrial integral membrane protein that binds pO(1-27) *in vitro*
PEP	processing enhancing protein
pO(1-27) and pO(16-27)	synthetic peptides corresponding to amino acids 1-27 and 16-27 of pOCT respectively
pO-DHFR or	hybrid proteins bearing the signal sequence of pOCT

and pO-UCP	C-terminlly fused mouse DHFR or UCP, respectively
S	sedimentation coefficient in Svedberg unit
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR	SRP receptor
SRP	signal recognition particle
SSR	signal sequence receptor
tRNA	transfer ribonucleic acid
UCP	uncoupling protein
VSV-G	vesicular stomatitis virus glycoprotein

CHAPTER 1

INTRODUCTION

1.1 PREFACE

Except for a limited number of proteins that are encoded and synthesized within mitochondria and chloroplasts, all other proteins are synthesized in the cytoplasm. Yet, many of them have to be transported across one or more intracellular membranes to a different cellular compartment or outside the cell. Mechanisms governing these diverse processes of intracellular protein transport have been the focus of much intensive research for the last decade. These include protein translocation across the endoplasmic reticulum (ER) and subsequent transport through the Golgi apparatus and delivery to the cell surface or lysosomes, protein import into mitochondria and chloroplasts, protein import into peroxisomes and the nucleus, and protein export in bacteria.

For the purpose of this thesis, I will attempt to discuss, in some detail, the literature on protein import into mitochondria. I have also included a brief discussion on protein translocation across the ER and bacterial plasma membranes. Finally, I have organized the mitochondrial literature into three parts directly related to the three chapters in this thesis that describe my research.

1.2 PROTEIN TRANSLOCATION ACROSS THE ER AND BACTERIAL PLASMA MEMBRANES

Most eukaryotic secretory proteins and bacterial proteins destined for export through the plasma membrane contain transient sequences (1) at the N-termini termed 'signal sequences' or 'leader sequences'. Although there is no consensus in amino acid sequence, these signal sequences all share the basic tripartite structure: a central segment of hydrophobic

amino acids preceded by a basic N-terminus and followed by a hydrophilic C-terminus containing the cleavage site (2). The similarity between protein translocation across the ER membrane and the bacterial plasma membrane is evident from the interchangeability of a bacterial leader sequence for a eukaryotic one and *vice versa*. Preproinsulin, when expressed as a fusion protein in bacteria, was translocated across the bacterial plasma membrane and accurately processed to proinsulin by leader peptidase (3); the leader sequence of either M13 procoat (4) or ß-lactamase (5), both bacterial proteins, was functional in canine microsomes *in vitro*.

Two hypotheses have been the most influential in studies on protein export in the two systems. The signal hypothesis (6), as originally formulated to account for protein secretion in eukaryotes, emphasizes the recognition of the signal sequence by a proteinaceous secretory apparatus, which then accomplishes the remainder of the process. The membrane trigger hypothesis (7), in contrast, emphasizes the participation of both the leader sequence and the mature portion of the precursor protein in assuming an export competent conformation. The latter hypothesis has gained most support from studies on post translational protein export from bacterial cytoplasm.

1.2.1 Protein Translocation Across the ER Membrane: The Signal Hypothesis

The signal hypothesis, as we understand it today, bears the basic concepts of the original one (6), but with much greater detail (8). Synthesis of a secretory protein is initiated on a free ribosome. As soon as, or shortly after, the signal peptide emerges from the ribosome, a ribonucleoprotein (the signal recognition particle, or SRP) binds to the

nascent signal sequence. SRP directs the nascent-chain-containing ribosome to the endoplasmic reticulum (ER) via an SRP receptor ('docking protein') on the ER membrane. Once the ribosome is bound to the ER membrane, through putative ribosome receptor(s), SRP is released, and will initiate another round of signal recognition process (8). The signal sequence, now free from the SRP, is thought to bind to another receptor on the ER membrane, the signal sequence receptor (SSR, ref. 9) and the translocation of the nascent polypeptide across the membrane occurs. The actual translocation process and the structure of the putative ER translocation apparatus, or the 'translocon' (8), are largely unknown. The signal peptidase located on the luminal side of the membrane cleaves off the signal peptide during or soon after nascent chain translocation (10).

1.2.2 Components of the ER Translocation Machinery

a) The Signal Recognition Particle

SRP is an 11S cytoplasmic ribonucleoprotein consisting of a 7S RNA (11) and 4 proteins (two moromers composed of a 19 kDa polypeptide and a 54 kDa polypeptide, and two heterodimers , one composed of a 9 kDa and 14 kDa polypeptide, and the other composed of a 68 kDa and 72 kDa polypeptide, respectively) (12, 13). The RNA, which is identical to the cytoplasmic 7SL RNA (11), contains repetitive *Alu*-like elements and forms a core to which the protein components are bound (13). Nucleolytic dissection of SRP into sub-particles (14) and reconstitution of SRP using purified components (15) have shown that the 9/14 kDa protein, together with the *Alu*-like elements of the 7SL RNA where the protein binds, is dispensable for signal recognition and translocation promotion. The separated domain of SRP containing the *Alu*-like elements and the 9/14 kDa

protein is apparently required for another function of SRP, a site-specific elongation arrest of nascent presecretory proteins assayed in a wheat germ translation system (16). The physiological relevance of this phenomenon is not yet clear (14, 17). Direct cross-linking (18, 19) has identified the 54 kDa protein of SRP as the signal binding component.

b) The SRP Receptor

The SRP receptor(SR), or docking protein, was initially identified as a 69 kDa ER membrane protein (SRα) with a large cytoplasmic domain (52 kDa), which recognizes SRP, and a small membrane anchoring domain (20-22). It was later found to contain an additional 30 kDa integral membrane protein (SRß) whose function is not yet known (23). The latter has been proposed to interact with, and thus stabilise, the large cytoplasmic domain of SRα, a function that might explain the rather surprising earlier observation that the proteolysed 52 kDa cytoplasmic fragment of SRα could be added back to the membrane and restore receptor function (24).

c) The Signal Sequence Receptor

The putative SSR has been recently identified by direct cross-linking approach (9). Taking advantage of the fact that the only two lysine residues in the precursor to bovine prolactin are present at the extreme amino terminus of the signal sequence, Rapoport and co-workers introduced a photoreactive group into the ε-position of these lysines in a cell free translation system in the presence of the modified lysyl-tRNA. Employing this probe in an *in vitro* translocation assay, they discovered that the signal sequence of the nascent preprolactin, upon release from SRP, became associated with an integral ER membrane glycoprotein with an apparent molecular weight of 35 kDa (9). This putative SSR may be

identical to the 35 kDa ER membrane glycoprotein recently purified by the same group (25). Antibodies raised against this purified protein inhibit protein translocation across the ER. Further studies on its exact function and its relationship to the putative 'translocon' (8) are as yet lacking.

d) Signal Peptidase

A functional signal peptidase complex has been isolated in two forms: one from canine pancreas contains 6 polypeptides (26), two of which were determined as differentially glycosylated forms of the same protein (27); the other from hen oviduct consists of two polypeptides (28). Neither the identities of catalytic subunit nor the exact nature of these complexes are as yet known.

1.2.3 Protein Export in Bacteria: The Membrane Trigger Hypothesis

Genetic studies have identified several bacterial secretion (*sec*) genes affecting protein export in bacteria, including *secA* (29), *secB* (30), and *prlA/secY* (31,32). *E. coli* leader peptidase was the first signal protease of any kind to be purified (33) and cloned (34). With the availability of an efficient *in vitro* translocation assay using inverted vesicles of bacterial inner membrane (35, 36), detailed biochemical studies on the functions of these different gene products have been possible.

a) The 'Trigger Factor' Cycle

ProOmpA is the precursor to a major outer membrane protein in *E. coli*. Its translocation across the inner membrane has been studied *in vitro* in a system consisting of inverted inner membrane vesicles and various purified components. The following model has been proposed by Wickner and co-workers to account for export of proOmpA and, presumably,

a subset of *E. coli* periplasmic and outer membrane proteins.

ProOmpA is synthesized on a 70S free ribosome and becomes associated with the ribosome bound 'trigger factor' (37), a 63 kDa soluble protein (38) with a binding affinity for the 50S ribosomal subunit (39). The association of proOmpA with a 'trigger factor', probably through the mature portion of proOmpA (40), renders the precursor competent for export (37, 38). This export-competent proOmpA-trigger factor complex then becomes associated with the inner membrane, probably through an interaction between the leader sequence and an integral membrane protein (e.g. the PrlA/SecY protein) Specific binding of the precursor to the plasma membrane releases trigger factor, which will enter the cytoplasmic pool and another round of conformation triggering process (39). Translocation across the inner membrane requires a membrane potential (41) and ATP hydrolysis (42), the latter function is performed by SecA, a peripherally bound inner membrane protein (43). Leader peptidase (34), an integral inner membrane protein, removes the leader sequence on the periplasmic side of the inner membrane (44).

b) <u>The SecB Stories</u>

Bassford and co-workers have analyzed export properties of various mutant precursors to maltose binding protein (MBP), an *E. coli* periplasmic protein, in strains of *E. coli* that were either $SecB^-$ (SecB absent) or $SecB^+$ (SecB gene present in multiple copies). They suggested that SecB promotes export of preMBP by conferring an export competent conformation ('antifolding') on the latter through interaction with the mature portion of the precursor (45). Purified SecB from an overproducing bacterial strain allowed the authors to confirm the 'antifolding' activity of SecB

(46). Independent studies from Gannon *et. al.* (47) and Lecker *et. al.* (40) supported the notion that SecB confers export competence through interaction with the mature portion of preMBP.

Watanabe and Blobel (48) do not agree with this. Using their *in vitro* assay consisting of inverted plasma membrane vesicles and wheat-germ translated precursor proteins, Watanabe and Blobel have purified an *E. coli* cytosolic export factor which was identified as a homotetramer of the SecB protein (49). They have subsequently proposed, from indirect evidence, that SecB binds to the leader sequence of preMBP. In this respect, SecB would represent the *E. coli* equivalent of SRP (48). Obviously, a 'SecB receptor' (the equivalent of SRP receptor) is still lacking.

1.2.4 Topogenesis of Integral Membrane Proteins

In contrast to exported proteins, integral proteins of both prokaryotic and eukaryotic plasma membranes do not completely traverse the membranes. Rather, they are anchored at these membranes by one or more transmembrane segments No separate rules are necessary to account for the biogenesis of integral membrane proteins. The membrane trigger hypothesis (7) favours a direct partition of precursors into the lipid bilayer. Studies on the phage M13 procoat protein have supported this notion (50, 51). Most other integral proteins studied so far require the same components of the export apparatus and thus are best accounted for by the signal hypothesis (6).

To accommodate the incomplete translocation of polypeptides across the ER membrane, the signal hypothesis was extended to include the concept of a 'stop-transfer sequence', i. e., a sequence characterized by a

stretch of about 20 hydrophobic amino acids that may interrupt membrane translocation of a polypeptide initiated by a signal sequence, and thus anchor the protein in the ER membrane (52).

a) Bitopic Integral Membrane Proteins

Bitopic integral membrane proteins (52) are proteins spanning the membrane once with either an exoplasmic N-terminal domain and cytoplasmic C-terminal domain (Type I) or *vice versa* (Type II). Integration of a Type I protein into the ER membrane is best illustrated by that of the vesicular stomatitis virus glycoprotein, or VSV-G (53, 54). VSV-G contains an N-terminal transient signal sequence and a single transmembrane segment towards the C-terminus of the protein which anchors the protein in the plasma membrane of the virus infected host cell. A mutant VSV-G protein without the transmembrane segment is secreted (53), while the membrane anchor of VSV-G, when fused C-terminal to the signal sequence of a secretory protein, converts the latter into a Type I ER membrane protein (54). Thus, the membrane topology of VSV-G is believed to result from interruption of the signal-sequence-initiated membrane translocation by the C-terminal transmembrane segment ('stop-transfer'). Such a stop transfer has also been identified in the case of murine surface immunoglobulin heavy chain (55).

A Type II protein usually does not contain a transient signal sequence. Rather, it contains a segment of hydrophobic amino acids at or towards the N-terminus that functions as a signal/anchor sequence. Such a sequence has been well defined in the case of human asialoglycoprotein (ASGP) receptor (56). Wild type ASGP receptor is anchored at the plasma membrane by a hydrophobic segment of amino acids, residues 39-58, with a

large C-terminal domain on the cell surface and a small N-terminus facing the cytoplasm. Membrane integration of ASGP receptor *in vitro* was SRP dependent indicating that ASGP receptor contained an 'internal' signal sequence. A mutant protein with the hydrophobic segment of amino acids deleted failed to be integrated into the membrane, while the same segment of amino acids, when fused N-terminal to a large fragment of a cytosolic protein, directed the hybrid protein into the ER membrane with the same orientation, i.e., the C-terminus facing the ER lumen. This integration again was SRP-dependent (56). The above results are best accounted for by the conclusion that the hydrophobic segment of amino acids functions as a signal/anchor sequence that initiated the SRP-dependent membrane integration as well as translocation of the C-terminus across the ER membrane (56). Similar signal/anchor sequences have been found in transferrin receptor (57) and human invariant chain of Class II histocompatibility antigen (58) by the same approach.

b) Polytopic Integral Membrane Proteins

For polytopic integral membrane proteins, i.e. proteins containing multiple transmembrane segments, (e.g. bovine opsin, ref. 59; human glucose transporter, ref. 60; and murine anion exchange protein Band 3, ref. 61), multiple signal and stop transfer (or signal/anchor) sequences may account for their complex membrane disposition (52). Bovine opsin is synthesized without a transient signal sequence. It contains four exoplasmic domains, four cytoplasmic domains, and seven transmembrane segments (59). Consequently, it could contain as many as four signal sequences (one for each exoplasmic domain) and four stop transfer sequences (one for each cytoplasmic domains). Alternatively, it could

contain multiple signal/anchor sequences (62). Among the six deletion mutants of bovine opsin, each containing a single transmembrane segment fused to the extreme N-terminus containing glycosylation sites (the latter lacking any targeting information and thus merely serving as a 'reporter'), five were able to function as signal sequences *in vitro* as indicated by SRP-dependent glycosylation of the mutant opsins (63). Two of the five were also anchored in the microsomal membrane, but only the mutant containing the first transmembrane segment of opsin was anchored in the membrane with the same orientation as in the native opsin (63). Thus, the internal topogenic sequences of bovine opsin may only function correctly in the context of the whole polypeptide. Nonetheless, multiple topogenic determinants were responsible for the complex membrane disposition (62, 63). Also in support of this proposal is the finding that human glucose transporter and hepatitis B surface antigen, both polytopic integral membrane proteins without transient signal sequences, contained multiple signal/anchor sequences (64 65).

A Similar proposal has been made to explain the biogenesis of the L and M subunits of the photosynthetic reaction center complex of *Rhodopseudomonas viridis* (66). The five transmembrane α-helices of each subunit, which has been definitively established by X-ray crystallography (67), are suggested to function, alternatively, as internal signal sequences and stop transfer sequences in generating the final topology of the polypeptides in the bacterial plasma membrane (66).

1.3 IMPORT OF PROTEINS INTO MITOCHONDRIA

1.3.1 Overview

The genetic contents of mitochondria from many species are known and

they include only a small percentage of the proteins comprising the organelles (68). The 16.6 kB mitochondrial genome of human, for instance, contains, in addition to genes for the two high molecular weight RNA species of mitochondrion-specific ribosomes and a complete set of tRNAs, about a dozen genes coding for some subunits of the oxidative phosphorylation chain (68, 69). All other proteins are encoded in the nucleus, and hence must be imported into mitochondria following synthesis in the cytoplasm.

Shortly after the proposal of the signal hypothesis, it was demonstrated that import of proteins into mitochondria was accompanied by proteolytic processing, presumably the removal of a mitochondrial 'signal sequence' (70-73). Protein import into mitochondria bears many similarities to protein export in bacteria and protein translocation across the membrane of the endoplasmic reticulum. Indeed, protein translocation into or across different intracellular membranes share a number of common characteristics (74).

Mitochondrial precursor proteins are synthesized by free ribosomes and imported post-translationally into one of the four compartments: the outer and inner membranes, the intermembrane space, and the matrix. Proteins of the outer membrane studied so far do not contain transient signal sequences. Most mitochondrial proteins destined for the other three compartments are synthesized as higher molecular weight precursors with transient mitochondrial signal sequences which are structurally distinct from the signal sequences of secretory proteins. Targeting of precursor proteins to mitochondria is mediated by proteinaceous structures on the mitochondrial surface, presumably receptors recognizing the mitochondrial

signal sequences. Import of proteins into or across the mitochondrial inner membrane requires a trans-inner membrane potential (inside negative). Translocation across the two membranes may occur at contact sites, where the two membranes are in close proximity, and translocation requires at least partial unfolding of the precursor proteins. The signal sequences are removed by the signal peptidase, a metalloprotease localized in the matrix (refs. 68 and 75 for recent reviews).

1.3.2 Precursor Proteins and Topogenic Sequences

a) Mitochondrial Signal Sequences

Like its counterpart in a pre-secretory protein, a mitochondrial signal sequence could direct C-terminally fused cytoplasmic proteins into mitochondria both *in vitro* (76, 77) and *in vivo* (77). A number of nuclear genes encoding mitochondrial proteins have been cloned and their signal sequences determined. Though there is little homology in primary structure among the different mitochondrial signal sequences, they do share some common characteristics very different from those of the signal sequences of pre-secretory proteins (2 [illegible], 79). They are rich in basic and hydroxylated amino acid residues and generally devoid of acidic ones. Most of them have the potential to form amphiphilic α-helices, with positive residues clustered on one side of the helix and hydrophobic ones on the other (80). Indeed, synthetic peptides corresponding to the signal sequences of either the rat pre-ornithine carbamyl transferase (pOCT) or the yeast pre-cytochrome oxidase subunit IV (pCOXIV) are more helical in a membrane-like environment (81, 82). The importance of amphiphilicity was also demonstrated by constructing completely artificial signal sequences that are positive amphiphiles and showing that they could indeed direct

attached proteins into the mitochondrial matrix (83, 84). It was believed that these positive amphiphiles may sense the inner membrane potential (negative inside) or even 'electrophorese' across this membrane (79). There is, however, little evidence that this was the case for the signal sequence of rat pOCT as indicated by studies employing artificial liposomes (85). Binding of the pOCT signal peptide to lipid vesicles was reversible and insensitive to a transbilayer potential (negative inside). It was suggested that amphiphilicity of the a mitochondrial signal sequence may facilitate its specific interaction with the cognate receptor(s) on the surface of mitochondria via an initial contact with the mitochondrial membrane (85). Subsequent studies showed that the presence of increasing amounts of liposomes in an *in vitro* import assay progressively slowed down mitochondrial import of pOCT, presumably by interfering with the precursor-membrane binding (86).

b) Topogenic Sequences of Mitochondrial Outer Membrane Proteins

The only outer membrane protein that has been studied in terms of topogenic sequences (52) is the yeast 70 kDa outer membrane protein (87). The 70 kDa protein is normally anchored in the outer membrane via its N-terminus with a large cytoplasmic C-terminal domain. When a stretch of hydrophobic amino acids towards the N-terminus (within amino acids 12-106) was deleted, the truncated protein was imported into the matrix, albeit with a low efficiency, presumably led by the extreme N-terminal 'signal sequence' (amino acids 1-11, which include 4 positively charged residues). The deleted segment seemed to contain a 'stop-transfer' sequence to prevent the wild type protein from translocating across the outer membrane (87). This suggestion was partially supported by gene fusion studies where

the N-terminal 21 amino acids of the 70 kDa protein were shown to direct an *E.coli* cytosolic enzyme to the mitochondrial matrix (88), although more direct evidence for a 'stop transfer' domain in the protein is still lacking. No such analysis is available on any other outer membrane protein. A hybrid protein bearing the plasma membrane anchoring sequence of the VSV-G protein immediately following the signal sequence of pOCT, the precursor to a matrix enzyme, has been imported into the outer mitochondrial membrane *in vitro*, which is also in support of such a mechanism (89).

c) Topogenic Sequences of AAC and UCP

ADP/ATP carrier protein, or AAC (90), is synthesized without a cleavable signal sequence (91). The protein contains about 300 amino acids, whose sequence, as first illustrated by protein sequencing of bovine AAC (92) and later deduced from the sequence of cloned cDNA from *Neurospora* (93), shows a striking triplicate structure. There are three repeats in the protein of about 100 amino acids each containing stretches of homologous residues (94). Each repeat is characterized by a pair of transmembrane α-helices connected by a large matrix domain which is overall positively charged. Unlike bitopic membrane proteins (VSV-G, ref. 54; ASGP receptor, ref. 56) where a single stretch of uninterrupted hydrophobic amino acids functions as a membrane anchor, the transmembrane segments in AAC are punctuated by scattered charged residues. Thus, the overall hydrophobicity is probably achieved by pairing of amphiphilic transmembrane helices as previously suggested for polytopic membrane proteins Band III and bacterial rhodopsin (95). Earlier studies on biogenesis of yeast AAC demonstrated that the N-terminal one third,

corresponding to the first repeat, could direct the C-terminally fused bacterial protein ß-galactosidase into mitochondria *in vivo* (96). More recent *in vitro* studies, however, suggested that the hybrid protein was in an intermediate location (97). *In vitro* studies on the *Neurospora* homolog, on the other hand, have suggested that the C-terminal two thirds contain sufficient information for specific targeting and import of AAC into isolated mitochondria (98).

Mitochondrial uncoupling protein, or UCP, is another polytopic integral inner membrane transporter protein. It is made without a transient signal sequence, and is highly related to AAC in that it contains a similar triplicate structure. The three repeats in UCP are homologous to each other and more distantly homologous to those in AAC (94). Unlike AAC, which is ubiquitous in mitochondria from all sources, UCP only exists in mitochondria of brown adipose tissue functioning in cellular thermogenesis (99). In conventional mitochondria under chemiosmotic respiratory control, there is an obligatory coupling on the one hand between electron flow down the respiratory chain and proton extrusion across the inner membrane and on the other hand between proton reentry through ATP synthase and ATP synthesis. Since the inner membrane of a conventional mitochondrion is largely impermeable to protons other than through ATP synthase, respiration is automatically coupled to ATP synthesis. In thermogenic brown adipocyte mitochondria, UCP exists as a homodimer in the inner membrane to allow protons reentry into mitochondrial matrix to bypass ATP synthase. This proton conductance by UCP couples respiration to heat production (99).

Our *in vitro* studies on import of various deletion mutants of rat

UCP have demonstrated that it contains N-terminal as well as internal mitochondrial targeting information. Furthermore, we have shown that the amino terminal one third contained sufficient information for both mitochondrial targeting and membrane integration. The internal targeting signal, located downstream of amino acid 101, did not result in insertion into the inner membrane independently. Thus, integration of the N-terminal one third may facilitate the integration of the remainder of the polypeptide (100).

In light of the proposed mechanism for integrating polytopic proteins into the ER membrane (62-64) and the triplicate structure of UCP and AAC (94), we proposed that targeting of UCP (or AAC) into the mitochondrial inner membrane follows three membrane insertion events in which each pair of transmembrane segments functions to stop translocation across the inner membrane initiated by a matrix-targeting-signal in the connecting ectodomain (100, 101).

d) Conformation of Mitochondrial Precursor Proteins

The specific mitochondrial targeting function clearly resides in the signal sequence of a precursor protein. The rest of the molecule, however, also contributes to this import process by assuming a conformation compatible with targeting and membrane translocation. Reagents that stabilized the tight folding of 'passenger' proteins (102, 103), or a passenger protein with intrinsic tight folding due to multiple intramolecular disulphide bonds (137), inhibited mitochondrial import of the respective artificial precursor proteins. This inhibition occurs probably due to the inability of the putative import machinery to accommodate the globular structure assumed by the passenger proteins. This

is in agreement with the earlier studies by Schelyer and Neupert (104) which suggested that precursor proteins assume an extended conformation during translocation across mitochondrial membranes. A loosely folded structure in the cytoplasm, as indicated by elevated sensitivity to low concentrations of proteases, has often been correlated with an 'import competent' conformation (105, 106). The exact nature by which this 'import competence' is achieved is not known. An interesting possibility is to maintain the availability of the signal sequence for interacting with its cognate receptor on the mitochondrial surface. Such a possibility has been suggested earlier by Hurt and Schatz (107) who discovered that a cryptic mitochondrial signal sequence 'masked' within the mouse cytosolic dihydrofolate reductase (DHFR) could be rendered functional when exposed on the surface of a recombinant protein.

1.3.3 Import Pathways

Protein import into the outer mitochondrial membrane does not require a trans-inner membrane potential and thus most likely involves direct insertion into the outer membrane from the cytoplasmic side (75). Proteins destined for the mitochondrial matrix are believed to traverse the two membranes at contact sites (104, 108). A major current question is how proteins are delivered to the intermembrane space and the inner membrane. Are they first translocated into the matrix, and then, following removal of the matrix targeting signals, 'exported' across the inner membrane as proposed in the 'conservative sorting' hypothesis (75)?; or do 'stop transfer' sequences prevent the precursor proteins from completely crossing the inner membrane as predicted in the 'stop transfer' hypothesis (68, 75)?.

a) The 'Conservative Sorting' Pathway

The 'conservative sorting' pathway is based on the endosymbiotic origin of mitochondria (109). It is suggested that, following transfer of genes from the endosymbionts to the host cell nucleus, a mechanism has evolved (the acquisition of the 'matrix targeting' signals) to transport the gene products back to the matrix space (equivalent to the bacterial cytoplasm) so that they can enter their conserved export pathway (75). Several proteins of the intermembrane space have been studied and they all follow the same import pathway as described below. The precursor is first completely translocated into the matrix via the translocation contact site. The imported precursor is processed to an intermediate form in the matrix, which then re-crosses the inner membrane to the intermembrane space directed by an 'export' signal. This intermediate processing is essential for the expression of the 'export' signal. Thus, in the presence of the signal peptidase inhibitor, o-phenathroline which exerts its effect by chelating the essential cation Zn^{++}, precursor forms accumulate in the soluble matrix. Two of the three proteins studied (cytochrome c_1 and cytochrome b_2) have bipartite signal sequences with the N-terminal part functioning as a 'matrix targeting' signal and the C-terminal part as an 'export' signal (110). The 'export' signal of the other protein, Fe/S protein of the respiratory complex III, most likely resides within the mature protein (111, 75). It has also been suggested that cytochrome p-450_{scc}, a mitochondrial inner membrane protein, follows the 'conservative sorting' pathway (75, 112), although in this case, no matrix-localized import intermediate was demonstrated (112).

b) The 'Stop Transfer' Hypothesis

Similar to the proposed mechanism for ER membrane protein integration (52), two topogenic determinants are proposed to exist in a precursor protein destined for either the inner membrane or the intermembrane space. A 'stop transfer' sequence C-terminal to a 'matrix targeting' signal functions to halt translocation of the polypeptides at the inner membrane during unidirectional transport from the cytoplasm to mitochondrial matrix. Interestingly, cytochrome c_1, which was shown to follow the 'conservative sorting' pathway (110), is the most studied example of this import pathway (113-115) While the presequence was able to direct attached 'passenger' proteins to the intermembrane space, the N-terminal part would direct them into the mitochondrial matrix (113, 114). Furthermore, in the presence of o phenanthroline, a hybrid protein [pC_1(1-64)-COXIV] bearing the presequence of cytochrome c_1 and the mature part of COXIV was imported into a membrane bound form with the bulk part of the protein exposed in the intermembrane space, rather than in the matrix as expected if it had followed the 'conservative sorting' pathway (refer to the above section). Presumably, the precursor protein was anchored at the inner membrane by the cytochrome c_1 presequence, since the authentic precursor of COXIV, which differs from pC_1(1-64)-COXIV only in the presequences, was found free in the matrix under the same conditions (115). Thus, the C-terminal half of the presequence which contained a stretch of 19 non-polar amino acids was proposed as a 'stop transfer' to interrupt translocation of the precursor protein across the inner membrane. Following two proteolytic processings (the first one cleaving the 'matrix targeting' signal in the matrix and the second one cleaving

the 'stop transfer' in the intermembrane space), the COXIV moiety was released in the intermembrane space (113-115).

The discrepancies between studies employing either the authentic cytochrome c_1 precursor (110) or hybrid proteins bearing the cytochrome c_1 presequence (113-115) is not yet explained but current thinking favours that cytochrome c_1 follows the 'conservative sorting' pathway (68).

The 'stop transfer' hypothesis was also examined by constructing a hybrid protein containing the well characterized 'stop transfer' sequence of VSV-G. This protein, pOCI containing the VSV-G sequence towards the C-terminus, was imported and anchored at the inner membrane by the VSV-G sequence with a short C-terminus left in the intermembrane space, a predicted orientation if the VSV-G stop transfer had functioned (89, 116).

c) Import Pathway of AAC and UCP

All the proteins that have been proposed to follow the 'conservative sorting' pathway have functional homologs in bacteria which are synthesized in the bacterial cytoplasm (equivalent to the mitochondrial matrix) and exported to the equivalent locations as their mitochondrial counterparts (75).

A number of mitochondrial proteins have evolved as the consequence of cellular compartmentalization, and thus have no counterparts in the prokaryote. Would the import pathway of these proteins differ from the 'conservative sorting' pathway? AAC and UCP are such proteins. Import of AAC has been intensively studied by manipulating the *in vitro* assay conditions so as to generate intermediates (117, 118). Although no intermediate was detected in the matrix, this obviously does not prove that AAC never exists in the matrix transiently.

Our earlier success in rerouting multiple transmembrane segments into the matrix by a matrix targeting signal (100) prompted us to take a very different approach to investigate the import pathway of UCP. By fusing a matrix-targeting signal to the N-terminus of UCP molecule, we rerouted UCP to the matrix (101). Upon removal of the signal sequence by the matrix located signal peptidase, the UCP moiety remained in the soluble matrix rather than inserting into the inner membrane from the matrix side. Our results suggested that UCP normally inserted into the inner membrane from the cytoplasmic side during unidirectional transport (101).

Thus, although import of a number of intermembrane space proteins are best accounted for by the 'conservative sorting' pathway, import of UCP and AAC may best be explained by direct insertion into the inner membrane from the cytoplasmic side.

1.3.4 Import Machinery

This remains the most challenging aspect of the mechanism of protein import into mitochondria despite much effort made in a number of laboratories. A complex proteinaceous import apparatus has been suggested since the early days of studies on mitochondrial biogenesis. Precursor proteins fractionated (119) or purified (120, 121) from *in vitro* translation systems were unable to enter mitochondria unless supplemented with reticulocyte lysate or yeast cytosol indicating the requirement of a cytosolic factor(s) for import. Surface digestion with low concentrations of protease abolished protein import into mitochondria suggesting the existence of proteinaceous receptor(s) on mitochondrial surface (119, 122). Proteins are believed to traverse mitochondrial

membranes through a proteinaceous 'translocator' whose nature is largely unknown. The mitochondrial signal sequences were known to be removed by a matrix localized metalloprotease activity (123).

a) Cytosolic Import Factor(s)

The rediscovery that ATP (or GTP) hydrolysis outside the inner membrane is necessary for mitochondrial import (105, 124-126) coupled to the earlier observation that precursor proteins need to be partially unfolded (102-104) suggests the existence of a cytosolic import factor(s) hydrolysing ATP (or GTP) to perhaps unfold precursor proteins. Such an 'unfoldase' was suggested earlier by Rothman and Kornberg (127). A similar activity has been proposed for the function of the 70 kDa heat shock proteins (hsp70s), a multigene family of stress induced proteins found in all organisms tested from *Escherichia coli* to man (128). Indeed, members of the ATP-binding hsp70s were found to stimulate post translational import of precursor proteins into either the yeast microsome or mitochondria both *in vivo* and *in vitro* (129-131). In addition to hsp70s, another cytosolic protein(s) with an essential sulfhydryl group(s), as indicated by its sensitivity to N-ethylmaleimide, is required for this import stimulating activity, although its identity remains unknown (130-132).

b) Import Receptors

Specific interaction of precursor proteins with mitochondria are believed to be mediated by mitochondrial surface receptors which are proteinaceous in nature (119, 122). Translocation of the specifically bound precursor proteins across the two mitochondrial membranes occurs through a hydrophilic, probably proteinaceous, structure (133, 134). Very

different approaches have been taken to identify components of these proteinaceous receptors and 'translocators'.

Antibodies raised against SDS-PAGE purified 45 kDa outer membrane proteins of yeast mitochondria were found inhibitory to precursor import into mitochondria (135). Furthermore, the antibodies were without effect on import if mitochondrial outer membrane was disrupted by osmotic shock, a situation whereby precursors seemed to by pass receptors on the outer membrane (136). It was concluded that a 45 kDa protein in the yeast mitochondrial outer membrane represented a surface component of the import machinery (135, 136). Subsequent studies in the same laboratory revealed that the responsible antibodies were against a 42 kDa protein, which contaminated the original 45 kDa preparation (163).

In separate studies, Vestweber and Schatz (137) employed an unusual probe to exploit components of the import machinery at the 'contact sites' (104, 108). The tripartite chimeric precursor consists of a well characterized hybrid precursor protein, pCOXIV-DHFR (the signal sequence of pCOXIV fused to mouse DHFR), and bovine pancreas trypsin inhibitor (BPTI) chemically cross-linked to the C-terminus of DHFR (137). When presented to mitochondria, the DHFR moiety was translocated across both membranes and folded in the matrix, while the BPTI moiety failed to be translocated and remained outside due to its tight folding caused by the multiple intramolecular disulfide bonds. Such a structure inhibited subsequent protein import into treated mitochondria, presumably by 'plugging' the translocation apparatus (137). Subsequent photo-cross-linking has demonstrated that the chimeric precursor physically associates with the 42 kDa outer membrane protein (163), thus strengthening their

argument for the physiological relevance of the antibody inhibition (135).

In view of the fact that the transient signal sequences of mitochondrial precursors contained sufficient information for targeting the precursors to mitochondria (76, 77, 138), a synthetic signal peptide corresponding to a large portion of the signal sequence of pOCT was employed as a probe to identify components of the putative import apparatus. The signal peptide, at a concentration (5 μM) that had no effect on the inner membrane potential, inhibited *in vitro* import of the corresponding precursor (pOCT), as well as two other precursors destined for the matrix and inner membrane respectively (139). Recently, Strauss and co-workers (140) found that another synthetic signal peptide, that of pre-malate dehydrogenase (pMDH), inhibited *in vitro* import of several precursor proteins at almost the same concentration (5 μM). Furthermore, mutant pMDH signal peptides replacing a single Leu^{13} with either Asn, Glu, or Pro had no effect at the same or higher concentrations. The same replacement in the precursor abolished *in vitro* import of the latter into mitochondria (141). This correlation suggests strongly that a synthetic signal peptide may indeed mimic certain functions of the signal sequence in the context of a precursor protein.

When a bifunctional cross-linker was employed in subsequent studies, an integral mitochondrial membrane protein with an apparent molecular weight of 30 kDa (p30) was found to associate with the synthetic signal peptide of pOCT (142). This association had several characteristics of a receptor-ligand interaction, thus implicating p30 as a candidate for a component of the import machinery (142). We have subsequently purified this signal peptide binding protein (p30) and identified it as the ADP/ATP

carrier (AAC) protein (143). AAC is an integral protein of the mitochondrial inner membrane functioning in adenosine nucleotide exchange between the cytoplasm and mitochondrial matrix, a function that is unrelated to protein import (125, 143). Yet, the apparent affinity of this protein to a signal peptide, as well as the correlation of this association with the loss of import ability by the mitochondria, are intriguing (143). Interestingly, in *Neurospora crassa*, an essential subunit of the cytochrome reductase complex has been found to play a role in mitochondrial signal sequence processing (154, see next section).

The availability of large amounts of purified, import competent porin, the precursor form of an integral mitochondrial outer membrane protein (144), allowed Neupert and co-workers to perform competition experiments against import of different groups of mitochondrial precursor proteins translated in a cell free system (145). Specific binding of precursor proteins of porin, AAC, or F_1ATPase ß subunit (F_1ß) to mitochondria could be divided into 2 stages. The precursor first bound to a protease-sensitive component on the mitochondrial surface, presumably a receptor protein. This 'receptor-bound' precursor itself was sensitive to protease, but could be chased into a location in the outer membrane protected against external protease. Furthermore, import competent porin was able to inhibit mitochondrial import of all precursor proteins tested except for apocytochrome c at about the same concentration, indicating the existence of a common component for all precursor proteins except for apocytochrome c. Import of apocytochrome c seems to follow a unique pathway very different from any other proteins studied so far (146). It was further determined that porin did not compete for generation of

receptor bound precursors of either AAC or $F_1\beta$, rather it competed at the second binding site which was protease insensitive. This site was thus named 'general insertion protein' or GIP (145). From these and the earlier observation that elastase digestion of mitochondrial surface abolished subsequent import of several precursors including that of AAC but not $F_1\beta$ (147), it was proposed that there are at least three classes of receptors: for porin, for AAC or for $F_1\beta$ (145). Based on antibody competition studies, the putative receptor for the precursor to $F_1\beta$ has been identified as a 19 kDa protein of the outer mitochondrial membrane (Neupert, *et. al.* unpublished).

c) Signal Peptidase

The matrix localized signal peptidase has been purified to homogeneity from *Neurospora crassa* (148), *Saccharromyces cerevisiae* (149) and rat liver (150). It consists of two subunits in all three cases. It has been determined for the fungal enzyme that one subunit is the catalytic subunit, the matrix processing peptidase (or MPP). MPP has weak processing activity by itself which is maximally enhanced by equimolar amounts of the other subunit, the processing enhancing protein (or PEP), and yet the two do not form a tight complex (148). cDNAs encoding the two subunits have been cloned in both *Neurospora* and yeast and their amino acids deduced (148, 151-153). The two subunits share strong homology to each other suggesting a common origin (75). In *Neurospora*, PEP was found in great molar excess over MPP and had both membrane and soluble distributions, while MPP was a soluble protein (148). This puzzle was solved by the recent discovery that PEP was identical to a subunit of cytochrome reductase complex (bc_1 complex), subunit I, which is an

essential part of the complex with an yet unknown function (154).

d) Molecular Chaperonins

From a temperature sensitive yeast mutant deficient in assembling the transformed human pOCT into a functional trimer, the responsible gene was cloned and identified as a member of the hsp60 family (155, 156). Mitochondria isolated from the yeast mutant grown at non-permissive temperature imported several precursor proteins into the matrix but failed to either assemble them to a functional complex (pOCT, $F_1ß$) or 'export' them to the intermembrane space (Fe/S protein of complex III, cytochrome b_2 refer to section 1.3.3.a). Thus, the hsp60 functions in interacting with imported precursors, conferring conformational competence required for either oligomeric protein assembly or further steps in the import pathway (155, 157). This function is similar to that of both *Escherichia coli* groEL product (158) and the chloroplast Rubisco subunit-binding protein (158), both members of the 'molecular chaperonin' family (159). Indeed, the three proteins are also structurally related (156).

1.4 PROSPECTS

We have learned a great deal about mitochondrial signal sequences due largely to the availability of cDNA clones of a number of mitochondrial proteins as well as the well developed *in vitro* DNA manipulation techniques. Components of the putative import machinery, both cytosolic and mitochondrial, are beginning to yield their identities. We are, however, far from understanding this complex process of protein import into mitochondria. With more and more components of the import machinery being identified and characterized in the future, one hopes that this import process will one day be reconstituted in a well defined

system.

Footnote: To avoid duplication, the literature cited in the introduction is included with the references for the General Discussions following Chapter 5.

CHAPTER 2

TOPOGENESIS OF MITOCHONDRIAL INNER MEMBRANE UNCOUPLING PROTEIN. RE-ROUTING TRANSMEMBRANE SEGMENTS TO THE SOLUBLE MATRIX COMPARTMENT

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 pre-carbamoyl phosphate synthetase was fused to UCP amino acids 169-307 or 52-307 (containing 3 and 5 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 employ more than one signal sequence to achieve this. In this respect, it may share certain mechanistic features in common 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.

INTRODUCTION

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 by-passing ATP synthase. The uncoupling of respiration from ATP synthesis results in production of heat (for a review, see ref. 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 3-fold repeat of about 100 amino acids. In the case of UCP, physical-chemical analyses (16) and computer modelling (2, 28) predict a protein which 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 6 transmembrane segments, whereas Aquila *et al.* (2) suggest a similar arrangement, but with an additional ß-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 post-translational 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 N-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 ER (5). At least in the case of simple bitopic transmembrane proteins, it has been proposed that two functionally distinct topogenic domains are employed: an N-terminal matrix-targeting signal and a distal stop-transfer sequence (11, 21, 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).

Compared to bitopic transmembrane proteins, UCP presents a different set of problems: first, it is a polytopic protein spanning the membrane at least 6 times and, secondly, 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 non-UCP 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.

EXPERIMENTAL PROCEDURES

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 earlier articles (3, 20). 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 (1000 Ci/mmol) for 30 minutes at 30°C. Aliquots were diluted 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_2HPO_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 minutes at 30°C, the mitochondria recovered by centrifugation in a microfuge operating for 5 minutes 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 constructs employed 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'-untranslated sequence (or at least the poly GC portion) from UCP cDNA was required for efficient translation to take place.

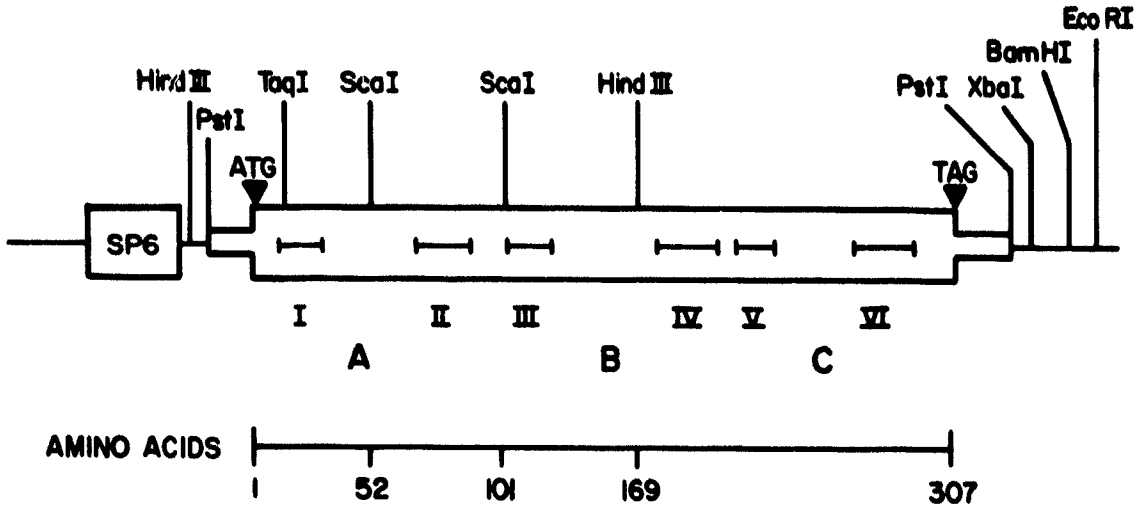
Fig. 1 indicates the positions of the transmembrane segments (I-VI) and extramembrane matrix domains (A-C) in the linear cDNA and polypeptide sequences (upper panel), as well as showing a simplified schematic illustration (lower panel) of the disposition of these regions in the mitochondrial inner membrane (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).

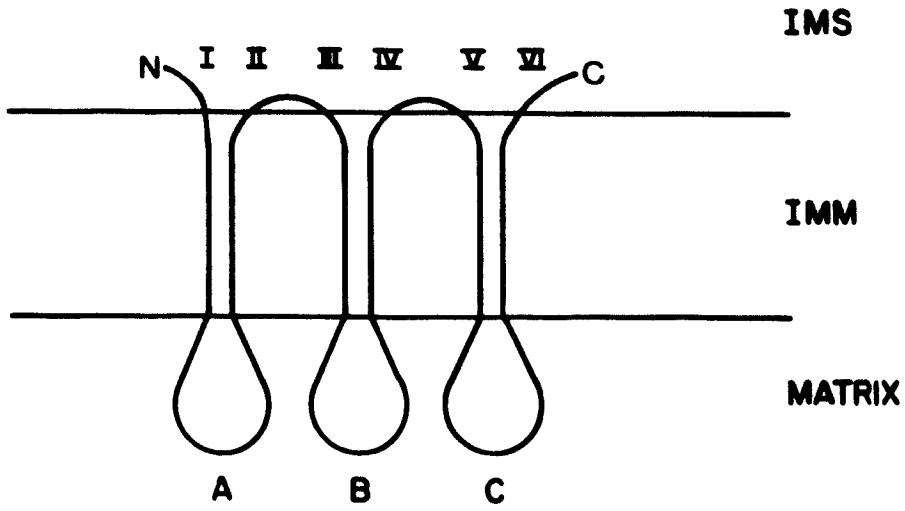
Import of UCP and UCPd1-12

Following 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 kDa on SDS gels, and a slightly smaller product migrating with a size of ~30 kDa (Fig. 2, lanes 7-12). Because the latter co-migrated 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 UCPd1-12 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

Fig. 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 refs. 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. pSPUCP encodes full-length UCP polypeptide (upper panel). UCP cDNA was excised from pUCPrat15 (27) with PstI and further digested with TaqI to remove the 5' untranslated region; the TaqI-PstI fragment was purified and inserted into the PstI site of the SP64 polylinker via an oligonucleotide adaptor (GACCATGGTAGAGTT), which also restored the correct UCP coding sequence. pSPUCPd1-12 encodes UCP lacking amino acids 1-12 and was constructed as above except that the adaptor, GGTGAGTT, was employed for ligation into pSP64. pSPUCP13-51-OCT and pSPUCP13-105-OCT encode UCP amino acids 13-51 and 13-105, respectively, attached to 209 carboxyl-terminal amino acids from OCT (20). A PstI-BglII fragment, encoding the amino-terminal 146 amino acids of pOCT, was deleted from pSP019 (20), and the deleted fragment replaced with either of two fragments purified from pSPUCPd1-12 after digestion to completion with PstI and partial cutting with ScaI; for pSPUCP13-51-OCT, the shorter PstI-ScaI fragment (encoding UCP amino acids 13-51) was inserted directly following blunting of the BglII 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 BglII and restores UCP up to amino acid 105. pSPUCP102-307 codes for a carboxyl-terminal fragment of UCP. A ScaI-PstI fragment coding for UCP amino acids 102-307 was inserted between the HindIII and PstI sites in the pSP64 polylinker, employing an oligonucleotide adaptor (AGCTTGGGCTGCAGACCATGGGGT) which creates a HindIII end (UCP101-307 begins with met-gly). pSPCPS-UCP169-307 and pSPCPS-UCP52-307 encode hybrid proteins containing the amino-terminal 96 amino acids from pCPS (20) fused to the carboxyl-terminal 169-307 or 52-307 amino acids of UCP. They were constructed by purifying a 596 bp PstI fragment from pHN291 (24) corresponding to the 5'-end of pCPS cDNA, inserting it into the PstI site in the polylinker of pSP64, linearizing at an internal NcoI site, filling in the cohesive ends with Klenow enzyme, and inserting the appropriate UCP cDNA fragment: for pSPCPS-UCP169-307, a HindIII fragment containing the 3' half of UCP cDNA was isolated from pSPUCP, blunt-ended with Klenow, and inserted into the blunt-ended NcoI site of the pCPS plasmid; for pSPCPS-UCP52-307, a ScaI-PstI fragment (blunted with T4 DNA polymerase) encoding UCP amino acids 52-307 was isolated from pSPUCP and inserted into the blunt-ended NcoI site of the pCPS plasmids. For *in vitro* transcription, the recombinant pSP64 plasmids were linearized with EcoRI.


Eco RI
BamHI
Hind III
TaqI
ScaI
ScaI
Hind III
PstI
XbaI
PstI
ATG
TAG
SP6
I
II
III
IV
V
VI
A
B
C
AMINO ACIDS
1
52
101
169
307


IMS
N
I
II
III
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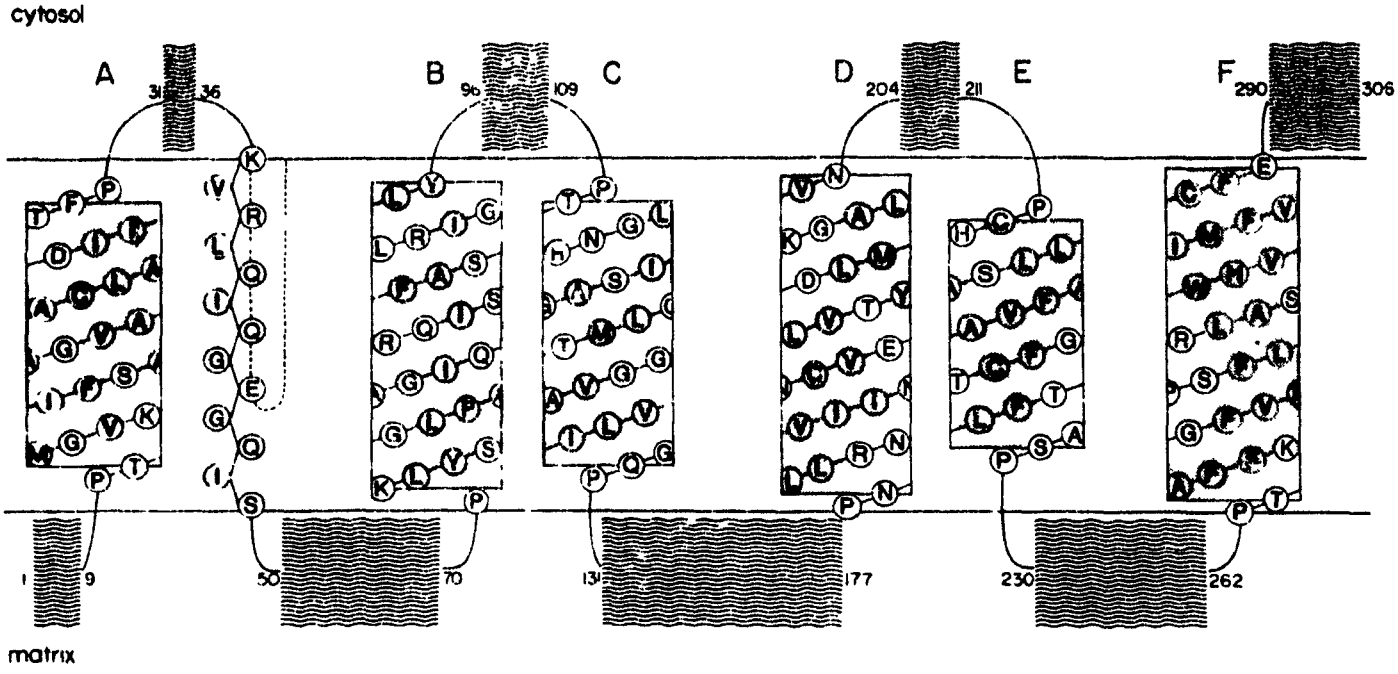
IMM
MATRIX
A
B
C



UCP SEQUENCE

Fig. 2. Import of pOCT and UCP by heart mitochondria *in vitro*. pSP019, pSPUCP, and pSPUCPd1-12 were transcribed and translated in a reticulocyte lysate in the presence of [^{35}S]methionine, after which import was carried out as described in Experimental Procedures; total products were analyzed by SDS-PAGE and fluorography. a, pOCT; b, UCP; c, UCPd1-12. Lanes 1 and 7, 20% of input pOCT and 33% of input UCP, respectively; lanes 2 and 8, mitochondrial pellets following import; lanes 3 and 9, prior to isolating mitochondria following import, mixtures were incubated at 4°C for 30 minutes with 100 μg/ml proteinase K (PROT.K), at which time phenylmethylsulfonylfluoride (final conc., 2 mM) was added and the mixture incubated for an additional 10 minutes; lanes 4 and 10, as in lanes 3 and 9 except that import was performed in the presence of 1.0 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP); lanes 5 and 11, following import, mitochondria were recovered, suspended (0.5 μg protein/μl) in 0.1 M Na_2CO_3, pH 11.5, sonicated vigorously, incubated on ice for 30 minutes, and the membranes recovered following centrifugation in an airfuge operating at 30 p.s.i. for 10 minutes; lanes 6 and 12, as in lanes 5 and 11 except that import was performed in the presence of CCCP. Lane 13, UCPd1-12. The arrowheads denote pOCT, mature OCT, 37K (an intermediate fragment routinely observed during pOCT import and processing *in vitro*), UCP, UCPd1-12, and M^{13} (resulting from internal initiation of polypeptide synthesis at UCP methionine-13).

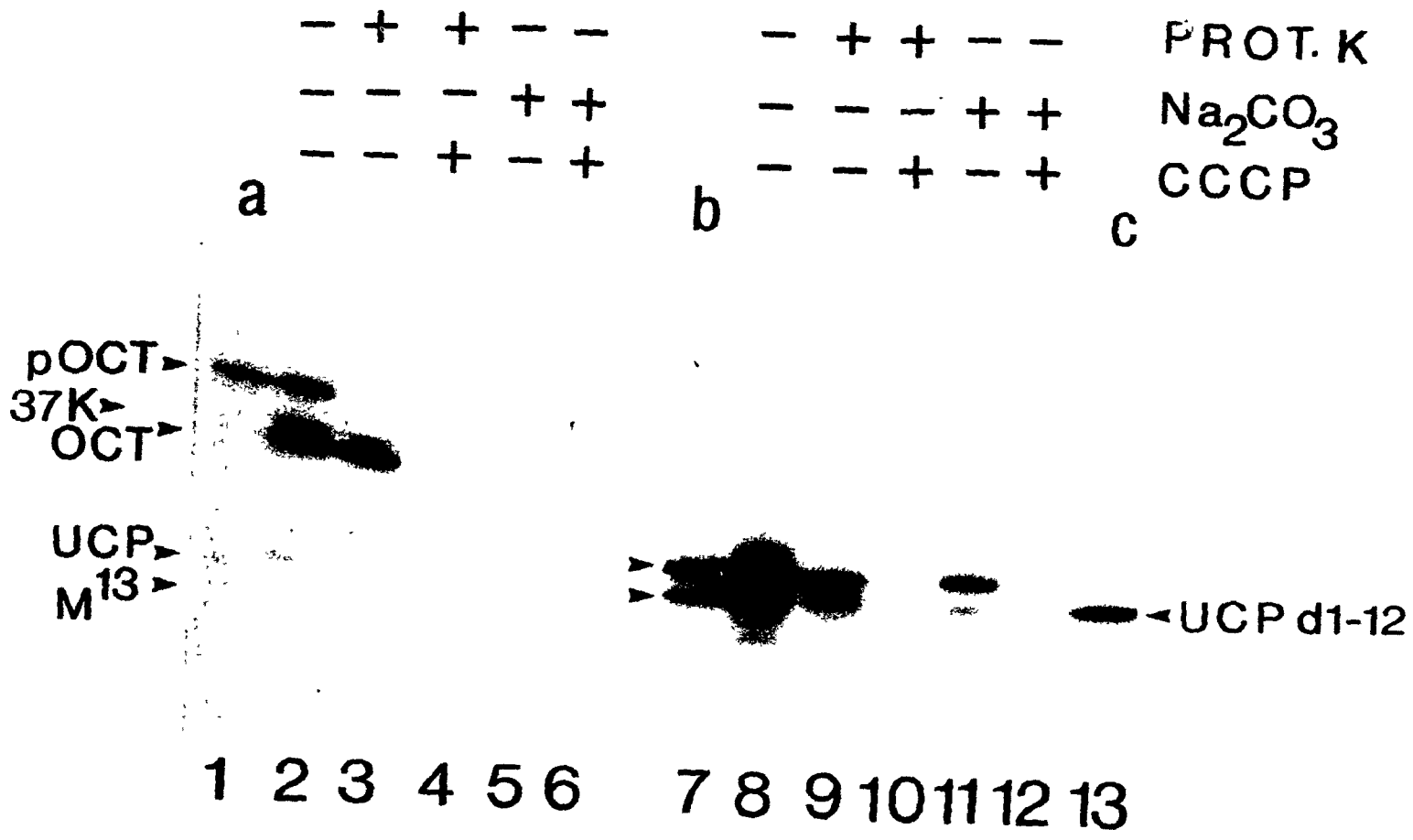
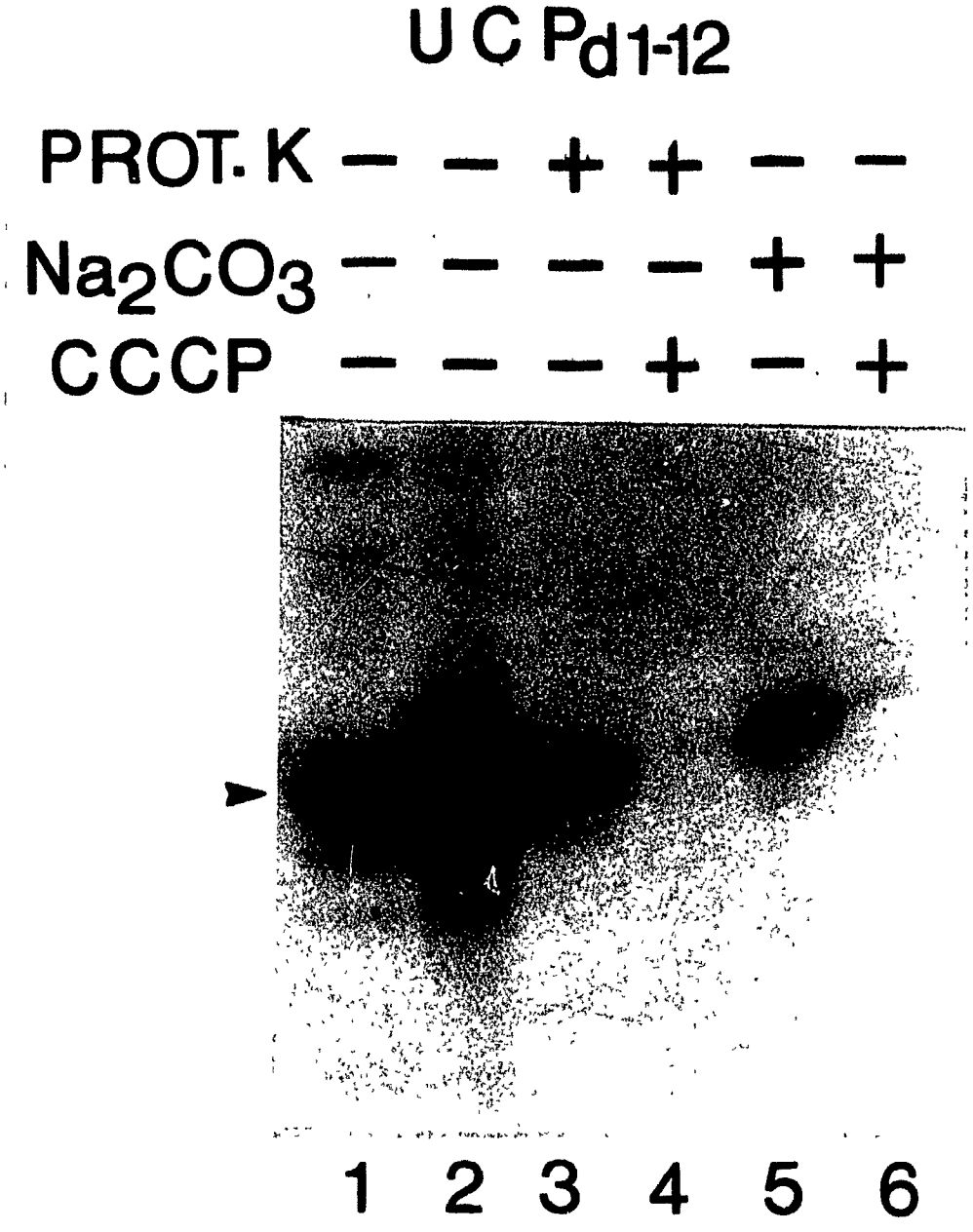

− + + − −
− − − + +
− − + − +
a
− + + − −
− − − + +
− − + − +
b
PROT. K
Na_2CO_3
CCCP
c
pOCT
37K
OCT
UCP
M13
UCP d1-12
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 3. Import of UCPd1-12. Conditions and treatments were the same as in Fig. 2. Lane 1, one-third of input; lanes 2-5, mitochondrial pellets. Arrowhead denotes UCPd1-12.


UCP$_{d1-12}$
PROT. K − − + + − −
Na_2CO_3 − − − − + +
CCCP − − − + − +
1 2 3 4 5 6

to a matrix protein (13, 22), was also documented. To distinguish between the large amount of high input levels of UCP and UCPd1-12 which sedimented with mitochondria following import incubations (Fig. 2, lane 8) and that fraction which entered the organelle and assembled into the inner membrane, two criteria were employed: acquisition of $\Delta\psi$-dependent resistance to exogenous proteinase K and $\Delta\psi$-dependent insolubility in 0.1 M Na_2CO_3, pH 11.5. Earlier studies (12, 25) have established that a mitochondrial electrochemical potential ($\Delta\psi$) is required for protein import into or across the inner membrane; thus, incubation in the presence of CCCP, an uncoupler which collapses the electrochemical gradient across the inner membrane, should render input UCP and UCPd1-12 entirely sensitive to exogenous 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 cf. lanes 11 and 12). Sonication of mitochondria in 0.1 M Na_3CO_2, pH 11.5, releases the mitochondrial soluble content (e.g., 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 following 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, UCPd1-12 (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 UCPd1-12 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 employed a C-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 UCPd1-12 Fig. 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 non-specific binding to membranes. In the case of UCP13-51-OCT, a large fraction of input polypeptide sedimented with mitochondria following 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

Fig. 4. UCP amino acids 13-51 fail to deliver a reporter protein into mitochondria. UCP13-51-OCT was produced following 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.

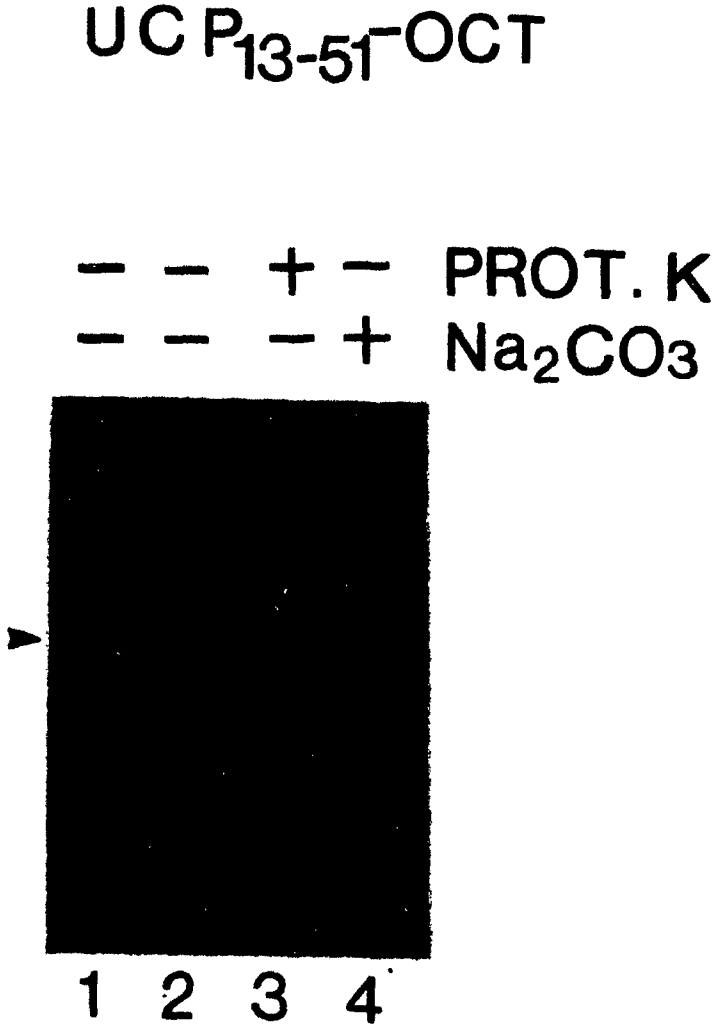
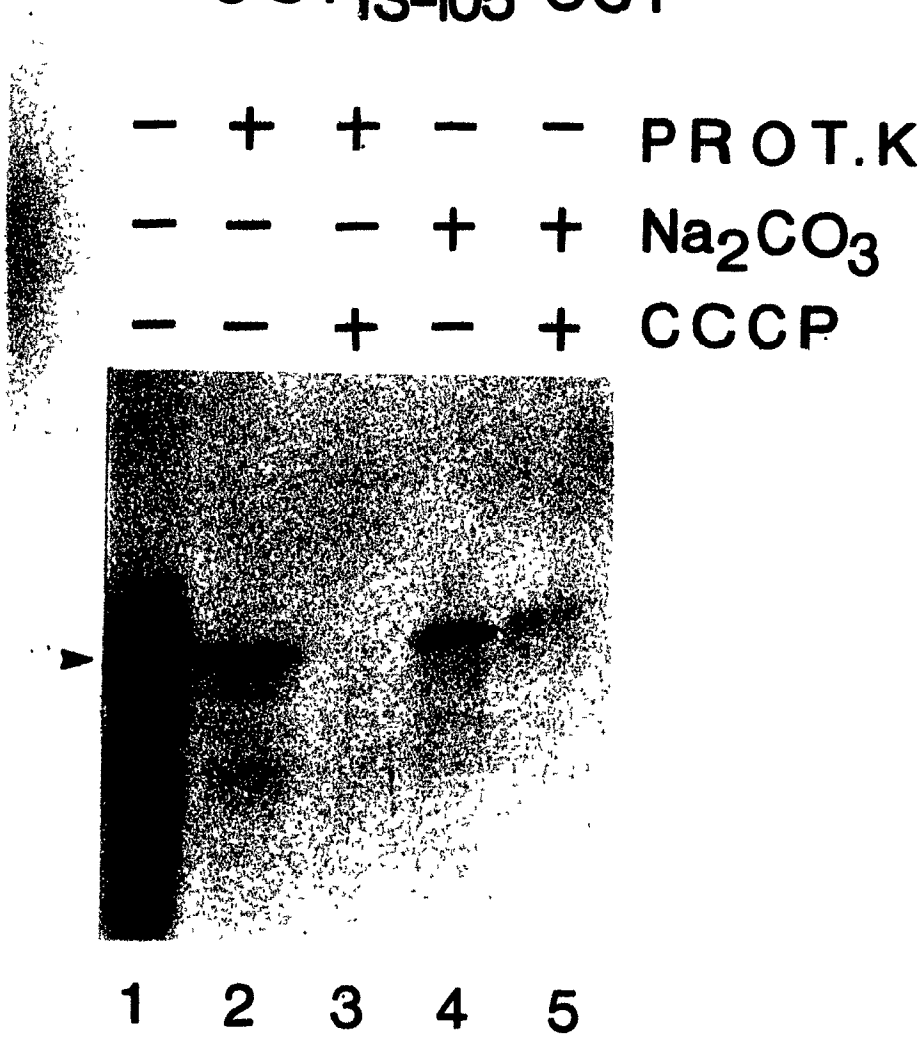

UCP13-51-OCT
- - + - PROT. K
- - - + Na2CO3
1 2 3 4

Fig. 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. Lanes 1, one-third of input; lanes 2-5, mitochondrial pellets. Arrowhead denotes UCP13-105-OCT.

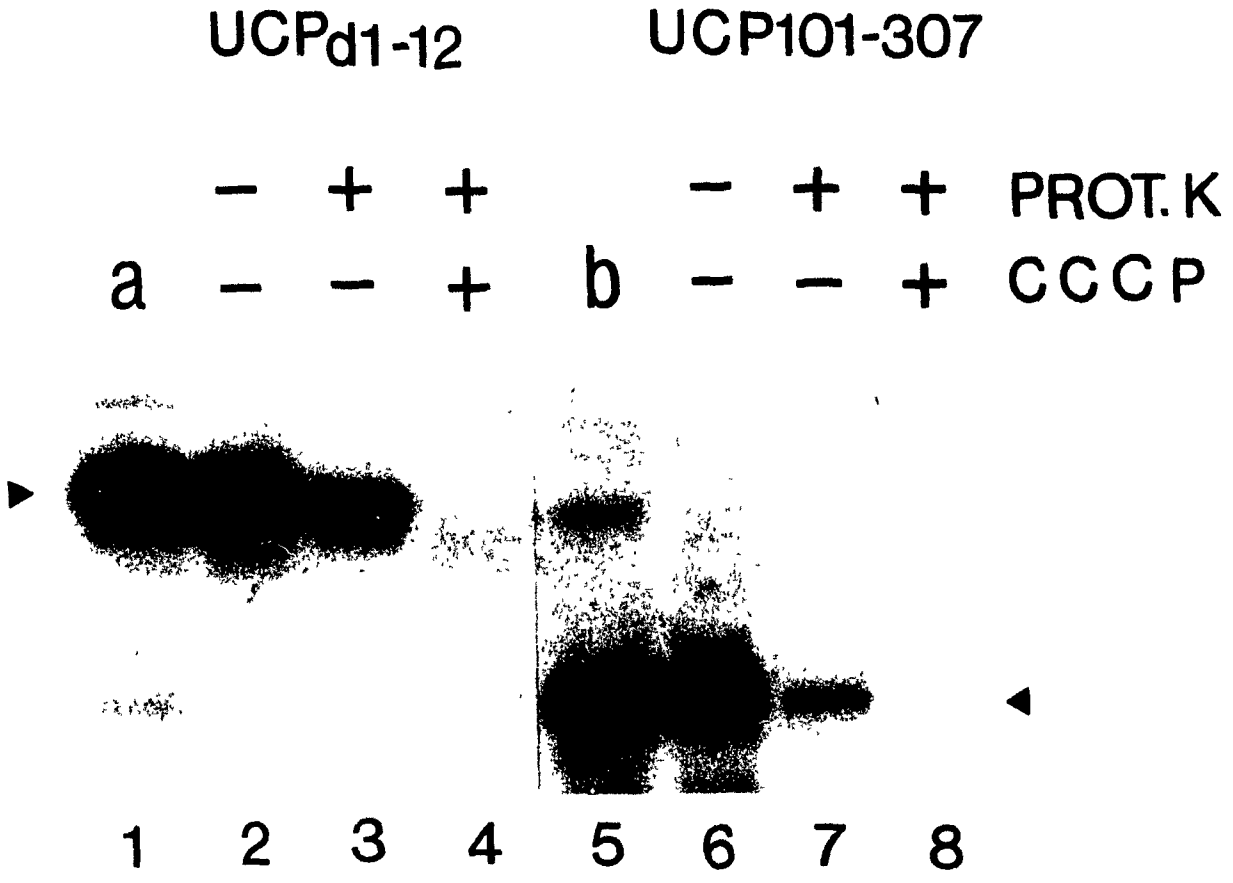

UCP13-105-OCT
- + + - - PROT.K
- - - + + Na2CO3
- - + - + CCCP
1 2 3 4 5

overall process. Indeed, deletion of this region (d2-51) was found to severely retard UCP import (data not shown).

A Large Carboxyl-terminal Fragment of UCP Is Inefficiently Imported.

To extend the finding 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 resulted 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. 6b). Following addition of mitochondria, a small portion was imported and rendered inaccessible to exogenous protease in a $\Delta\psi$-dependent manner (Fig. 6, cf. lanes 7 and 8). Compared to 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, cf. lanes 3 and 7), as determined by densitometric analysis. Furthermore, in a number of separate experiments, we routinely found that UCP102-307 following import was not resistant to extraction with alkaline Na_2CO_3 (not shown), indicating its lack of integration into the mitochondrial inner membrane bilayer.

Fig. 6. Import of UCPd1-12 and UCP102-307. The two polypeptides were produced by transcription-translation of pSPUCPd1-12 (a) and pSPUCP102-307 (b). 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.


UCPd1-12
UCP101-307
– + + – + + PROT. K
a – – + b – – + CCCP
1 2 3 4 5 6 7 8

A 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 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 3 or 5 transmembrane segments (Fig. 1) were fused behind a strong matrix-targeting signal derived from pCPS; 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 (20, 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 following treatment with alkaline Na_2CO_3 (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 Na_2CO_3 observation, demonstrate that pCPS-UCP169-307 was imported across the inner membrane and deposited in the

Fig. 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 Na_2CO_3, pH 11.5. p, precursor, m, processed product.

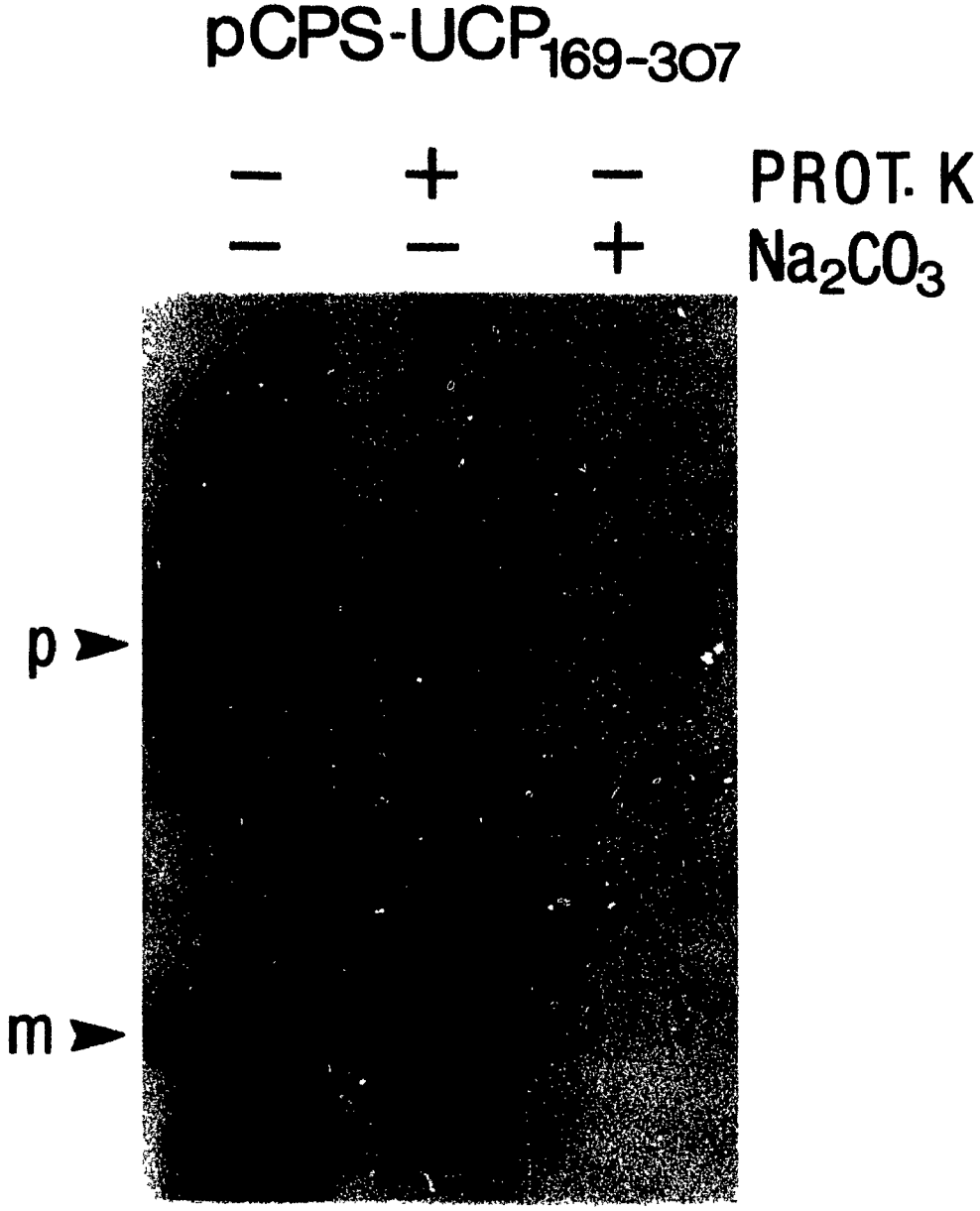

pCPS-UCP169-307
– + – PROT. K
– – + Na2CO3
p
m

Fig. 8. Radiosequence analysis of [^{3}H]leucine-labelled product following import and processing of pCPS-UCP169-307. The import described in Fig. 7 was repeated except that [^{3}H]leucine replaced [^{35}S]methionine in the translation mixture; following import and proteinase K treatment, the product designated m in Fig. 7 was electroeluted and subjected to automated Edman degradation on an Applied Biosystems 470A gas-phase sequencer, 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.

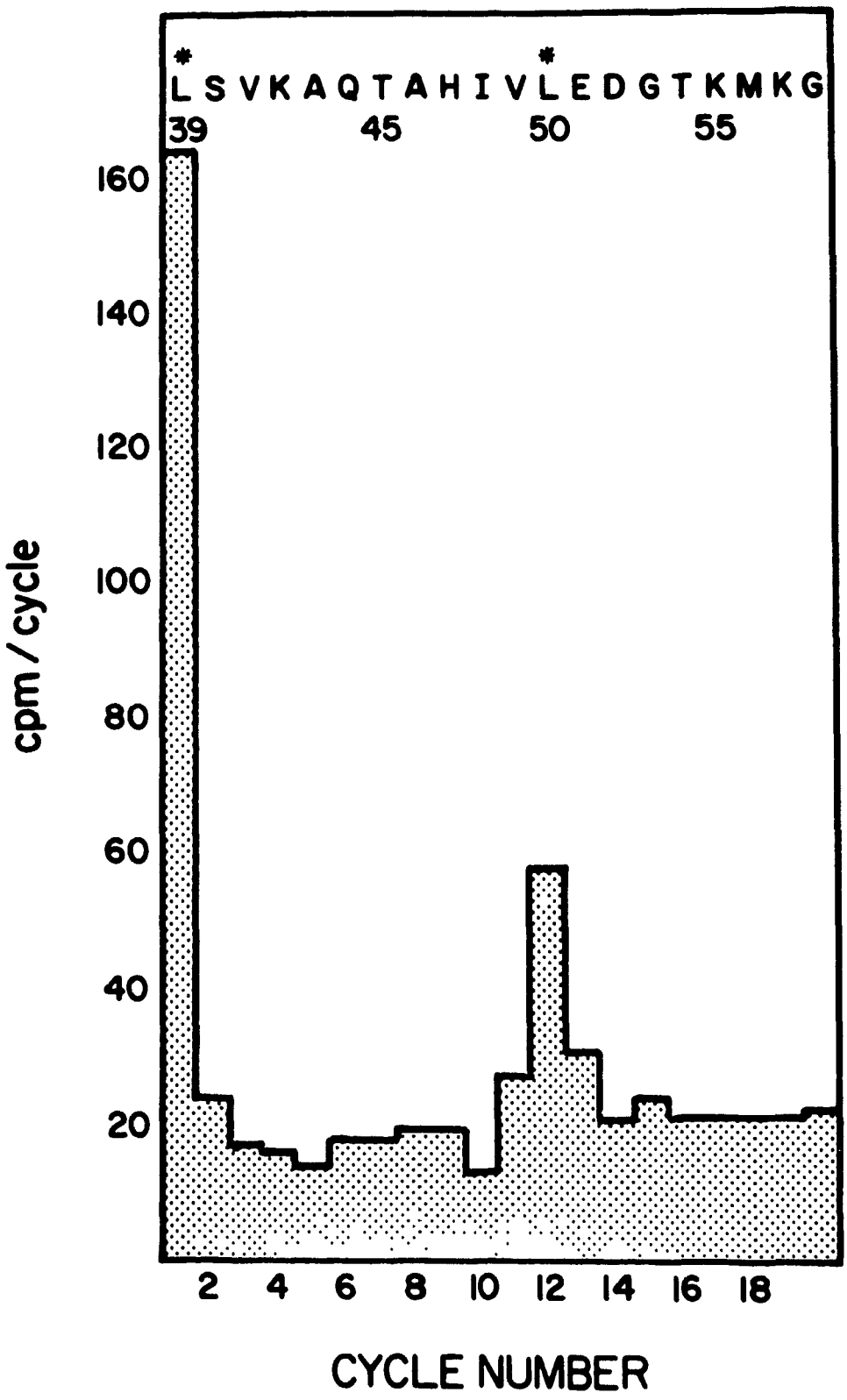
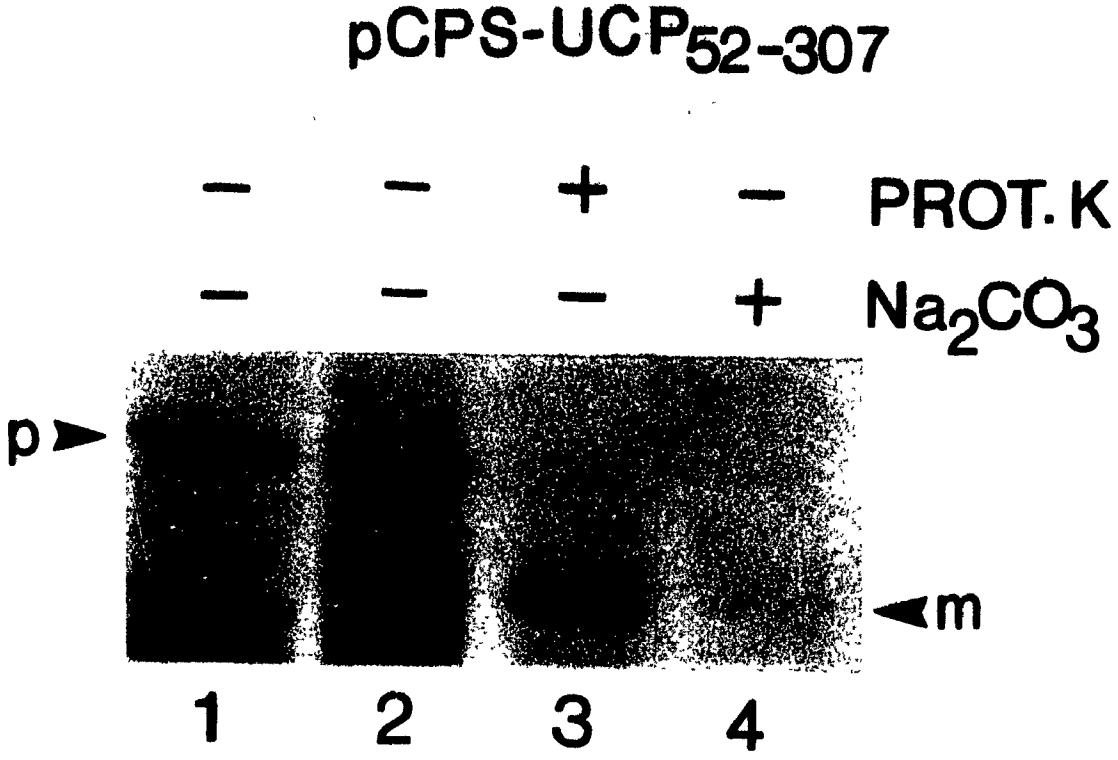

* *
L S V K A Q T A H I V L E D G T K M K G
39 45 50 55
160
140
120
100
80
60
40
20
cpm / cycle
2 4 6 8 10 12 14 16 18
CYCLE NUMBER

Fig. 9. Import and processing of pCPS-UCP52-307. 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 designate precursor and processed pCPS-UCP52-307, respectively.


pCPS-UCP52-307
– – + – PROT. K
– – – + Na2CO3
p
m
1 2 3 4

soluble matrix 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., UCPd1-12) 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 following 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 two-fold: (1) to identify regions in the UCP primary translation product that contain topogenic information for import and insertion into the mitochondrial inner membrane, and (2) 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-terminal fragment so that the second signal sequence can help direct insertion. Because UCP is composed of a three-fold 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 (21, 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 ecto-domains 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-UCP69-307, 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.

ACKNOWLEDGMENTS: We thank Hélène Gagné for excellent technical assistance and Dr. Stephen Randall for comments on the manuscript.

[1]**ABBREVIATIONS**: UCP, uncoupling protein; ER, endoplasmic reticulum; pOCT and OCT, precursor and mature ornithine carbamyl transferase; pCPS and CPS, precursor and mature carbamoyl phosphate synthetase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

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CHAPTER 3

AN AMINO TERMINAL SIGNAL SEQUENCE ABROGATES THE INTRINSIC MEMBRANE-TARGETING INFORMATION OF MITOCHONDRIAL UNCOUPLING PROTEIN

ABSTRACT

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 *et al.* J. Cell Biol. 107, 503-509 [1988]) identified internal regions of the molecule that are critical for targeting and membrane insertion. Here, we demonstrate that the ability of UCP to insert into the inner membrane is abrogated when the molecule is fused behind the matrix-targeting signal of pre-ornithine carbamyl transferase; 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.

INTRODUCTION

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 three-fold repeat of ~100 amino acids, with each repeat predicted to consist of a pair of transmembrane segments connecting an ectodomain which, in the case of AAC and UCP, is exposed to the matrix (2-4). The transmembrane segments exhibit amphiphilic characteristics and, therefore, are probably stabilized in the membrane as paired helical structures (2).

Analysis of UCP (5) and AAC (6-8) by deletion mutagenesis and hybrid protein constructions suggest the existence of at least two mitochondrial-targeting signals within the polypeptide - one located within the N-terminal third of the molecule (i.e., within the first repeat domain) and the other downstream of this position. Neither protein is made as a higher molecular weight precursor (2, 5, 9, 10). In the case of UCP, the amino terminal third is responsible for both targeting and membrane insertion (5), and as well may be required to help mediate insertion of the rest of the molecule (5). We recently suggested that UCP follows a coordinate insertion pathway during import into mitochondria, in which the three pairs of membrane-spanning segments are threaded into the inner membrane led by matrix-targeting signals located in the ectodomains (5). 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 three-fold repeat structure of UCP, 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 carbamyl transferase. The hybrid protein was imported to the matrix compartment where the pOCT 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 [^{35}S]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 pSPpO-UCP, the Plasmid Encoding pO-UCP

pSPUCP (5) was linearized with SphI and then partially digested with BglII to delete a fragment encoding the N-terminal 16 amino acids of UCP. It was replaced with two fragments, an SphI - PvuII fragment from pSP019 (12) encoding the entire signal sequence of pOCT as well as the first 4 amino acids of the mature enzyme, and a PvuII - BglII oligonucleotide adaptor, CTGCAACCCACCGGGGTCAA, 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 OCT.

Succinate Cytochrome c Oxidoreductase Activity

Mitochondria (50 μg protein) were added to 1 ml of assay medium (exactly as described in ref 13) to initiate the reaction. After 2 minutes, 10 μl of 1 N HCl was added and the absorbance at 550 nm determined. Total enzyme activity was routinely observed to be $\sim$100 nmol cytochrome c reduced/mg 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 UCP_{d1-12}, respectively, are imported to the inner membrane of rat heart mitochondria *in vitro*, as judged by their acquisition of $\Delta\psi$-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 ($\Delta\psi$-dependent) and product adventitiously and perhaps cryptically associated with the surface of the organelle ($\Delta\psi$-independent) (5). As illustrated previously, the deletion mutant UCP_{d1-12} 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 pOCT 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 pOCT signal sequence (32 amino acids) from the hybrid protein would yield a "mature" product in which 5 amino acids of mature OCT (SQVQL) replace 9 amino acids (MVSSTTSEV) from the N-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 UCP_{d1-12}, except of course that processing of UCP_{d1-12} 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 N-terminal pOCT signal sequence presumably gained access to the matrix compartment where the Zn^{++}-dependent pOCT processing enzyme is located (14). As expected, therefore, import of pO-UCP in the presence of o-phenanthroline, a Zn^{++} 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 Na_2CO_3, pH 11.5 (not shown). This analysis was extended to include the full-size precursor that was allowed

Fig. 1. pO-UCP is imported and processed by rat heart mitochondria *in vitro.*

Plasmids encoding pO-UCP and UCP_{d1-12} were transcribed, and translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Import was carried out for 30 minutes, 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 CCCP. Samples were divided into two aliquots. One aliquot (lanes 3, 5, 8, and 10) was incubated with trypsin (final concentration 50 μg/ml) at 4°C for 30 minutes 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 UCP_{d1-12}, respectively. p and m: precursor and mature forms of pO-UCP, respectively.

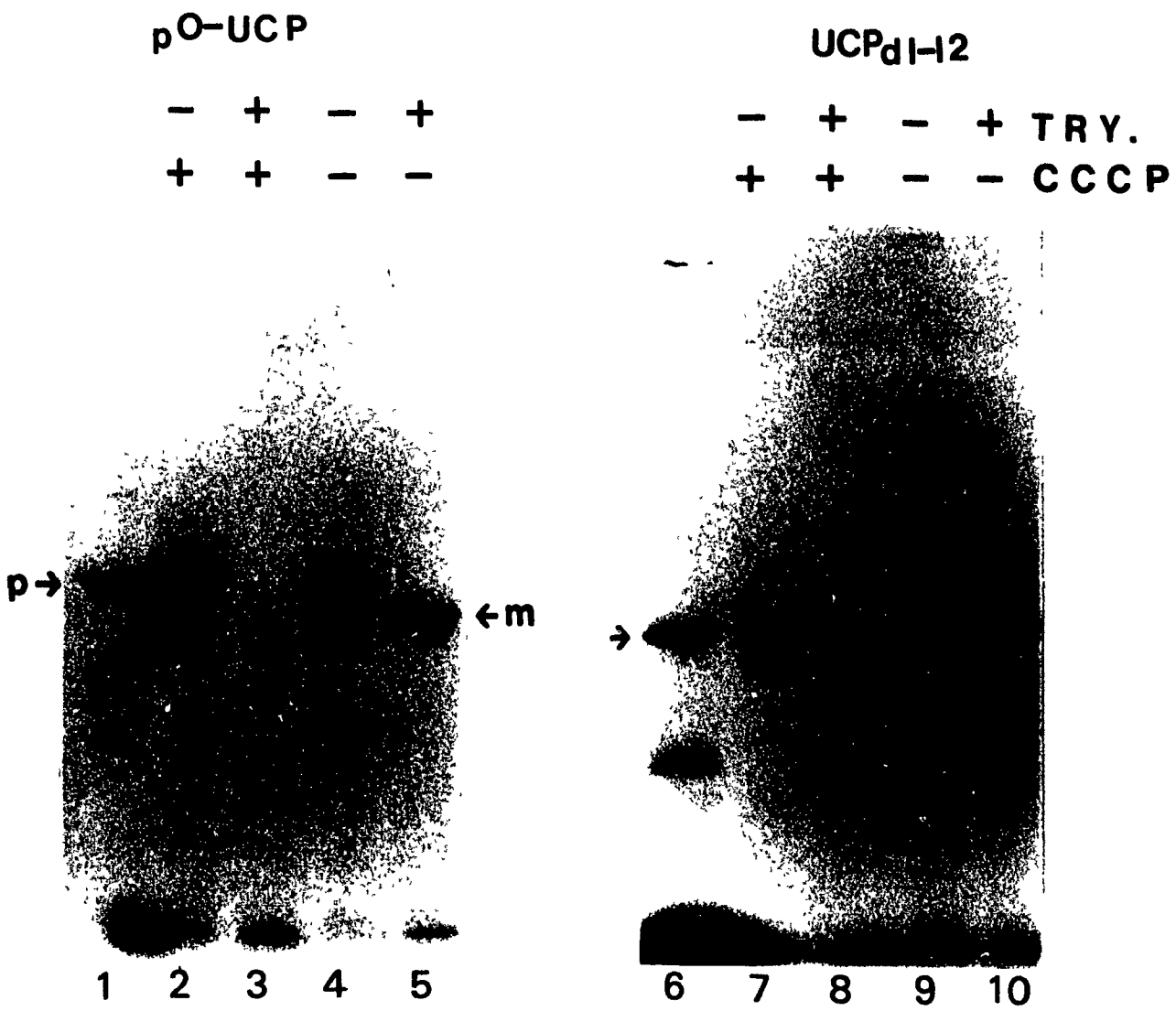
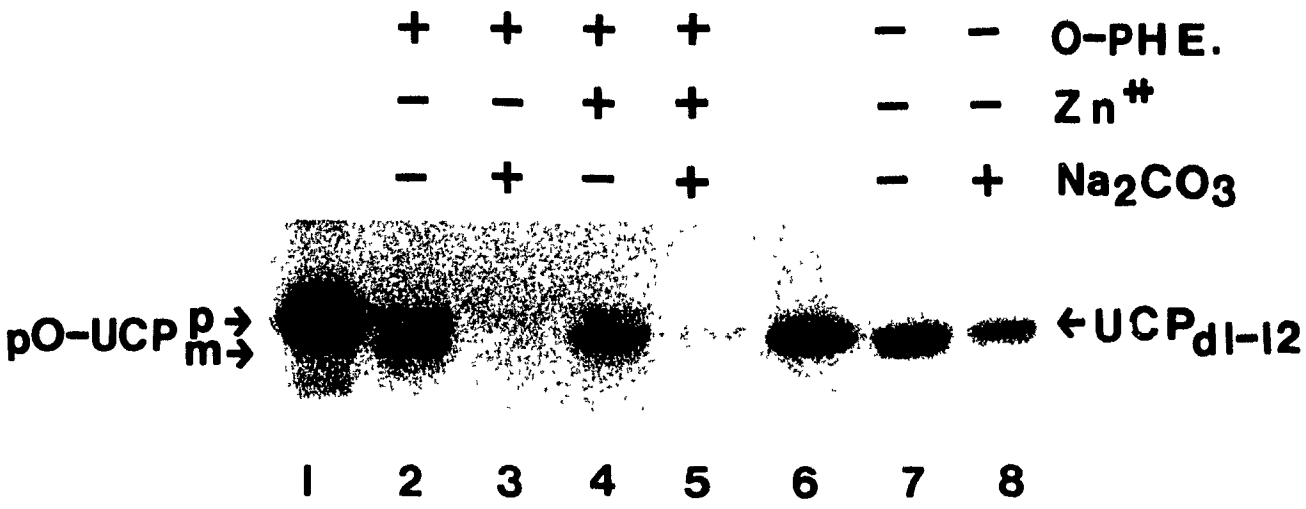

pO-UCP
UCPdl-12
- + - +
+ + - -
- + - + TRY.
+ + - - CCCP
p
m
1 2 3 4 5
6 7 8 9 10

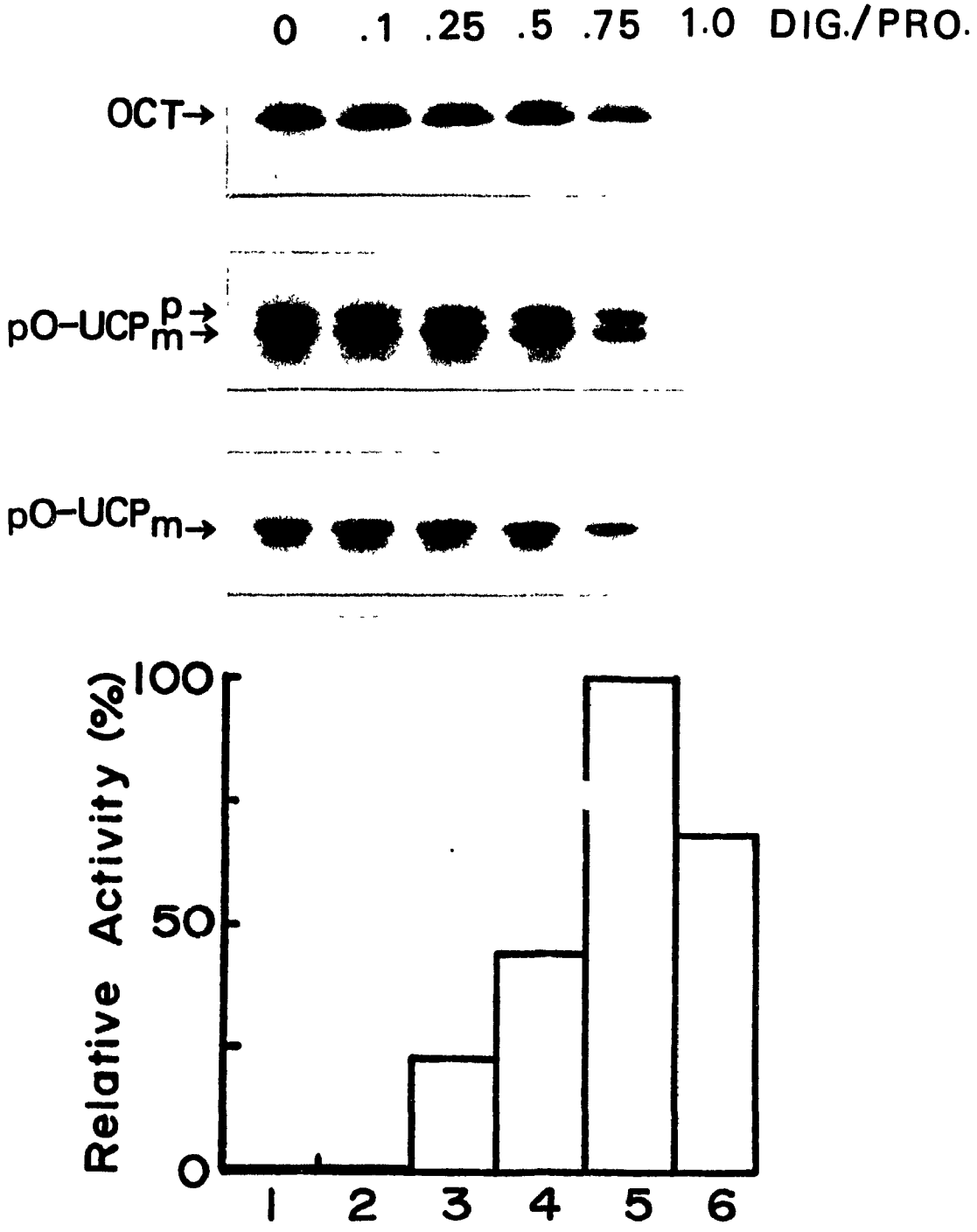
Fig. 2. Precursor and processed pO-UCP are released by alkaline extraction following import into mitochondria. Lanes 2-5. Import of pO-UCP was performed as described in Fig. 1, but in the presence of 1 mM o-phenanthroline and 5 mM EDTA. After trypsin treatment (Fig. 1), mitochondria were collected by 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 $ZnCl_2$ to a final concentration of 1.5 mM. Both aliquots were incubated at 30°C for 30 minutes. Excess Zn^{++} (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 minutes. Samples were divided: mitochondria in one aliquot (lanes 3 and 5) were extracted with 0.1 M Na_2CO_3, pH 11.5, and the membranes recovered by high speed centrifugation (5); 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 UCP_{d1-12}; lanes 7 and 8, import of UCP_{d1-12} followed by trypsin treatment as described in Fig. 1; lane 7, mitochondrial pellet; lane 8, membrane pellet following extraction with 0.1 M Na_2CO_3, pH 11.5.


+ + + + − − O-PHE.
− − + + − − Zn++
− + − + − + Na2CO3
pO-UCP p m
←UCPdl-12
1 2 3 4 5 6 7 8

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 Zn^{++}, 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 UCP_{d1-12} 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 Na_2CO_3, pH 11.5, and the membranes recovered by high speed centrifugation (Fig. 2). The percent recovery of imported UCP_{d1-12} 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 gradient across the mitochondrial inner membrane (Fig. 1), that processing was carried out by the Zn^{++}-dependent signal peptidase (Fig. 2), and that the resulting processed product was released by alkaline extraction (Fig. 2), suggest that it accumulated in the soluble matrix compartment rather than being retained in the inner membrane. This conclusion was extended by the results presented in Fig. 3, which rule out the possibility that processed pO-UCP was located in the intermembrane space; rather, it co-localized with OCT, a matrix marker. Following import of pO-UCP or pOCT, mitochondria were treated with various concentrations of digitonin to disrupt the outer membrane, and the sensitivities of OCT, processed pO-UCP, and pO-UCP (which was allowed to accumulate inside mitochondria in

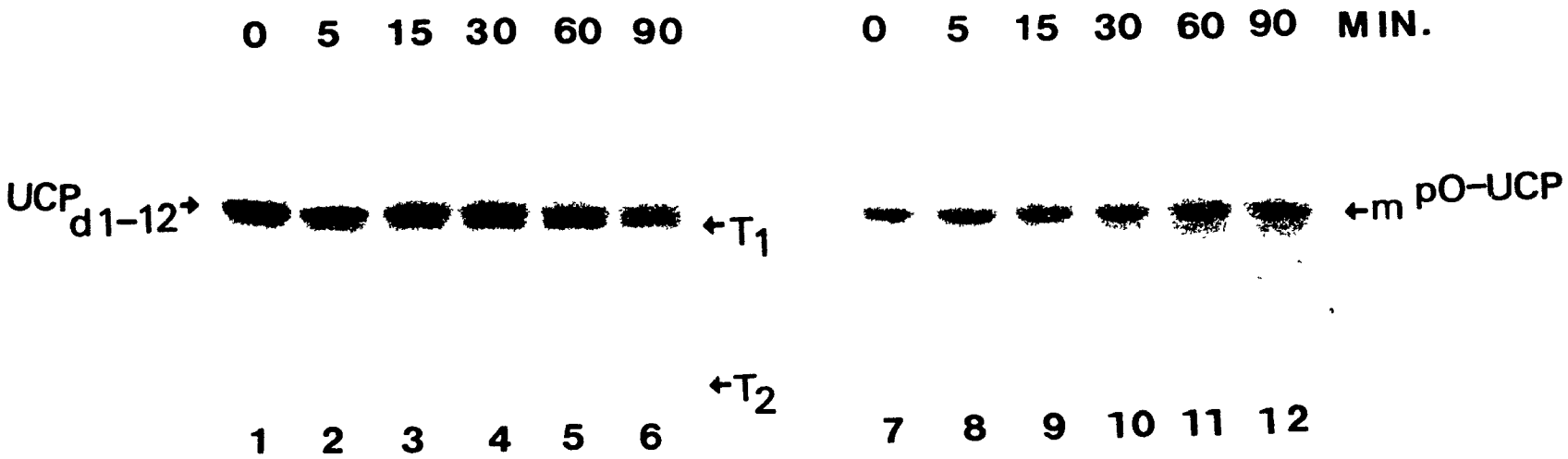
Fig. 3. Effect of digitonin on the accessibility of precursor and mature forms of pO-UCP to trypsin. Following import of pOCT and UCP_{d1-12} into mitochondria, digitonin (twice recrystallized from ethanol) was added to a final concentration as indicated. Incubation was at 4°C for 15 minutes after which the mitochondria were collected by centrifugation. Mitochondria were resuspended in import medium (11) and the resuspension 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 in 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. Number 1-6 on the bar graph correspond to treatments with 0, 0.1, 0.25, 0.5, 0.75, and 1.0 mg digitonin/mg mitochondrial protein, respectively.


0 .1 .25 .5 .75 1.0 DIG./PRO.
OCT→
pO-UCP$_m^p$→
pO-UCP$_m$→
Relative Activity (%)
100
50
0
1 2 3 4 5 6

the presence of o-phenanthroline) to exogenous trypsin were determined (Fig. 3). All three polypeptides were equally resistant to trypsin, up to a concentration of 0.75 mg digitonin/mg mitochondrial protein; in contrast, disruption of the outer membrane by 0.5-0.75 mg digitonin resulted in the loss of latency of succinate cytochrome c oxidoreductase activity, as indicated by exogenous substrate (cytochrome c) gaining access to the enzyme which is located on the outer aspect of the inner membrane (13). The partial sensitivity to trypsin of the 3 polypeptides that was observed at 0.75 mg digitonin presumably reflected partial damage to the inner membrane; by 1.0 mg digitonin, 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 (T_1) was ~30 kDa in size and was generated by removal of ~2 kDa from the C-terminus of UCP; a minor fragment (T_2) of ~25 kDa arose with a time delay from T_1 (15). As shown in Fig. 4, fragments similar to those demonstrated in ref. 15 (T_1 and T_2) were obtained when rat heart mitochondria containing newly-imported UCP_{d1-12} were treated under similar conditions; treatment of frozen-thawed mitochondria with trypsin for progressively longer times led to the disappearance of full-size UCP_{d1-12} and the appearance of T_1 and T_2 (Fig. 4, lanes 1-6). This is in contrast to the situation for processed pO-UCP

Fig. 4. The processed form of pO-UCP lacks a trypsin sensitive domain extending into the intermembrane space. Import of UCP_{d1-12} and pO-UCP was performed as in Fig. 1. After a brief digestion (5 minutes on ice) by trypsin (50 µg/ml) to remove precursor proteins on the surface of the mitochondria, the protease was inhibited by 1 mg/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. T_1 and T_2, tryptic fragments of UCP_{d1-12} (see text).


0 5 15 30 60 90
0 5 15 30 60 90 MIN.
UCP d1-12
←T1
←T2
←m pO-UCP
1 2 3 4 5 6
7 8 9 10 11 12

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 UCP_{d1-12} 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 minutes (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 (5). 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 pOCT. In this context, the fusion protein is translocated to the soluble matrix compartment of mitochondria *in vitro* where it is processed by the Zn^{++}-dependent matrix processing enzyme. Presumably, the presence of the pOCT 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 pOCT 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.

[1]ABBREVIATIONS

UCP, uncoupling protein; pOCT, preornithine carbamyl transferase; OCT, processed pOCT; pO-UCP, a fusion protein containing the N-terminal 37 amino acids of pOCT fused to amino acids 10-307 of UCP; CCCP, carbonylcyanide m-chlorophenylhydrazone; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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CHAPTER 4

A SYNTHETIC SIGNAL PEPTIDE ASSOCIATES WITH ADP/ATP CARRIER PROTEIN FOLLOWING MITOCHONDRIAL IMPORT ACROSS THE OUTER MEMBRANE *IN VITRO*

ABSTRACT

Pretreatment of intact mitochondria with low concentrations of a synthetic peptide corresponding to amino acids 1-27 of preornithine carbamyl transferase (pOCT) under chemical cross-linking conditions resulted in the subsequent inability of the organelle to import proteins destined either for the mitochondrial matrix or inner membrane; the treatment was without effect on the electrochemical potential across the inner membrane and, under the conditions employed, neither free peptide alone nor crosslinking agent alone affected import. The major protein product of irreversible peptide binding under cross-linking conditions was a mitochondrial protein of 30 kDa (p30). Pre-digestion of the surface of mitochondria with trypsin abolished subsequent peptide-p30 interactions, and the dose response of this interaction to trypsin was similar to the dose response of protein import to trypsin. However, p30 itself was not the trypsin-sensitive component. At saturation of peptide binding to p30 in the intact organelle, only about 10-20% of the total p30 population was found attached to peptide. Employing a biotinylated peptide as ligand, the peptide-p30 complex was purified to homogeneity by affinity chromatography, employing immobilized streptavidin. Structural analysis of the derived p30 molecule suggested an identity with the ADP/ATP carrier protein (AAC), an abundant family of proteins of the mitochondrial inner membrane. This identity was confirmed immunologically. While there is no evidence to date suggesting that AAC plays a direct role in protein import into mitochondria, our results demonstrate an obvious affinity of this protein for a mitochondrial signal sequence.

INTRODUCTION

Although the details are lacking, a variety of indirect studies have provided strong circumstantial evidence that mitochondrial import and sorting of precursor polypeptides are mediated by a complex machinery composed of a number of different proteins that function at various steps along the import pathway. Certain of these components must function by a signal recognition process since it is only this information within a precursor polypeptide that is required to target either normal or foreign proteins into the organelle (for recent reviews, see refs. 1,2). Because such topogenic signals are encoded by a discrete amino acid sequence within any given precursor protein, it may be possible to exploit synthetic peptides corresponding to these sequences as probes to identify specific signal recognition components of the mitochondrial import machinery.

A major question, however, is whether or not such targeting sequences within the context of a short peptide faithfully mimic the structure-function features of the sequence as it exists in the full-length precursor molecule. The fact that a number of different foreign proteins are taken up by mitochondria when fused to mitochondrial signal sequences (3-5) suggests that the signal may normally function independently of the rest of the molecule. Nevertheless, one potential source of aberrant behaviour may derive from the amphiphilic structure of mitochondrial signal sequences (7-11), a property that can give rise to non-specific membrane perturbations at high concentrations of peptide (12, 13). In the case of a well-characterized synthetic peptide corresponding to amino acids 1-27 of rat preornithine carbamyl transferase (pOCT[1]),

however, the problem is less severe: the peptide exhibits a relatively weak binding affinity to anionic lipid membranes (7, 11), it does not penetrate into the lipid bilayer to any significant extent, even in the presence of a large electrochemical potential (inside negative) across the membrane (11), and it does not collapse the potential across the inner mitochondrial membranes unless present in concentrations in excess of 10 μM (12). Taken together, these findings imply that penetration of the pOCT signal sequence across mitochondrial membranes during import is mediated by membrane proteins rather than by direct passage through an exclusively lipid domain. The synthetic pOCT signal peptide, therefore, appears to be a suitable candidate for use as a ligand probe to identify such proteins. Indeed, earlier work has shown that this peptide, designated pO(1-27), can be cross-linked to a mitochondrial membrane protein with a predicted size of 30 kDa; binding of pO(1-27) to the 30 kDa protein (p30) was specific, saturable, and reversible (14).

In the present study, we have purified p30 to homogeneity and have identified the protein as the ADP/ATP carrier (AAC), a relatively abundant protein of the mitochondrial inner membrane that performs the critical function of adenine nucleotide exchange between the cytosol and mitochondrial matrix (15). In mammals, AAC is encoded by a small family of genes (16) and in yeast by at least 2 genes (17); whether or not all members of the family are functionally equivalent is not known, nor is it known whether the protein can perform functions other than nucleotide exchange. Certainly, it is well established that interference with the nucleotide exchange function of AAC has no direct effect on the ability of mitochondria to import proteins (18). Nevertheless, the results of

this study clearly demonstrate an affinity of ACC for a mitochondrial targeting sequence.

EXPERIMENTAL PROCEDURES

General

Earlier publications describe the procedures used for transcription-translation of recombinant plasmids (5), import of translation products by purified rat heart mitochondria *in vitro* (19), chemical cross-linking of pO(1-27) to intact mitochondria *in vitro* (14), and analysis of import products and peptide-protein conjugates by SDS-PAGE and radioautography (14, 19). Additional details are provided in the Figure legends.

Biotinylation and Iodination of pO(1-27)cys

1.1 mg of maleimidobutyrylbiocytin (CalBiochem) was dissolved in 25 μl dimethylsulfoxide. The solution was combined with 225 μl 10 mM Hepes, pH 7.4, and immediately transferred to an Eppendorf tube containing 0.7 mg NH_2-pO(1-27)cys-amide (Fig. 1) and incubated at room temperature for 1 hour; un-incorporated biotin was inactivated by continuing the incubation at 4°C overnight after which time the mixture was frozen in small aliquots. The biotinylated peptide was labelled with ^{125}I (14), as required.

Cross-linking and Purification of Peptide-p30 Complexes

Solutions containing biotinylated peptide (0.8 mM) were thawed, adjusted to 35 mM 2 mercaptoethanol, incubated at room temperature for 20 minutes, and mixed with a small amount of ^{125}I-peptide-biotin. Crosslinking to purified, intact mitochondria was carried out as described earlier (14), with the following modifications: the crosslinking mixture contained (final concentration) 5 μM pO(1-27)cys-biotin, mitochondria (0.5 mg protein/ml), 0.25 M sucrose, 1 mM Mg acetate, 40 mM KCl, 10 mM K phosphate buffer, pH 7.4, and 50 μg/ml [bis(sulfosuccinimidyl)suberate]

(BS^3). The mixture was incubated at 12°C for 1 hour. Mitochondria were recovered by centrifugation, resuspended (1 mg protein/ml) in 0.1 M Na_2CO_3, pH 11.5, vigorously sonicated, and incubated on ice for 30 minutes to release non-integral membrane proteins. Membranes were collected by sedimentation at 50,000 g for 40 minutes. Following solubilization of the pellet in 2% (w/v) SDS, the soluble fraction was mixed with immobilized streptavidin (Pierce Chemical Co.; 1.5 mg mitochondrial protein/100 μl packed volume) in Triton medium [1% (w/v) Triton X-100, 10 mM EDTA, 0.02% (w/v) NaN_3, phosphate buffered saline, pH 7.4] After rotating overnight at 4°C, the beads were washed twice with Triton medium containing 0.5% SDS, once with Triton medium containing 1.0 M NaCl, and twice again with Triton medium containing 0.5% SDS. The beads were suspended in 2% SDS, 0.4 M urea, 0.1 mM EDTA, and 20 mM Na phosphate buffer, pH 7.4, and the peptide-p30 complex released by boiling.

Ligand Blotting

Mitochondrial proteins were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose paper. Ligand blotting was performed essentially as described in ref. 20. The blot was incubated sequentially for 1 hour at room temperature in each of buffers A, B, and C: buffer A is 10 mM K phosphate, pH 7.4, 30 mM $MgCl_2$, 1 mM EDTA, 0.1% (w/v) Triton X-100, 1 mM dithiothreitol, 5% (v/v) glycerol, and 3 M guanidinium HCl; buffer B is buffer A without guanidinium HCl; buffer C is 10 mM K phosphate, pH 7.4, 1 mM Mg acetate, 40 mM KCl, 5 mM KI, 0.1% Triton X-100, 1 mM dithiothreitol, 5% glycerol, and 0.05% (w/v) Tween 20. ^{125}I-p0(1-27) was diluted in buffer C (1×10^6 cpm/ml; 10^6 cpm/nmol peptide) and a minimum volume applied to the blot. After incubating at

room temperature for 1 hour, the blot was washed in large volumes of buffer C at room temperature for 45 minutes and air-dried. ^{125}I-peptide-protein complexes were visualized by autoradiography.

RESULTS AND DISCUSSION

Fig. 1 outlines the strategy that was employed in this study to develop a ligand probe that might be useful for the purification of mitochondrial protein(s) that interact with a synthetic mitochondrial-targeting (signal) sequence. The peptide, pO(1-27), corresponds to amino acids 1-27 of rat pOCT; it has been extensively investigated with respect to its structural and membrane surface-seeking properties (7, 11), its ability to inhibit import of heterologous precursor proteins (12), and its interaction with a 30 kDa mitochondrial protein, designated p30 (14). pO(1-27) contains 3 primary amino groups (positions 1, 11, and 16) which provide a means of covalently attaching the peptide to target protein(s) via the water soluble crosslinking agent, BS^3 (14), as well as containing a *tyr* at position 27 that can be labelled with ^{125}I (14). Additionally, we have modified pO(1-27) to include a *cys* residue at its C-terminus (11) (in place of *gly* at position 28 of pOCT), thus providing a thiol group for covalent attachment to maleimidobutyryl biocytin. Due to the high binding affinity that exists between biotin and streptavidin, peptide-protein conjugates can be purified by one-step affinity chromatography.

Cross-linking

When ^{125}I-pO(1-27) was incubated with intact mitochondria at 1, 12 and 30°C in the presence of BS^3, and total mitochondrial proteins subsequently analyzed by SDS-PAGE and radioautography, a predominant cross-linked product (34 kDa) was observed, yielding a predicted size for the target protein (i.e., assuming the addition of a single peptide moiety) of ≈30 kDa (Fig. 2). Similar results were obtained using ^{125}I-pO(1-27)cys (e.g., see Fig. 6). Also present in the gel was free peptide,

Fig. 1. Strategy for purifying protein-peptide complexes. pO(1-27)cys peptide amide (11) corresponds to amino acids 1-27 of pOCT, plus a cys in place of gly at position 28. The peptide can be modified to contain a maleimidobutyryl biotin group via the thiol of *cys*^{28}, and can be labelled with ^{125}I at *tyr*^{27}. Upon addition of the modified peptide to intact mitochondria, followed by chemical cross-linking with BS^3, covalent peptide-protein conjugates can be purified using immobilized streptavidin, as described in Experimental Procedures.

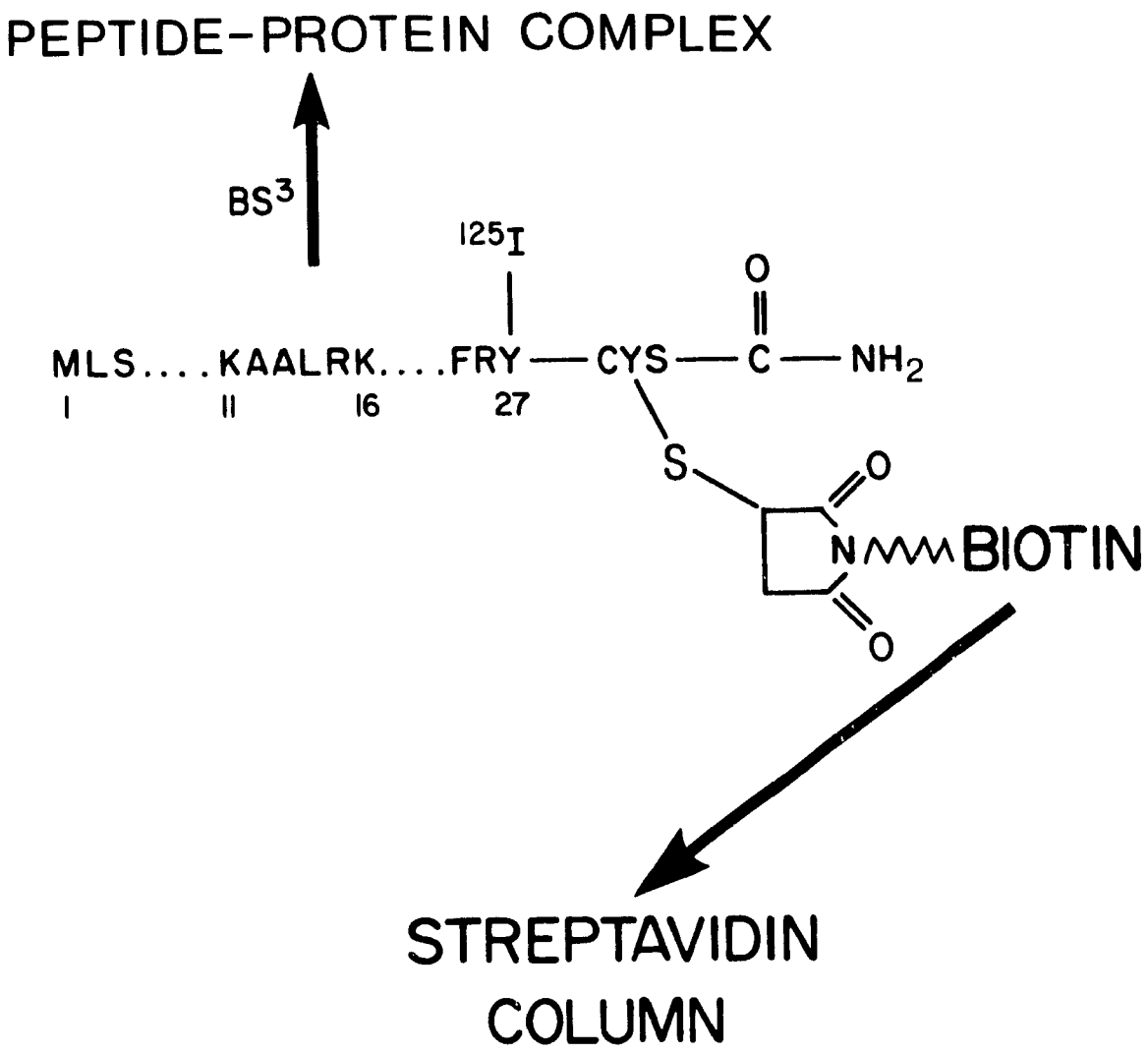
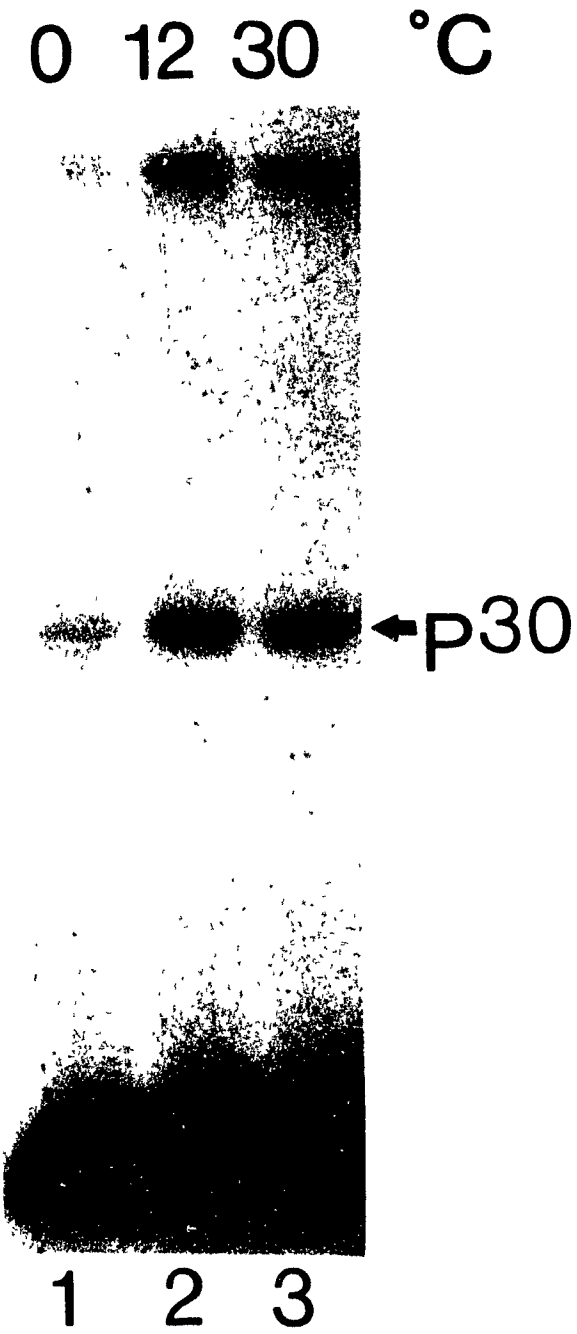

PEPTIDE-PROTEIN COMPLEX
BS3
125I
O
MLS....KAALRK....FRY — CYS — C — NH2
1 11 16 27
S
O
N
BIOTIN
O
STREPTAVIDIN
COLUMN

Fig. 2. Chemical cross-linking of pO(1-27) to mitochondrial p30. Mitochondria (0.5 mg protein/ml) were incubated with 5 μM ^{125}I-pO(1-27) (10^6 cpm/nmol) in the presence of 50 μg/ml BS^3 for 30 minutes at the indicated temperatures (see Experimental Procedures). The mitochondria were collected by centrifugation at 12,000 g for 5 min and analyzed by SDS-PAGE and autoradiography. The arrow denotes a cross-linked pO(1-27)-protein complex of ~34 kDa, with the protein component having a predicted size of ~30 kDa. Free peptide migrates in these gels near the front, while aggregated material collects at the spacer/resolving gel interface at the top.


0 12 30 °C
p30
1 2 3

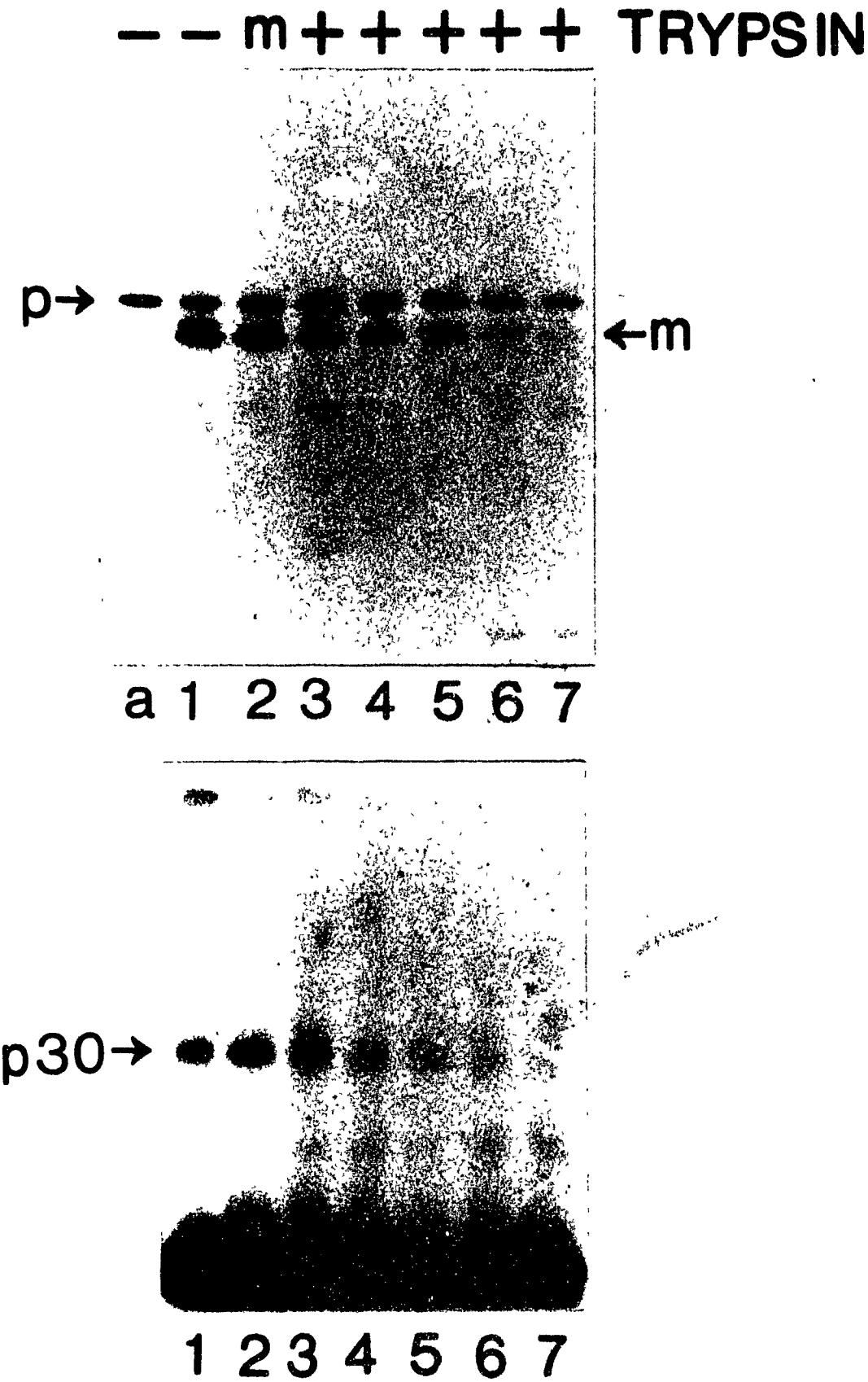
which migrated near the gel front, as well as product which did not enter the resolving gel and probably represented large aggregates of the peptide-p30 complex (Fig. 2). Cross-linking of pO(1-27) to p30 in intact mitochondria was equally effective at 30°C and 12°C, but was reduced at 0°C (Fig. 2) suggesting either that access or binding of free peptide to p30 was restricted at this temperature or that the efficacy of the cross-linking agent was affected.

That peptide gains access to p30 via a proteinaceous pathway is suggested by the results shown in Fig. 3. Pretreatment of mitochondria with exogenous trypsin blocked pOCT import (Fig. 3, upper panel) and prevented peptide from cross-linking to p30 (Fig. 3, lower panel). Analysis of original radioautograms by laser densitometry (not shown) revealed that the dose response of these two events (precursor import and peptide cross-linking) to trypsin was similar; 50% inhibition occurred at approximately 2-4 μg trypsin/mg mitochondrial protein (not shown). Thus, the correlation between the sensitivity of import to trypsin and the trypsin-sensitivity of peptide cross-linking to p30 suggests that peptide is gaining access to p30 via the import pathway.

Covalent Attachment of pO(1-27) to p30 Correlates with Inhibition of Precursor Import into Mitochondria

To explore the possibility that an irreversible association between pO(1-27) and target protein(s) in intact mitochondria might plug the protein translocation machinery and block the ability of the organelle to import precursor proteins, conditions were first sought in which pretreatment of mitochondria with either pO(1-27) alone or BS^3 alone was ineffective in inhibiting import. This was achieved by subsequently

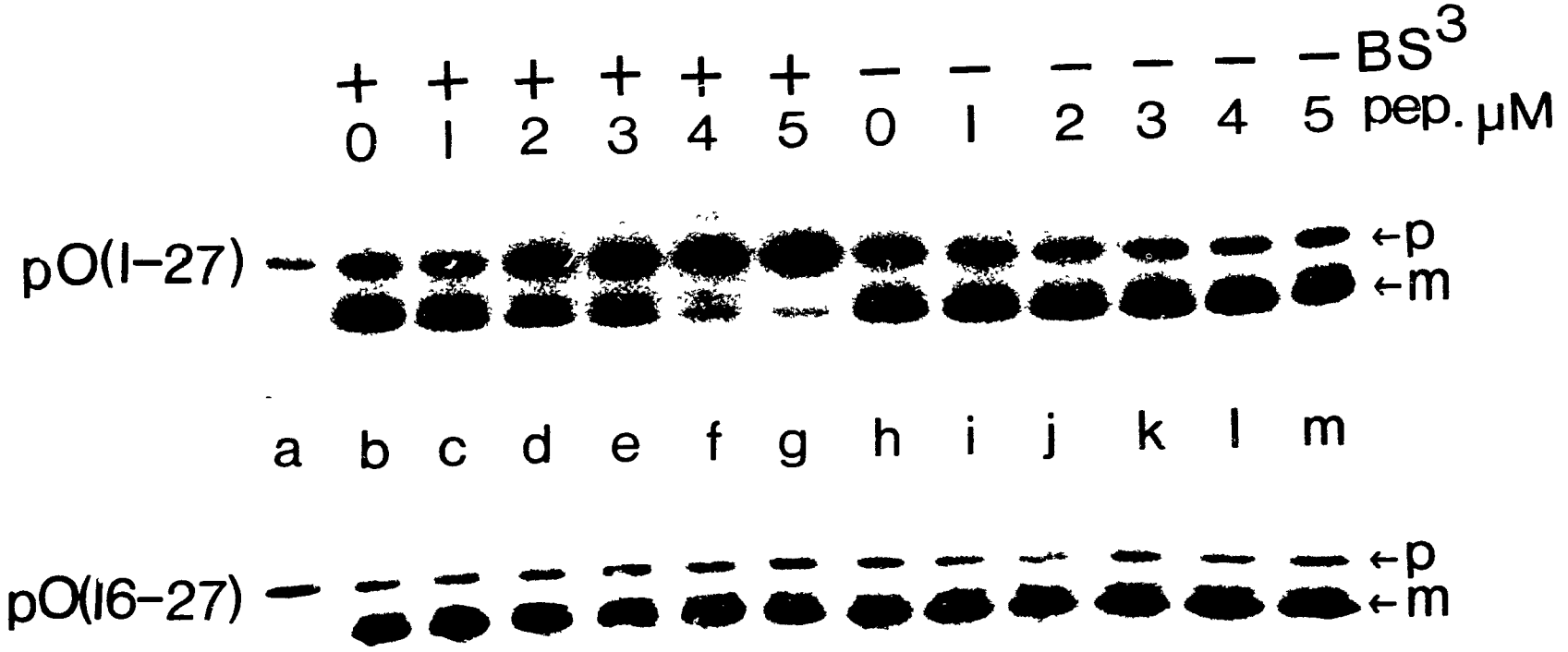
Fig. 3. Cross-linking of pO(1-27) to p30 is sensitive to pretreatment of mitochondria with trypsin. Mitochondria (1 mg protein/ml) were incubated for 30 min. at 0°C either with trypsin or trypsin which had been pre-inhibited with soybean trypsin inhibitor (m = mock trypsin, lane 2), exactly as described previously (14). Mitochondria were collected and suspended in mitochondrial import medium (ref. 19; 0.5 mg protein/ml) containing 500 μg/ml soybean trypsin inhibitor and either [^{35}S]pOCT translation product in reticulocyte lysate (top panel) or 5 μM ^{125}I-pO(1-27) (lower panel). Following import at 30°C or cross-linking at 0°C, mitochondria were recovered and analyzed by SDS-PAGE and autoradiography. Lane 2, 100 μg/ml trypsin pre-inhibited with 2 mg/ml soybean trypsin inhibitor; lanes 1, and 3-7, 0, 1, 5, 10, 40, 100 μg trypsin/ml, respectively; lane a, 10% of input pOCT. The positions of pOCT(p), processed product (m), and p30 are indicated.


– – m + + + + + TRYPSIN
p→
←m
a 1 2 3 4 5 6 7
p30→
1 2 3 4 5 6 7

washing mitochondria with 1.0 M KCl for 15 minutes at 0°C prior to testing the mitochondria for import. Pretreatment of mitochondria with 50 μg/ml BS^3 at 0°C for 30 minutes in the absence of pO(1-27) was not inhibitory to the import of pOCT (Fig. 4, upper panel, cf. lanes b and h). Furthermore, pretreatment with up to 5 μM of free peptide alone under these conditions was also non-inhibitory (Fig. 4, upper panel, lanes h-m). However, when both pO(1-27) (up to 5 μM) and BS^3 were included in the pretreatment (i.e., resulting in peptide-p30 cross-linking, Fig. 2 lane 1), import of pOCT was inhibited (Fig. 4, upper panel, lanes c-g). Similar results were observed for import of uncoupling protein, an integral protein of the inner membrane (not shown). The concentration of pO(1-27) that was required to give near-maximal inhibition of pOCT is similar to the concentration that is saturable for peptide-p30 cross-linking (14). In contrast, substituting pO(16-27) for pO(1-27) was without effect (Fig. 4, lower panel). pO(16-27) is a peptide corresponding to amino acids 16-27 of pOCT which has a similar charge:mass ratio as pO(1-27); unlike pO(1-27), however, pO(16-27) does not compete for import of precursor proteins into mitochondria (12) and does not cross-link to p30 (14).

The inhibition of import of pOCT by pO(1-27) described in Fig. 4 was not due to an effect on the electrochemical potential across the mitochondrial inner membrane (assayed as described in ref. 12) (not shown). However, it is noteworthy that cross-linking of 5 μM peptide to mitochondria inhibited subsequent import of pOCT by >90% (Fig. 4, upper panel, lane g), despite the fact that under such conditions (5 μM peptide at 0°C) cross-linking to p30 is saturated but not all of the p30 population is associated with peptide (Fig. 2, cf lanes 1 and 3). Thus, inhibition of import correlates with cross-linking of peptide to only part

Fig. 4 Cross-linking of pO(1-27) to intact mitochondria inhibits the subsequent ability of the well-washed organelle to import pOCT. Mitochondria were suspended in import medium (0.5 mg protein/ml) containing the indicated concentrations (0-5 μM) of pO(1-27) and incubated at 0°C for 30 min in the presence (lanes b-g) or absence (lanes h-m) of 50 μg BS^3/ml. Glycine was added to a final concentration of 5 mM to terminate the cross-linking reaction, and the incubation was continued for 5 min. Mitochondria were collected, suspended in import medium containing 1.0 M KCl, and incubated at 0°C for 15 min. The mitochondria were recovered and suspended in import medium (0.5 mg mitochondrial protein/ml) supplemented with reticulocyte lysate containing [^{35}S]-pOCT. Import was performed and analyzed as described in Fig. 3. Lane a, 10% of input pOCT. The positions of pOCT (p) and processed product (m) are indicated.


+ + + + + + − − − − − − BS3
0 1 2 3 4 5 0 1 2 3 4 5 pep. μM
pO(1–27)
←p
←m
a b c d e f g h i j k l m
pO(16–27)
←p
←m

of the total available p30 population. Furthermore, it cannot be ruled out that inhibition of import correlates with peptide binding to a different protein(s) which is present at a concentration much lower than p30 (cf. Fig. 2) but which has a binding affinity for pO(1-27) that is similar to that of p30.

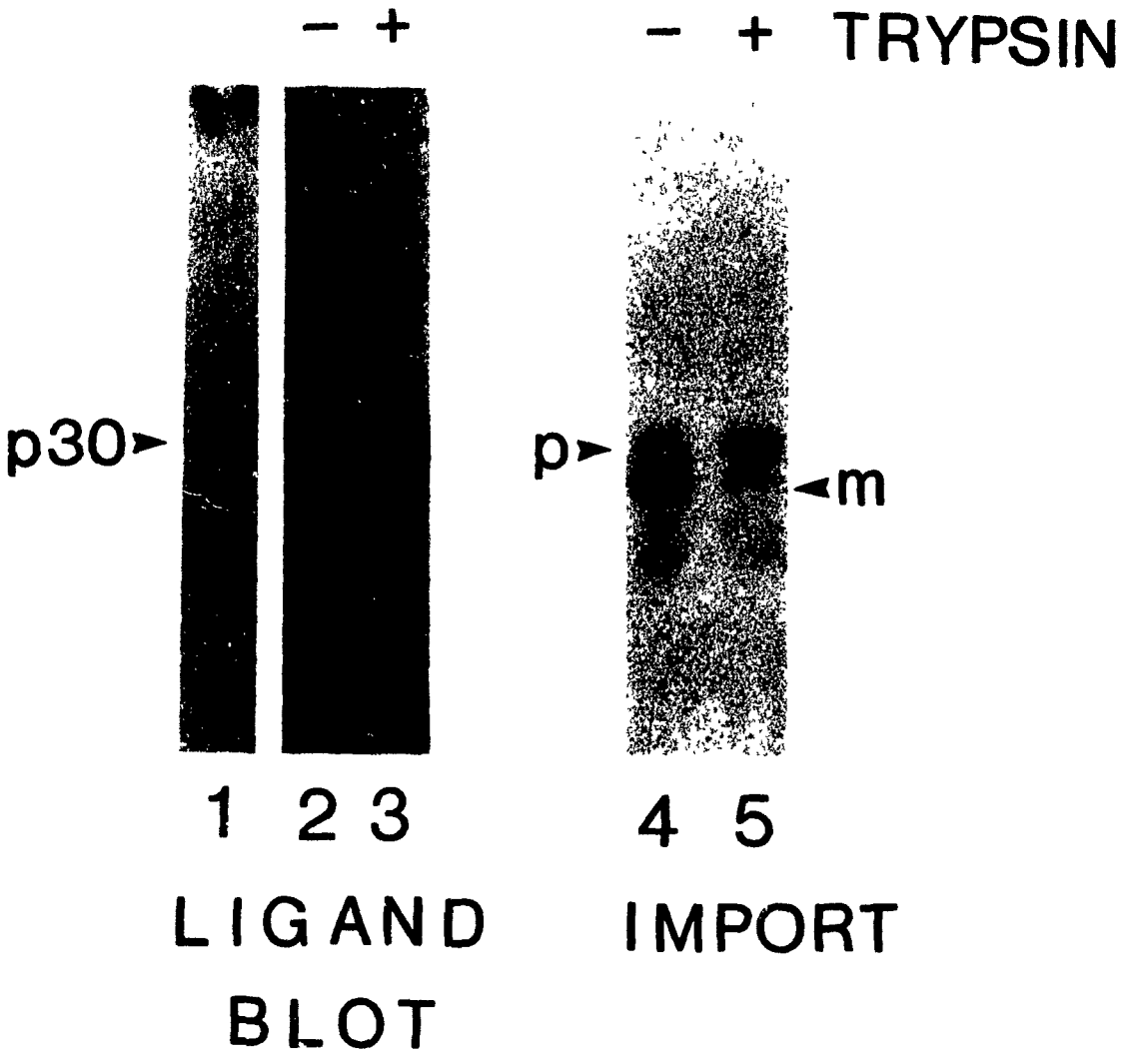
Finally, inhibition of import of pOCT by cross-linking peptide to mitochondria resulted in an accumulation of full-size precursor which co-sedimented with mitochondria upon subsequent centrifugation of the import reaction mixture (Fig. 4, upper panel, lanes c-g). Cross-linked mitochondria that had subsequently been treated with low concentrations of trypsin (40 μg/ml), however, did not demonstrate such an accumulation of bound precursor (data not shown), but rather yielded a background level of co-sedimenting precursor similar to that observed in Fig. 3, upper panel, lanes 6 and 7. Cross-linking of pO(1-27) to mitochondria, therefore, did not prevent binding of pOCT to trypsin-sensitive sites on the surface of the organelle, but instead interfered with some other step of the import pathway.

Ligand Blotting

Fig. 5 shows that pO(1-27) can also bind to p30 under conditions where total mitochondrial proteins were resolved by SDS-PAGE and transferred to nitrocellulose paper, after which the proteins were "renatured" following a treatment with detergent and guanidine-HCl. That the 30 kDa protein recognized by pO(1-27) in intact mitochondria (Fig. 2) and on nitrocellulose blots (Fig. 5) was one and the same was confirmed immunologically and by peptide mapping (discussed later).

In contrast to the situation using intact mitochondria, however, pretreatment of mitochondria with exogenous trypsin did not affect the

Fig. 5. Treatment of intact mitochondria with trypsin does not degrade p30. Mitochondria were treated with 40 μg/ml trypsin (lanes 3 and 5) or with 40 μg/ml trypsin pre-inhibited with soybean trypsin inhibitor (lanes 2 and 4) as described in Fig. 3. The mitochondria suspensions were split in half, and the mitochondria recovered and analyzed either for import of [^{35}S]pOCT or for ligand blotting with ^{125}I-pO(1-27) (see Experimental Procedures). Lane 1 shows a ligand blot in which the prior incubations in buffers A, B, and C (protein renaturation conditions, see Experimental Procedures) were omitted. The positions of p30, pOC^{m} (p), and processed product (m) are indicated.

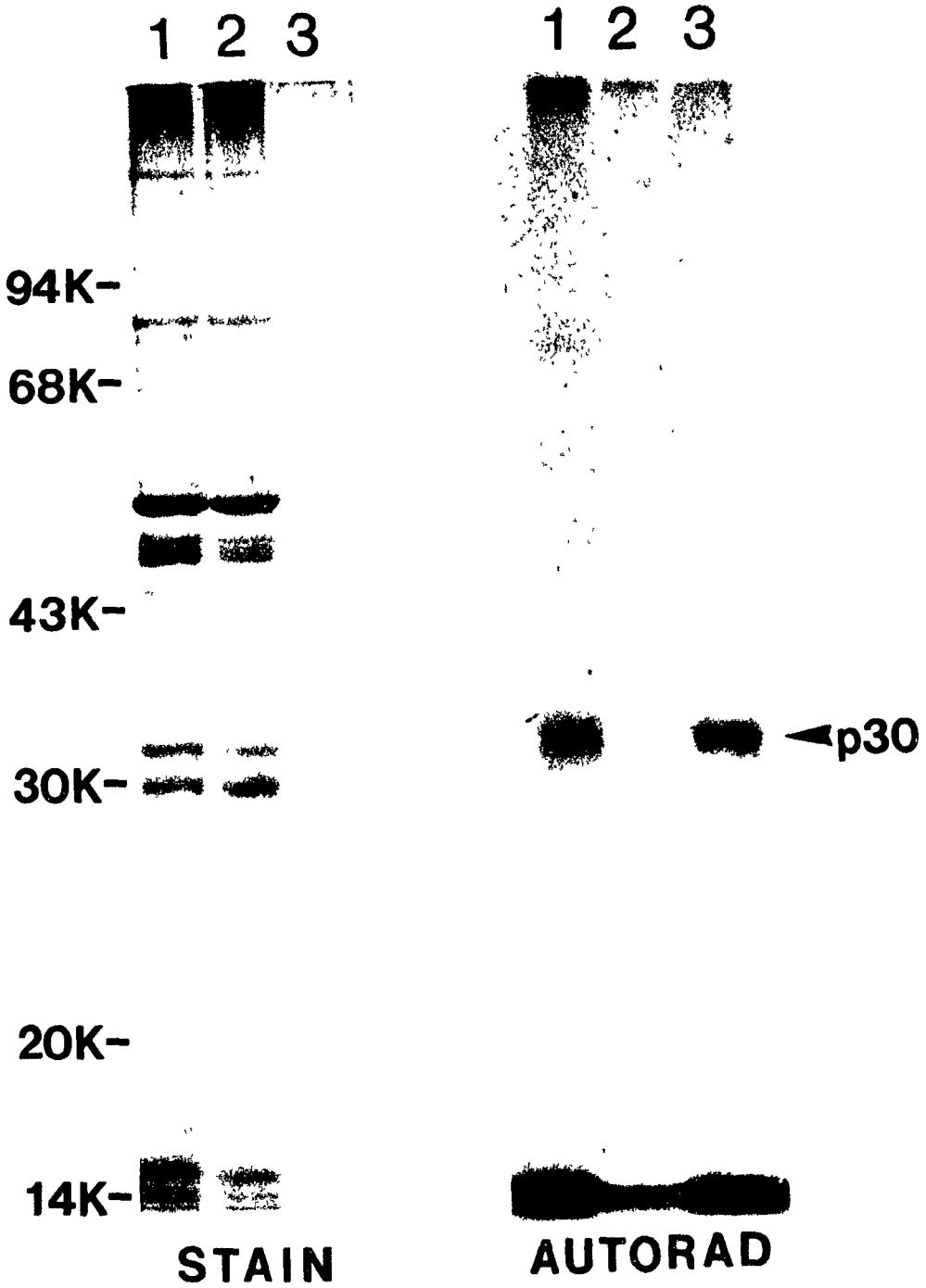

- +
- + TRYPSIN
p30
p
m
1 2 3
4 5
LIGAND BLOT
IMPORT

level of peptide-p30 interactions on a nitrocellulose blot (cf Fig. 5, lane 3 with Fig. 3, lower panel). We interpret this to mean that, whereas trypsin pretreatment of mitochondria prevented both import of pOCT (Figs. 3 and 5) and access of pO(1-27) to p30 (Fig. 3) in the intact organelle, p30 itself is not the trypsin-sensitive component; rather, trypsin presumably inactivates a surface protein that is requisite for pO(1-27) to gain access to p30 (e.g., a surface receptor). Finally, calculations based on the specific radioactivity of pO(1-27) and on the assumption that p30 binds a single peptide moiety, revealed that the amount of p30 that binds peptide on a nitrocellulose blot (Fig. 5) is 5- to 10-times higher than the amount of p30 that cross-links to peptide in the intact organelle (Fig. 2).

Purification of p30 and Identification as an ADP/ATP Carrier Protein(s)

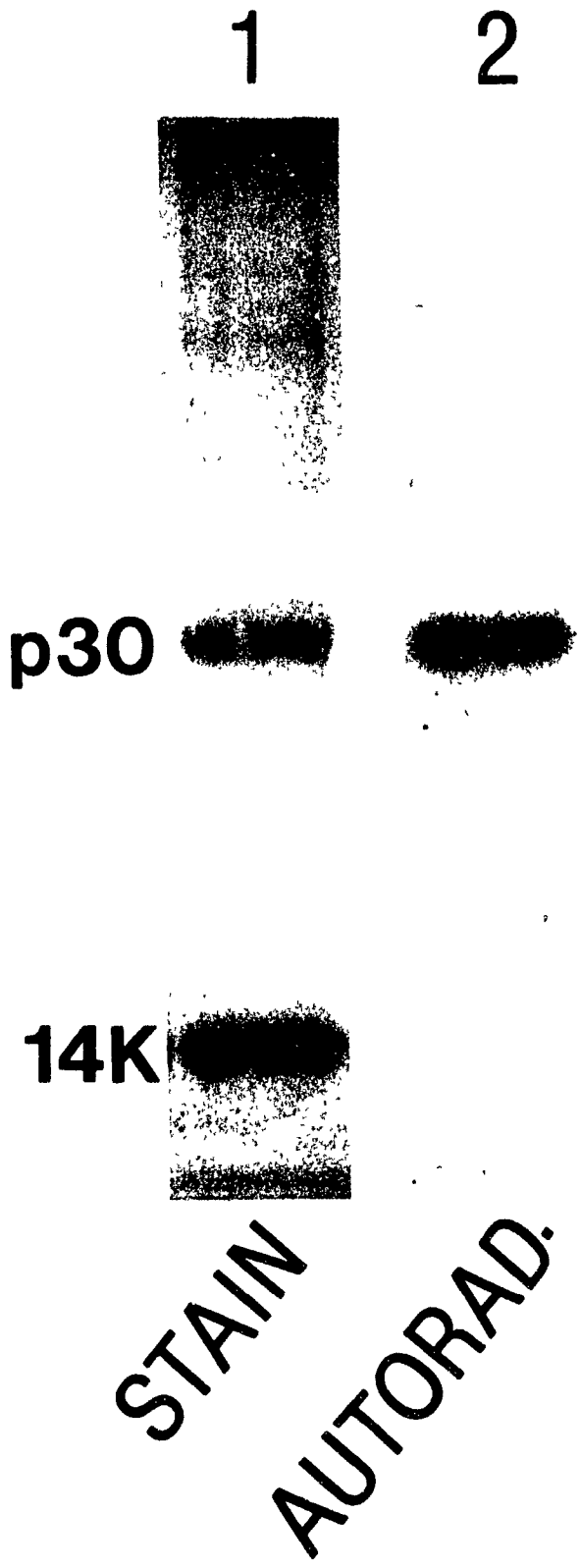
Cross-linking of ^{125}I-pO(1-27)*cys*-biotin to intact mitochondria yielded a reaction profile very similar to that obtained with the unmodified peptide (cf. Fig. 2 and Fig. 6A, right panel, lane 1), i.e., p30 was the predominant product. As shown previously (14), we found that the peptide-p30 complex was recovered exclusively with the alkali-insoluble fraction of total mitochondrial proteins. When this fraction was solubilized in SDS-Triton medium and applied to a streptavidin column, the ^{125}I-pO(1-27)cys-biotin-p30 complex was retained with high specificity, and could be recovered (together with free peptide) by elution in hot SDS-urea (Fig. 6A). Also present in the eluate, however, was a 14 kDa protein (Fig. 6B) that was also observed when the column alone was treated with hot SDS and, presumably, represented subunits of

Fig. 6. Purification of p30. The p30-peptide complex was purified employing pO(1-27)cys-biotin, as described in Experimental Procedures. PANEL A. Protein samples (equivalent aliquots) were resolved by SDS-PAGE and either stained with Coomassie blue (STAIN) or autoradiographed (AUTORAD). Lane 1, total mitochondrial membrane protein that had been mixed with immobilized streptavidin following cross-linking of intact mitochondria with ^{125}I-pO(1-27)*cys*-biotin and alkaline extraction; lane 2, unbound proteins in flow-through from immobilized streptavidin; lane 3, hot SDS-urea eluate from immobilized streptavidin.


1 2 3
1 2 3
94K-
68K-
43K-
30K-
20K-
14K-
p30
STAIN
AUTORAD

PANEL B. Thirty-times more of the sample shown in lane 3 of Panel A was subjected to SDS-PAGE and analyzed either by staining for protein (lane 1) or by autoradiography (lane 2). The 14 kDa band derives from immobilized streptavidin as a consequence of boiling in SDS-urea.

Fig 6, PANEL B. Thirty-times more of the sample shown in lane 3 of Panel A was subjected to SDS-PAGE and analyzed either by staining for protein (lane 1) or by autoradiography (lane 2). The 14 kDa band derives from immobilized streptavidin as a consequence of boiling in SDS-urea.

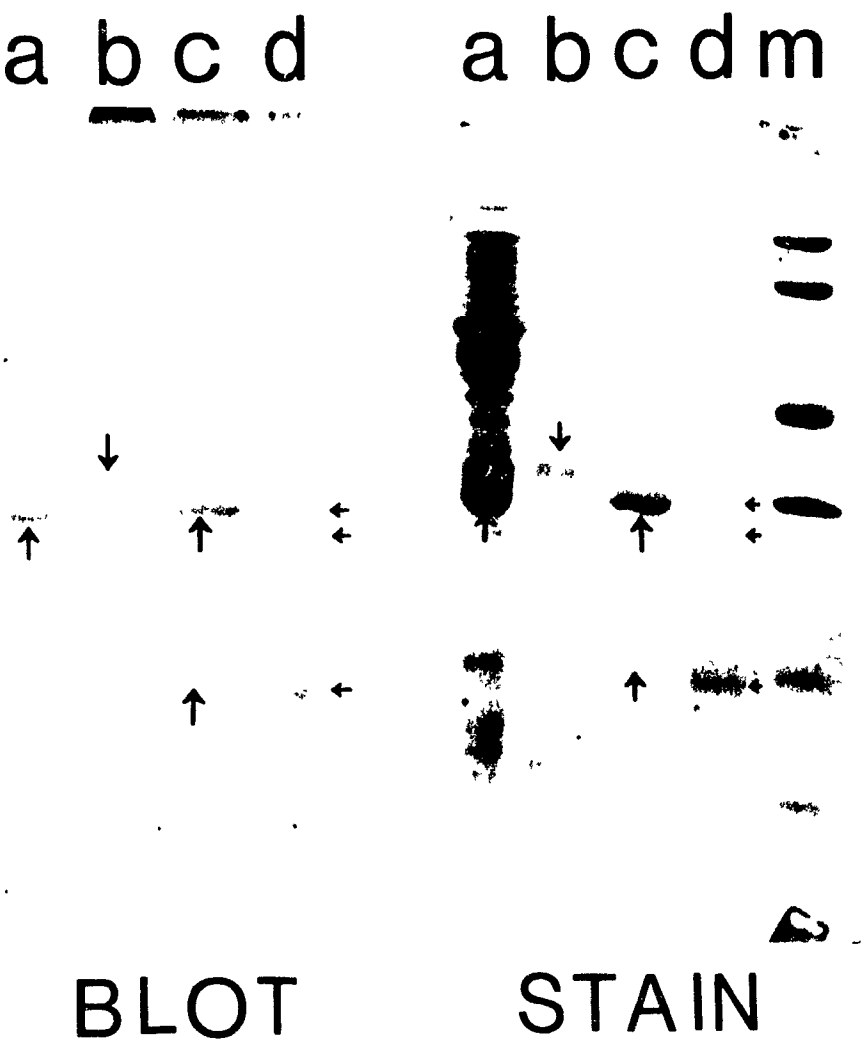

1
2
p30
14K
STAIN
AUTORAD.

streptavidin that were removed from the column under these conditions.

During the course of our subsequent analysis of the p30 moiety recovered from the purified peptide-p30 complex, e.g., p30 localization and peptide mapping, it became clear that the ADP/ATP carrier protein (AAC, Mr = 30 K, refs. 21 and 22), an abundant family of proteins of the mitochondrial inner membrane, was a likely candidate for its identity. To test this prediction, the purified peptide-p30 complex was examined with antibody against AAC and was found to react on a Western blot (not shown). Conversely, when anti-AAC was adsorbed to the purified p30-peptide complex and the affinity-purified antibodies obtained, they were found to react on Western blots with AAC (not shown) and as well with a single alkali-insoluble mitochondrial protein of molecular size 30 kDa (i.e., presumptive AAC) (Fig. 7, left panel, lane a) and, of course, with the p30-peptide complex (apparent size:34 kDa, Fig. 7, left panel, lane b).

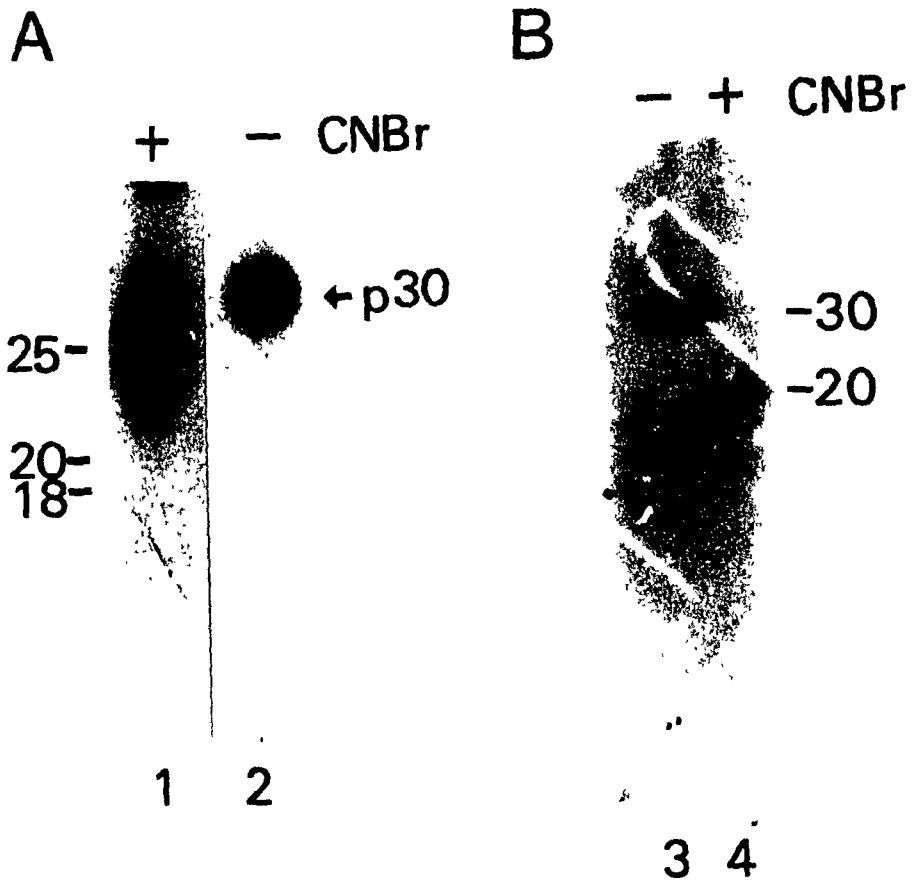
The predominant 30 kDa protein of mitochondrial membranes (i.e., AAC) was purified and treated with formic acid ± CNBr; in the absence of CNBr, a small amount of a lower fragment was generated presumably due to the presence of an acid-labile *asp-pro* at positions 203 and 204 of AAC (21, 22) (Fig. 7, lanes C, lower arrow). Partial cleavage with CNBr yielded a predominant fragment of ~20 kDa which is due to the presence of the first methionine from the N-terminus in AAC at position 200 (21, 22), and as well yielded a product slightly smaller than full-size AAC (Fig. 7, right panel, lane d). Anti-AAC which was affinity purified against the peptide-p30 complex reacted with both fragments, to an extent equivalent to its reactivity toward the uncleaved protein (Fig. 7, left panel, cf.

Fig. 7. p30 is the ADP/ATP carrier protein (AAC). The p30-peptide complex was obtained from immobilized streptavidin as illustrated in Fig. 6, Panel B, lane 1, and subjected to SDS-PAGE and electroblotting onto nitrocellulose. A nitrocellulose strip containing the complex was excised, and after blocking, was incubated with anti-AAC antiserum and the reacting antibodies purified as described in ref. 23. The purified antibodies were diluted 100-fold and incubated under standard conditions with a nitrocellulose blot containing: lane a, total alkali-insoluble membrane proteins from purified mitochondria (ref. 24); lane b, purified peptide-p30 complex (downward pointing arrow); lane c, the major 30 kDa polypeptide shown in lane a (upward pointing arrow) which was obtained by gel excision and electroelution; lane d, the 30 kDa protein shown in lane c following cleavage with CNBr in 70% formic acid (horizontal arrows). A duplicate blot was stained with Coomassie blue (STAIN). The latter also contained molecular weight markers (lane m): phosphorylase b (94 kDa); albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20 kDa); and α-lactalbumin (14 kDa).


a b c d
a b c d m
BLOT
STAIN

lanes c and d), indicating that antibody was directed against the predominant protein in the 30 kDa region of mitochondrial membranes (i.e., AAC) rather than reacting with a minor species in this region. More complete digestion by CNBr yielded quantitative conversion of full size AAC to the 20 kDa fragment (plus smaller fragments that were not resolved by the present SDS-PAGE analysis). When probed with ^{125}I-pO(1-27), the 20 kDa CNBr fragment was reactive (Fig. 8, lane 4), indicating that the N-terminal two-thirds of AAC contains the pOCT signal binding site. This predicts that cleavage with CNBr of the peptide-p30 complex obtained by cross-linking ^{125}I-pO(1-27) to p30 in intact mitochondria should yield a radioactive fragment that is slightly larger than 20 kDa due to the presence of the peptide (i.e., ~24 kDa). That this is the case is shown in Fig. 8, lane 1; under the conditions employed, CNBr did not cleave the peptide moiety (which contains *met* at position 21), presumably because of cross-linking in the immediate vicinity of *met*21.

Fig. 8. The 20 kDa CNBr fragment of AAC contains the pOCT signal binding site. PANEL A. ^{125}I-peptide-p30 was purified (Experimental Procedures) and digested with CNBr in 70% formic acid. Polypeptides were precipitated in trichloroacetic acid and analyzed by SDS-PAGE and radioautography. Lane 1, CNBr digest of ^{125}I-peptide-p30; lane 2, purified ^{125}I-peptide-p30. PANEL B. Total mitochondrial membrane proteins were solubilized in SDS and treated with (lane 4) or without (lane 3) CNBr. The two samples were analyzed by ligand blotting with ^{125}I-pO(1-27), as described in Experimental Procedures. The positions of protein size markers in kDa are indicated.


A
+
−
CNBr
←p30
25-
20-
18-
1
2
B
−
+
CNBr
-30
-20
3
4

CONCLUSIONS

Previous studies have shown that a synthetic peptide corresponding to amino acids 1-27 of the pOCT signal sequence inhibits import of precursor proteins destined for different mitochondrial compartments (12) and, as well, can form a complex with a mitochondrial membrane protein with a predicted size of 30 kDa (p30) (14); in neither case does a control peptide, pO(16-27), elicit the pO(1-27) effect (12, 14). Here, we show: (1) that pO(1-27) requires a trypsin-sensitive protein on the surface of mitochondria in order to gain access to p30 (Figs. 3 and 5), (2) that the formation of an irreversible adduct between p30 and pO(1-27) correlates with inhibition of import of a normal precursor protein (Fig 4), and (3) that p30 corresponds to an abundant integral protein of the mitochondrial inner membrane, AAC (Figs. 7 and 8). While there is no evidence to implicate the nucleotide transport function of AAC in mediating protein translocation into mitochondria (e.g., atractyloside is not an inhibitor of import, ref. 18 and data not shown), it is interesting that a synthetic signal peptide establishes a specific interaction with this protein following import via a trypsin-sensitive pathway across the outer membrane. Although this interaction, when rendered irreversible by chemical cross-linking, correlates with the inhibition of subsequent import of a normal precursor protein (Fig. 4), it cannot be ruled out that inhibition arises because of the interaction of peptide with another minor protein (i.e., one that is not easily detectable by gel analysis, cf. Fig 2). Although functions for AAC other than nucleotide transport across the inner membrane have yet to be established, it is intriguing that AAC is encoded by a gene family in mammals (16), and by at least 2 genes in yeast

(17). Whether or not the affinity that we have demonstrated here between a signal peptide and AAC *in vitro* is relevant to import *in situ* remains to be established.

ACKNOWLEDGEMENTS

We thank Laura Gillespie for preliminary studies on p30 purification and for carrying out the experiment described in Fig. 6, panel A, and William Sheffield for carrying out unpublished experiments. We are grateful to Stephen Randall, Ilona Skerjanc, John Bergeron, William Sheffield, and John Silvius for many helpful discussions, and thank John Silvius for advice concerning biotinylation of pO(1-27)*cys*.

[1]ABBREVIATIONS

pOCT, preornithine carbamyltransferase, pO(1-27), peptide corresponding to amino acids 1-27 of pOCT (the sequence is NH_2-MLSNLRILLNKAALRKAHTSMVRNFRY-amide); AAC, ADP/ATP carrier; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, BS^3, bis(sulfosuccinimidyl)suberate; EDTA, ethylenediamine tetraacetate.

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CHAPTER 5

GENERAL DISCUSSIONS

The research described in this thesis is divided into two parts: biogenesis of UCP, a polytopic integral inner membrane protein of brown fat mitochondria (Chapters 2 and 3), and identification of a mitochondrial signal sequence binding protein (Chapter 4).

5.1 BIOGENESIS OF UNCOUPLING PROTEIN

Our studies have concentrated on the *in vitro* import of UCP into the inner membrane of a heterologous mitochondrion. The following characteristics suggest the fidelity of our assay system: 1), Import was dependent on the electrochemical potential across mitochondrial inner membrane as indicated by its sensitivity to the protonophore CCCP; 2), The imported UCP generated similar tryptic fragments in mitoplasts to that published for UCP in mitoplasts prepared from brown fat mitochondria (Chapters 2, and 3).

Our studies have also demonstrated that UCP contains at least two mitochondrial targeting signals. The N-terminal one third, corresponding to the first of three repeats in UCP, is sufficient to direct a fusion protein into the mitochondrial inner membrane, albeit with a lower efficiency, in a membrane potential dependent manner *in vitro*; the C-terminal part, containing the other two repeats, is also able to enter mitochondria but not integrate into the inner membrane independently (Chapter 2). For AAC, it has been suggested that the N-terminal repeat was only able to enter an intermediate location in mitochondria rather than insert into the inner membrane (97).

How does a polytopic integral membrane protein, like UCP, achieve its complex membrane topology? We favour the hypothesis that multiple topogenic sequences within the precursor protein operate coordinately to

interact with a proteinaceous 'translocator' in the target membrane. Such a mechanism has been proposed for integration of polytopic membrane proteins into the ER membrane (52, 62-65), as well as the bacterial plasma membrane (66). Considering the tripartite structure of UCP and the amphiphilic nature of the UCP transmembrane α-helices, we propose that import of UCP follows three sequential membrane insertion events. We further propose that a matrix-targeting signal contained within each ectodomain of UCP initiates translocation by interacting with the 'translocator' and this translocation is interrupted by a pair of transmembrane α-helices. Such an insertion mechanism, as described above, requires the correct folding of UCP transmembrane segments to achieve proper membrane anchoring.

The possibility of abrogating such intrinsic membrane anchoring properties of UCP transmembrane segments was suggested by our observation that the C-terminal multiple transmembrane segments of UCP were rerouted to the matrix when fused C-terminal to a matrix targeting signal sequence derived from pre-carbamyl phosphate synthase (100). Indeed, a UCP molecule with an intact three pairs of transmembrane segments was also rerouted to the mitochondrial matrix by an N-terminally fused matrix targeting signal sequence, that of pOCT (101). The mechanism for this abrogation is not yet clear One possibility is that the presence of a signal sequence at the N-terminus of UCP may have caused a conformational change in the fused UCP molecule so that the latter was no longer compatible for membrane anchoring, since the overall hydrophobicity of UCP transmembrane domains are most likely achieved by pairing of amphiphilic α-helices. Alternatively, the hybrid protein may have followed an import pathway

distinct from what for UCP, thus the 'stop transfer' signals of UCP, i.e., the pairs of transmembrane segments, were not recognized How do we reconcile this latter possibility with the earlier observation that the synthetic signal peptide pO(1-27) (139), or purified pO-DHFR (a hybrid precursor protein containing the signal sequence of pOCT fused to mouse DHFR, Sheffield and Shore, unpublished), competed for import of both pOCT and UCP *in vitro*? It is possible that pOCT (or hybrid proteins containing the pOCT signal sequence) and UCP both bind to a common component [e.g. 'GIF' (145)] following which the two diverge into their own pathways pO(1-27), or pO-DHFR, inhibited import of both precursor proteins by occupying this common component (discussed in more detail later). The component(s) that recognizes the 'stop transfer' signals in UCP is further down the import pathway which pO-UCP never encounters due to the dominance of the matrix targeting signal sequence

Our discovery that a matrix targeting signal sequence can abrogate the intrinsic membrane targeting signal of UCP allowed us to examine the import pathway of UCP. The hybrid precursor protein, pO-UCP, was allowed to accumulate in the mitochondrial matrix following import in the presence of a Zn^{++} chelator, a situation that inhibits the signal processing in the matrix. Relieving this inhibition by adding Zn^{++} back to the *in vitro* import assay resulted in conversion of the precursor to a mature form, which represents a UCP molecule with three intact pairs of transmembrane segments. The fact that this mature form remained in the matrix suggests an incompatibility of UCP insertion from the matrix side of the inner membrane, implying that UCP normally inserts into the inner membrane from the cytoplasmic side.

At present, we can not rule out the possibility that an insertion incompetent conformation of UCP resulted from interference by the matrix targeting signal sequence, and that this conformation persisted in the matrix even after the signal sequence was removed.

The difference in import pathways between UCP (and probably AAC) and all the precursor proteins that are suggested to follow the 'conservative sorting' pathway may be reflected in the following differences in the precursor proteins. First, UCP and AAC do not have homologs in bacteria, while all those proteins that are supposed to follow the 'conservative sorting' pathway (Fe/S protein, cytochrome c_1, *etc.*) have homologs in the equivalent location in bacteria. Second, neither UCP nor AAC contains a cleavable signal sequence, while all these other precursor proteins do. Such signal sequences are suggested to be acquired by these proteins through evolution to transport them back to the matrix to enter their 'conserved' export pathway (75, also refer to 1.3.3a in Chapter 1).

5.2 IDENTIFICATION OF A MITOCHONDRIAL SIGNAL PEPTIDE BINDING PROTEIN

The early observation made in a number of laboratories that treatment of mitochondria with low concentrations of proteases abolished the ability of the organelle to import proteins suggested the existence of proteinaceous 'receptors' on mitochondrial surface. A number of different approaches have been used in search of these import 'receptors', among which has been the employment of a synthetic signal peptide [pO(1-27)]. pO(1-27) has been studied intensively as for its membrane induced helical structure and membrane surface seeking properties (81, 85). It was demonstrated that its presence at a concentration that had no effect on

the trans-inner membrane potential of mitochondria inhibited *in vitro* import of several precursor proteins destined for either the matrix or the inner membrane (139). Subsequent chemical cross-linking demonstrated a specific interaction of this synthetic signal peptide with a mitochondrial integral membrane protein with an apparent molecular weight of 30 kDa (p30) (142). Further studies described in Chapter 4 have established a correlation between the irreversible association of pO(1-27) to p30 and the inhibition of protein import into mitochondria *in vitro*. Although we cannot rule out the possibility that inhibition of import was due to an association of pO(1-27) with another protein(s) which exists at much lower concentration than p30 and thus undetected, we consider this very unlikely, since our purified p30 preparation, which was enriched more than 1000 fold with signal-peptide-containing proteins, did not contain readily detectable contaminants (fig. 6B, lane 2 in Chapter 4). Furthermore, binding of pO(1-27) to p30 was saturable and the saturation concentration corresponded well with maximum inhibition of import (139, 142, and Chapter 4). The simplest interpretation of our results was that p30 represents a component of the putative import machinery.

Unexpectedly, p30 was identified as a member of the AAC family (Chapter 4). Our identification is based on the following criteria: 1), Antibodies raised against purified AAC and further affinity-purified against p30 reacted with p30 and the 30 kDa mitochondrial membrane protein with comparable intensities indicating that the antibodies were reacting with the same protein. 2), With most of the Coomassie blue stained 30 kDa material being converted to 20 kDa by CNBr digestion (a characteristic peptide map of AAC), there was a concomitant conversion of antigenicity

from 30 kDa to 20 kDa, indicating that the p30-affinity-purified antibodies were against the major component (i.e., AAC) of the 30 kDa band. 3), This peptide map was also obvious by either direct digestion of the purified peptide-p30 complex or by ligand blot analysis of CNBr digested mitochondrial membrane proteins. A more definitive identification of p30 by partial amino acid sequencing of purified p30 was unsuccessful due to modification of the N-terminus by the cross-linker.

As demonstrated earlier by Chen and Douglas (125), and by my own unpublished work, the adenine nucleotide exchange function of AAC is not required for *in vitro* import as long as ATP is provided in the assay medium and mitochondria are energized by a functional electron transport chain.

Since AAC is one of the most abundant mitochondrial proteins and pO(1-27) may not mimic the signal sequence in the context of a precursor protein, the association of pO(1-27) with AAC may simply represent an event totally unrelated to protein import. In this case, the inhibition of import by pO(1-27) in the presence of a bifunctional cross-linker may be attributed to the association of pO(1-27) with a yet undetected protein(s) or to an unknown mechanism that is unrelated to peptide-protein association. However, it is possible that the association of pO(1-27) with AAC indeed contributes to the inhibition of protein import. How do we interpret this latter possibility? Very little is known as to the fate of the signal sequence of a precursor protein during or after translocation of the polypeptide into mitochondria. Ono and Tuboi (160) have recently presented evidence that a synthetic signal peptide, corresponding to the signal sequence of pre-ornithine aminotransferase, was imported into

mitochondria *in vitro*. A significant percentage (33%) of the internalized peptide was associated with the inner membrane (vs. 54% for the matrix). Our results indicate that the interaction of pO(1-27) with a trypsin sensitive surface component of mitochondria is a prerequisite for its interaction with AAC. I suggest that this trypsin sensitive component is an import receptor that interacts with the signal sequence of pOCT during import of the latter into mitochondria. During subsequent translocation of pOCT across the membranes via a putative proteinaceous 'translocator', the signal sequence may permanently anchor in this 'translocator' to extrude the mature part into the matrix as a loop. The only part of the signal sequence that may ever be exposed on the matrix side of the inner membrane is the C-terminus containing the cleavage site. The pO(1-27) that is associated with AAC may have fulfilled all the duties of the signal sequence of pOCT. A portion of AAC may be part of this 'translocator' to provide ATP for the immediate need of energy to perhaps unfold the polypeptide for efficient import *in vivo*, although we know this function of AAC is dispensable in vitro when ATP is provided in the assay medium. Alternatively, different members of the AAC family may have as yet unknown functions in addition to adenine nucleotide exchange. An irreversible binding of pO(1-27) to AAC at this 'translocator' elicited by a bifunctional cross-linker thus provides a mechanism to inhibit further translocation of precursor proteins.

It is interesting that our inhibition experiment presented in figure 4 of Chapter 4 suggested that the peptide inhibited precursor import at a step beyond the surface receptor. The peptide also inhibited import of UCP (139, Chapter 4) and pMDH (139). In these two respects, AAC

seems to share properties that 'GIP' exhibits (145, refer to 1.3.4b). It would be most interesting to examine if the irreversible binding of the signal peptide to AAC would inhibit the generation of 'GIP' bound intermediates. Obviously, identification of 'GIP' will be most welcome.

Independent evidence would be useful to clarify the physiological relevance of the peptide-AAC association. Recently, Schatz's laboratory (161) has isolated inner membrane vesicles and demonstrated that they are comparable to intact mitochondria in import efficiency. Presumably, protein import across these inner membrane vesicles by-passes the outer membrane components of the import machinery. It would be interesting to examine the possibility of inhibiting import into these vesicles by anti-AAC antibodies, which would not be able to penetrate the outer membrane in intact mitochondria.

A more definitive answer as to whether or not AAC is involved in protein translocation into mitochondria may be achieved by a genetic approach. Lawson and Douglas (162) have recently identified 2 AAC genes in the yeast *Saccharomyces cerevisiae*. A double deletion mutant (*aac1/aac2*) should reveal if AAC plays a role in protein translocation into mitochondria.

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ORIGINAL CONTRIBUTION TO KNOWLEDGE

The following findings presented in this thesis represent original contribution to knowledge:

1). The demonstration of *in vitro* import of UCP into the inner membrane of rat heart mitochondria with a similar membrane topology to that of the UCP in brown fat mitochondria.

2). The demonstration of multiple mitochondrial targeting signals in UCP.

3). The demonstration of abrogation of the intrinsic membrane targeting signal in UCP by an N-terminally fused matrix-targeting signal sequence resulting in the relocation of UCP to the soluble matrix.

4). The demonstration of the inability of UCP insertion into the inner membrane from the matrix side.

5). The demonstration of a correlation between the irreversible association of pO(1-27) to p30 and the loss of the ability of mitochondria to import precursor proteins.

6). The development of a pO(1-27)-p30 binding assay on nitrocellulous paper following SDS-PAGE.

7). Purification of p30 to near homogeneity by an affinity approach.

8). The identification of p30 as a member of the ADP/ATP carrier family.