Import of Proteins into Mitochondria:

Properties of Precursor Proteins

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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September, 1989

ABSTRACT

The mechanism of protein translocation into mitochondria has been investigated by studying properties of precursor proteins destined for the mitochondrial matrix. Characterization of the amphiphilic properties of the signal sequence for pre-ornithine carbamyltransferase has led to the conclusion that precursors can not translocate across the inner membrane via a lipid route alone (i.e. in the absence of proteins). A correlation was established between the rate of precursor import and the degree of hydrophobicity of a short region in the presequence, suggesting that precursor binding to the two-dimensional phospholipid surface of the outer membrane may enhance the rate of diffusion to the translocation apparatus.

The conformations of the mature portions of two hybrid proteins, pOCAT and pODHFR, were examined at various steps on the import pathway. The bulk population of these precursors remained in a near-native conformation prior to precursor engagement of the import apparatus. Unfolded polypeptide translocation intermediates, the formation of which requires ATP, an intact signal sequence, and a protease-sensitive component of the outer mitochondrial membrane, have been detected in association with submitochondrial fractions containing sites of contact between the inner and outer mitochondrial membranes.

Résumé

Le mécanisme de translocation protéique dans la mitochondrie a été étudié en examinant les propriétés de précurseurs destinés à la matrice mitochondriale. La caractérisation des propriétés amphiphiliques de la pré-séquence de ornithine carbamyle transférase a mené à la conclusion que les précurseurs ne peuvent traverser la membrane mitochondriale interne en utilisant une voie lipidique seule (i.e. en l'absence de protéines). Une corrélation a été établi entre la vitesse à laquelle le précurseur est importé et le degré d'hydrophobicité d'une courte région dans la pré-séquence. Ceci suggère la possibilité que le précurseur s'attache à la surface lipidique de la membrane mitochondriale externe, ce qui augmenterait la vitesse de diffusion à travers l'appareil translocationnel. La conformation de deux protéines hybrides, pOCAT et pODHFR, a été étudié à diverses étapes du processus de leur transport et translocation. La majeure partie de la population de ces précurseurs retiennent une conformation semblable à celle de la protéine active, jusqu'à ce qu'ils s'engagent dans l'appareil de translocation. Un intermédiaire de conformation différente a été détecté, associé avec une fraction sub-mitochondriale contenant les sites de contact entre la membrane externe et interne. La formation de cette intermédiaire nécessite ATP, une pré-séquence intacte, et un élément, protéase-sensible, présent sur la surface externe de la mitochondrie.

Acknowledgements

I would like to thank all of the people who have helped me in completing this work, especially the following:

Dr. Gordon Shore, for his generosity, enthusiasm, and supportive guidance.

Dr. John Silvius, for his help with the biophysical aspects of this work, as well as for his generous time and advice.

Dr. Steve Randall, for his daily contributions to this work and many stimulating discussions.

Monique Lagacé, for translating my abstract and for helping to make an enjoyable working atmosphere.

Bill Sheffield and Johné Liu, for their helpful comments.

Mai Nguyen, for teaching me cassette mutagenesis.

Laura Gillespie, for numerous interesting discussions.

Carol Miyamoto, for many helpful hints when I first started out.

Edi, my husband, for the countless ways he has helped and encouraged me throughout everything.

Dr. Morris Kates, my father, for his inspiration.

Pirkko Kates, my mother, for her continual support.

NSERC, for their financial assistance.

PREFACE

In accordance with the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research of McGill University, the following regulations are cited in full:

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As approved by the Department of Biochemistry, two

papers that have already been published have been incorporated into this thesis:

Chapter 2 — Skerjanc, I.S., Shore, G.C., and Silvius, J.R. (1987) EMBO J. 6: 3117-3123.

Chapter 3 — Skerjanc, I.S., Sheffield, W.P., Silvius, J.R., and Shore, G.C. (1988) J. Biol. Chem. 263: 17233-17236.

The work described in Chapter 4 is being prepared for publication.

Dr. John Silvius prepared the fluorescent derivatives of the synthetic peptide used in Chapter 2. Bill Sheffield prepared the mitochondrial matrix extract used in Chapter 3 and the constructs of pOCAT and pODHFR used in Chapter 4. Dr. Mai Nguyen provided the clone for pOCT used in Chapter 3 and Chapter 4. With these exceptions, the work described is entirely my own.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CCCP	carbonylcyanide 3-chlorophenylhydrazone
cDNA	complementary deoxyribonucleic acid
CL	cardiolipin
EDTA	ethylenediamine tetraacetate
ER	endoplasmic reticulum
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
hsp70	70 kilodalton heat shock proteins
kDa	kilodaltons
K_m	Michaelis-Menten constant
LamB	the lambda-receptor protein
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
pCoxIV	cytochrome oxidase subunit IV precursor
pCoxIV-DHFR	a hybrid protein containing the presequence of yeast cytochrome oxidase subunit IV fused to mouse dihydrofolate reductase
pCPS	precarbamyl-phosphate synthetase I
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PreMBP	precursor to maltose binding protein (MalE)

pre-PhoE	E. coli outer membrane pore protein precursor
proOmpA	E. coli outer membrane protein A precursor
pOCAT	a hybrid protein containing the pOCT signal sequence fused to chloramphenicol acetyltransferase
pOCT	precursor to ornithine carbamyl transferase
pODHFR	a hybrid protein containing the pOCT signal sequence fused to dihydrofolate reductase
pO(1-27)	a synthetic peptide corresponding to amino acids 1-27 of pOCT
pO(16-27)	a synthetic peptide corresponding to amino acids 16-27 of pOCT
PS	phosphatidylserine
SDS	sodium dodecyl sulfate
SRP	signal recognition particle
$TPMP^+$	triphenylmethylphosphonium iodide
TCA	trichloroacetic acid

CHAPTER 1

GENERAL INTRODUCTION

1.1 Import of Proteins into Mitochondria

Mitochondria are not created de novo but are derived from the growth and division of pre-existing organelles (Palade 1983; Storrie and Attardi 1973; Luck 1963). Although some proteins are encoded by mitochondrial DNA and synthesized in the matrix (Tzagoloff et al. 1979), the majority of mitochondrial proteins are encoded by nuclear genes and synthesized as precursors in the cytoplasm of the cell (Maccecchini et al. 1979; Shore et al. 1979; Hay et al. 1984). Some precursors contain amino-terminal extensions, termed signal sequences, and are imported into either the intermembrane space, the inner membrane, or the matrix. Outer membrane proteins do not seem to be synthesized as higher molecular weight precursors (Hay et al. 1984; Hartl et al. 1989). Translocation in vivo is post-translational (reviewed in Nicholson and Neupert 1988) and translocation across the inner membrane requires an electrochemical potential (Kolansky et al. 1982; Gasser et al. 1982; Schleyer et al. 1982). The signal sequence is cleaved upon insertion into the matrix by a processing peptidase, which has recently been purified and cloned (Witte et al. 1988, Hawlitschek et al. 1988; Yang et al. 1988; Jensen and Yaffe 1988). Subsequent assembly of matrix proteins into oligomeric complexes requires a nuclear encoded mitochondrial heat-shock protein, hsp60

(Cheng et al. 1989; Reading et al. 1989).

Although a great deal has been learned about the overall import pathway in the last few years, the major components of the mitochondrial translocation apparatus remain unknown. This thesis focuses on what has been learned about mechanisms of import by examining properties of mitochondrial precursor proteins. Specifically, I examine how properties of the signal sequence, as well as sequence and conformation of the mature portion of the protein, influence the import efficiency of natural and artificial mitochondrial precursors.

Topics not covered in this introduction include the extensive mutagenesis studies performed on signal sequences, import of precursors to the outer membrane, intermembrane space, and the inner membrane, sorting and stop transfer signals, analysis of distinct steps on the import pathway of various precursors, and details of precursor processing. Information on these topics may be obtained from several recent review articles (Attardi and Schatz 1988; Verner and Schatz 1988; Pfanner et al. 1988a; Eilers and Schatz 1988; Roise and Schatz 1988; Hartl et al. 1989).

1.1.1 Mitochondrial Targeting Sequences Direct Import

The first indication that the signal peptides of mitochondrial precursors carry important targeting information came from the observation that mature F_1-

ATPase α-,β-, and γ-subunits, could not be imported into isolated mitochondria, as opposed to their corresponding precursors (Maccecchini et al.1979). Subsequent gene fusion studies created various hybrids of signal sequences fused to nonmitochondrial proteins and demonstrated that an N-terminal signal sequence is sufficient to direct these "passenger" proteins to the organelle (Hurt et al.1984; Horwich et al.1985; Nguyen et al.1986). Thus, it was concluded that signal sequences contain the necessary information required to target proteins to mitochondria.

The observation that chemically synthesized signal peptides inhibit precursor import (Gillespie et al.1985; Chu et al. 1989), indicates that these peptides retain important features of the signal sequence in the native precursor and are capable of interacting with essential components of the import apparatus. These peptides were found to inhibit the import of precursors destined for either the matrix or the inner membrane, suggesting that proteins destined for different intramitochondrial compartments may share, at least in part, a common import pathway. This type of degeneracy in the import pathway has also been documented by the demonstration that an excess of one precursor can effectively compete for both import and specific binding of other precursors, even those that are destined for different intramitochondrial compartments (Mori et al. 1985; Pfaller and Neupert 1987;

Pfaller et al. 1988). These findings suggest that it is highly unlikely for each precursor to have its own specialized translocation apparatus and thus there must be a significant degree of similarity between signal sequences which target proteins to various compartments.

Approximately 85 mitochondrial precursors have been cloned and sequenced to date (Hartl et al. 1989). Surprisingly, comparison of the signal sequences of these precursors has revealed no consensus in primary structure, although they are generally enriched in basic, hydroxylated, and hydrophobic residues, evenly distributed along the length of the signal peptide. Since various signal peptides appear to direct precursor import via the same pathway, it is reasonable to postulate that these signals must have similar three-dimensional conformations in order to recognize and interact with the translocation apparatus.

1.1.2 Mitochondrial Signal Sequences are Amphiphilic

A common secondary structure of signal peptides may involve the formation of an amphiphilic α-helix, a structure which has also been postulated for the signal sequences of various bacterial secretory proteins (Briggs and Gierasch 1984; Briggs et al. 1985; Tamm et al. 1989; see section 1.2.1). Amphiphilic structures tend to bind to the lipid surface of membranes, because hydrophobic residues can easily associate with the phospholipid acyl

chains while hydrophilic residues remain in an aqueous environment. Further, the basic residues of the signal peptides may form electrostatic interactions with the negative charges on the phosphate head groups.

The first evidence that the signal peptides of mitochondrial precursor proteins interact with membranes was provided by the observation that relatively low concentrations of synthetic signal peptides can cause uncoupling of oxidative phosphorylation and leakage of enzymes from mitochondria (Ito et al. 1985; Gillespie et al. 1985). These results suggest that the peptides can bind membranes in such a way as to perturb the bilayer structure. Circular dichroism studies have shown that the pCox IV and pOCT signal peptides have little α-helical content in aqueous solution but undergo a significant induction into an α-helical conformation in membrane-like environments (Epand et al. 1986; Roise et al. 1986, 1988). When these signal peptides are folded into a model α-helix, it is found that the hydrophobic and hydrophilic residues are clustered on opposite faces. Theoretical calculations by von Heijne (1986) have shown that the majority of 23 mitochondrial signal sequences examined were capable of folding into amphiphilic α-helices. However, it has been shown recently that functional presequences may be amphiphilic without being α-helical (Roise et al. 1988, discussed below).

Signal peptides have been shown to insert spontaneously into phospholipid monolayers (Roise et al. 1986, 1988; Tamm 1986) and bind to liposomes containing anionic phospholipids (Skerjanc et al. 1987; Myers et al. 1987). Also, such peptides have been found to cause disruption of unilamellar liposomes (Roise et al. 1986, 1988; Skerjanc et al. 1987; Chapter 2 of this thesis). More recently, several full length proteins were shown to bind acidic liposomes, including the precursors of adrenodoxin, cytochrome P-450(SCC), malate dehydrogenase, pOCT, and pCoxIV-DHFR (Skerjanc et al. 1988; Chapter 3 of this thesis; Ou et al. 1988; Endo and Schatz 1988). Thus, it is clear that the ability to bind to membranes is a common feature of all the mitochondrial presequences examined to date.

Even artificial presequences, which behave like natural presequences, have been shown to be amphiphilic (Ito et al. 1985; Allison and Schatz 1986). For example, artificial sequences fused to subunit IV of cytochrome oxidase were able to be imported into mitochondria (Allison and Schatz 1986), and synthetic signal peptides corresponding to these functional artificial presequences were found to insert into phospholipid monolayers and to disrupt vesicles with similar efficiencies as the wild type Cox IV signal (Roise et al. 1988). Although one of the artificial signal peptides examined was found by

circular dichroism measurements not to be α-helical in the presence of detergent micelles, it was still found to be amphiphilic, as measured by its ability to insert into phospholipid monolayers and to disrupt phospholipid vesicles. Based on these studies, it may be concluded that the amphiphilicity of the signal sequence is essential and may be achieved either through an α-helical or an alternative conformation.

1.1.3 Possible Roles for Signal Sequence Amphiphilicity

How is the amphiphilicity of targeting sequences important in the overall mechanism of mitochondrial import? One possible hypothesis, originally postulated by Roise et al. (1986) and von Heijne (1986), involves membrane translocation via a lipid pathway. In this model, the signal sequence initially binds to the membrane, senses the potential across the inner membrane, and is effectively pulled across the bilayer by the electrochemical potential. Since signal peptides are positively charged and the electrochemical potential across the inner mitochondrial membrane is negative inside, the energy gained by the peptide passing down the gradient would be substantial and could compensate for any unfavorable polar/apolar interactions that would occur in the process (von Heijne 1986). The ability to respond to a membrane potential applied to lipid vesicles has been demonstrated for melittin (Kempf et al. 1982) and for

hydrophobic model peptides, consisting of 5 or 6 amino acids with a net positive charge at the amino terminus (de Kroon et al. 1989).

Roise et al. (1986) found that a synthetic peptide, corresponding to the COX IV presequence, caused leakage of a self-quenching fluorescent dye, carboxyfluorescein, from lipid vesicles. In the presence of a transbilayer potential (inside negative) the peptide was found to induce an enhanced rate of leakage. A similar result was also shown using the signal peptide to pOCT (Skerjanc et al. 1987; Chapter 2 of this thesis). However, from this type of experiment, it was unclear as to whether the dye leaks out of the vesicles at a faster rate because the peptide has a higher affinity for vesicles in the presence of a potential, or simply because the dye itself is negatively charged and would move at a faster rate through the bilayer defects created by the peptide.

A more direct way to test the above hypothesis is to measure the affinity of the signal peptide for lipid vesicles in the presence and absence of a membrane potential. If the peptide can translocate across the bilayer, one would expect to see a substantial increase in lipid affinity in the presence of a potential. Using two different measurements of binding affinity, Skerjanc et al. (1987; Chapter 2 of this thesis) found no effect of a transbilayer potential on the affinity of the pOCT signal

peptide for liposomes. Thus, it seems unlikely that the signal sequence of pOCT can undergo transfer into or across the lipid bilayer even in the presence of a strong transmembrane potential. The peptide probably interacts with the membrane in such a way that no positively charged residue can insert into the hydrophobic core of the bilayer and sense the electrochemical potential, although high concentrations of the peptide can insert into and disrupt the vesicle bilayer, perhaps cooperatively, in a potential-independent manner. A major conclusion from these results is that translocation of the signal sequence across the inner membrane probably requires the presence of specific translocation proteins.

In contrast, translocation of apocytochrome c across the outer membrane may occur through the lipid phase. It has been shown that apocytochrome c can be digested by proteases encapsulated inside pure lipid vesicles (Rietveld and de Kruijff 1984; Dumont and Richards 1984; Rietveld et al. 1985). However, apocytochrome c seems to be the only mitochondrial precursor capable of crossing lipid bilayers in this manner.

While membrane translocation of the presequence via a lipid route seems unlikely, the lipid-associating properties of mitochondrial presequences may still play an important role in the import process. Given the moderately high affinity of the signal peptides for

liposomes and the reversibility of the interaction (Skerjanc et al. 1987; Chapter 2 of this thesis), we proposed a model in which the precursor binds to the lipids of the outer membrane and uses this 2-dimensional surface to enhance diffusion to a putative receptor, as postulated for other surface-seeking entities (Sargent and Schwyzer 1986). Evidence in support of this model has been shown by Skerjanc et al. (1988; Chapter 3 of this thesis) in which a pOCT presequence mutation produces both a 4-5 fold slower rate of import and a correspondingly lower affinity for anionic liposomes, when compared to the wild-type precursor. This suggests that regions of the signal sequence may contribute to the overall rate of import by functioning as membrane surface-seeking entities. This model is consistent with all the data obtained to date.

Another possible role for the amphiphilic signal sequence might involve the facilitation of direct interactions between the precursor and proteinaceous components of the translocation apparatus. Identification and purification of these components will be required before this possibility may be further assessed.

1.1.4 Precursor Interactions with Mitochondrial Proteins

The first evidence suggesting the existence of an import receptor came from the observation that import was sensitive to pretreatment of mitochondria with low

concentrations of proteases (Gasser et al.1982; Argan et al. 1983; Riezman et al. 1983; Zwizinski et al. 1984). Subsequently, the binding of several precursors to mitochondria has been shown to be specific and sensitive to proteases (Riezman et al. 1983; Zwizinski et al. 1984; Pfaller and Neupert 1987). Further, high affinity binding sites have been solubilized in an impure form from both whole mitochondria and purified outer membrane preparations and subsequently reconstituted into liposomes (Riezman et al. 1983; Ono and Tuboi 1985; Pfaller and Neupert 1987).

The finding that mitochondrial translocation intermediates remain accessible to protein denaturants such as urea, suggests that import occurs through a hydrophilic membrane environment, probably mediated by proteinaceous structures (Pfanner et al. 1987b). Possible components of the translocation apparatus have been identified. For example, the signal peptide of pOCT has been found to bind specifically to mitochondria and to cross-link to a 30 kDa integral membrane protein (Gillespie 1987). Cross-linking has been shown to inhibit subsequent import of pOCT and the 30 kDa protein has been purified (Liu, X. and Shore, G.C. 1989, manuscript in preparation). Import into yeast mitochondria has been shown to be inhibited, relative to controls, by Fab fragments against a highly purified 45 kDa protein of the

outer membrane (Ohba and Schatz 1987). Further analysis will be required to determine the exact nature of these components and their role in import.

Pfaller et al. (1988) have shown that although precursors are capable of competing for the import of proteins targeted to different compartments, different classes of precursors seem to have different characteristic receptor sites. Competition studies together with studies of the protease sensitivity of both import and mitochondrial binding of different precursors, has led to the proposal that there are at least three distinct import receptors, which converge at a common membrane insertion site, termed general insertion protein (Pfaller et al. 1988; Hartl et al. 1989). Conclusive evidence for this model remains to be obtained via identification and purification of these proteins.

1.1.5 Conformational Requirements of Precursors:

a) Precursors Must at Least Partially Unfold to be Imported

The observation that pCPS, a 165 kDa precursor, failed to be imported in vitro, while a 33 kDa hybrid protein of the CPS signal sequence fused to mature OCT succeeded in being imported efficiently, suggests that the mature portion of precursors can influence import efficiency (Nguyen et al. 1986). This effect of the mature portion could be due to specific conformational

requirements for import. A great deal of evidence has accumulated suggesting that these requirements involve at least partial unfolding of the precursor. The first indication of this possibility came from the isolation of precursor polypeptide translocation intermediates which span the outer and inner membranes in such a way that the signal sequence is cleaved by the matrix peptidase but the carboxy-terminal portions of the precursor remain accessible to exogenous proteases (Schleyer and Neupert 1985). Since the distance across the two membranes at contact sites is larger than the diameter of the properly folded precursor, it would seem that the precursor protein must at least partially unfold to form this intermediate. Further, Eilers and Schatz (1986) have found that the import of a pCoxIV-DHFR fusion protein was inhibited in the presence of methotrexate, a high affinity inhibitor of DHFR which stabilizes its tertiary structure. Similar results were obtained by Chen and Douglas (1987b) using a hybrid protein of F_1-ATPase ß-subunit precursor fused to yeast copper metallothionein. Further, the import to the intermembrane space of a natural precursor, cytochrome c peroxidase, has been found to be inhibited by the binding of its heme group (Kaput et al. 1989).

Recently, direct evidence for an unfolded translocation intermediate has been obtained using an artificial hybrid protein, pOCAT, which is composed of the

signal sequence of pOCT fused to chloramphenicol acetyltransferase (Skerjanc, I.S., Randall, S.K., Sheffield, W.P., Silvius, J.R., and Shore, G.C., manuscript in preparation; Chapter 4 of this thesis). This precursor has a measurable enzyme activity when translated in reticulocyte lysate and thus a loss of specific enzyme activity can be correlated to conformational changes that involve at least partial unfolding of the precursor. When pOCAT translation products were imported into isolated mitochondria and the mitochondria were subsequently sonicated and subfractionated on a sucrose density gradient, a small amount of the precursor was found to enter the gradient and co-migrate with vesicles of an intermediate density between the outer and inner membranes. This fraction of precursor had about a 16 fold lower specific enzymatic activity than did either the soluble pOCAT or a fraction of the precursor that co-migrated with the outer membrane-derived vesicles. This at least partially unfolded precursor is believed to be a <u>bona fide</u> translocation intermediate because its formation requires an intact signal sequence, a trypsin-sensitive component of mitochondria, and ATP. Taken all together, the results of several workers strongly suggest that precursors must pass through an unfolded intermediate during the import process.

Unfolding seems to be a rate limiting step for the import of pCoxIV-DHFR since the efficiency of import was greatly increased by denaturing the precursor with urea (Eilers et al.1988) or by destabilizing its tertiary structure via site-directed mutagenesis (Vestweber and Schatz 1988c).

b) How Extensive is Precursor Unfolding?

The above findings do not define to what extent mitochondrial precursors must unfold in the process of being imported. Recently, it has been shown that the precursor to aspartate aminotransferase is imported efficiently, even when bound to the coenzyme pyridoxal 5'-phosphate (Nishi et al.1989; Altieri et al.1989). Perhaps this precursor exists in a conformation compatible with import, even when the ligand is bound, or perhaps the ligand does not inhibit the unfolding required for import.

To test the tolerance of the import machinery towards precursors exhibiting chain branching or residual folded structure, pCoxIV-DHFR was coupled to various molecules in an attempt to block the precursor in the translocation apparatus. Interestingly, it was found that coupling the precursor to cytochrome c (Vestweber and Schatz 1988a) or to a double-stranded 24-base pair piece of DNA (Vestweber and Schatz 1989) did not inhibit import whereas coupling to bovine pancreatic trypsin inhibitor abolished import (Vestweber and Schatz 1988b). Examination of the

unfolding properties of cytochrome c, which is about 12 kDa and covalently linked to a protoheme group, as compared to trypsin inhibitor, which is 6 kDa and contains internal disulfide bonds, may lead to valuable information about the translocation apparatus. Thus, a clear picture of what is involved in precursor unfolding remains to be defined. For the purpose of this thesis, however, the term unfolding will be used to designate any loss of the native folded conformation of a precursor.

c) Possible Mechanisms of Unfolding:

Where and how do mitochondrial protein precursors unfold? Several possible mechanisms exist to explain this process. First of all, it is possible that the import apparatus itself may mediate the unfolding of precursor proteins, either spontaneously or in an energy-dependent manner. Second, it is possible that a component of reticulocyte lysate actively unfolds the precursor, perhaps in an ATP-dependent manner. Finally, the lipids of the outer mitochondrial membrane could provide an amphiphilic surface upon which precursors might unfold. Although there is evidence for the latter two possibilities, the exact mechanism of unfolding for all precursors remains controversial.

d) Evidence for ATP-dependent Unfolding

Several studies have demonstrated a requirement for reticulocyte lysate components in mitochondrial import

(Argan et al. 1983; Miura et al. 1983; Ohta and Schatz 1984; Argan and Shore 1985; Pfanner and Neupert 1987; Chen and Douglas 1987a; Ono and Tuboi 1988; Murakami et al. 1988b). Cytosolic factors could be required to actively unfold precursors, to maintain the precursor in an import competent conformation, or simply to target precursors to mitochondrial receptors. Various degrees of purification of the import-stimulatory activity from reticulocyte lysate have been reported, identifying proteins of rather different molecular mass (Firgaira et al. 1984; Ohta and Schatz 1984; Argan and Shore 1985; Ono and Tuboi 1988). Recently, Murakami et al. (1988a) have actually identified at least two distinct stimulatory activities in yeast post-ribosomal supernatants. These include an NEM-sensitive protein(s) and the recently purified Ssalp/Ssa2p (Chirico et al. 1988), which is a mixture of two hsp70-related proteins of 98% homology. An NEM-sensitive stimulatory activity has also been identified in reticulocyte lysate (Randall and Shore 1989). Further, depletion of a subset of 70kDa stress proteins in yeast mutants was found to cause accumulation of mitochondrial precursors in vivo (Deshaies et al. 1988).

The finding that heat shock proteins, which are ATPases, stimulate import supports the postulate of Rothman and Kornberg (1986) that ATP-dependent "unfoldases" are present in the cytosol. Further support

for this model derives from the requirement of NTP hydrolysis for import of several mitochondrial precursors, including the F_1-ATPase ß-subunit, ADP/ATP carrier, F_o-ATPase subunit 9, porin, and for a highly purified pCoxIV-DHFR fusion protein (Pfanner et al. 1987a; Eilers et al. 1987; Chen and Douglas 1987c).

Detailed analysis of the ATP requirement for several precursors also seems to support the unfoldase model. Verner and Schatz (1987) have found that the import of incomplete precursor chains of pCoxIV-DHFR, formed by interrupting translation with cycloheximide, requires a potential across the inner membrane but does not require ATP. The nascent chains were found to be incompletely folded and thus would not be expected to require energy-dependent unfolding of precursor proteins. Although in vitro synthesized porin requires ATP for import, a water-soluble form of porin, obtained by treating the membrane-derived porin with acid and base, was found to be imported in the absence of NTPs and to be in a more unfolded conformation (Pfanner et al. 1988b). Pfanner et al. (1987a) found, by dissecting out different steps of import, that translocation of precursor domains from the cytosol onto or into the mitochondrial outer membrane generally required NTPs. In addition, hybrid proteins composed of a common signal sequence fused to different mature protein parts, required different levels of NTPs

for import. Finally, the sensitivity of precursors in reticulocyte lysate to exogenous protease was decreased by removal of NTPs and increased by their readdition (although this could potentially be explained by the presence of ATP-dependent proteases in reticulocyte lysate). In sum, a large number of indirect observations are consistent with the presence of an ATP-dependent "unfoldase" in reticulocyte lysate.

A related observation suggests that an ATP-requiring activity in lysate may be important for preventing oligomerization of precursors prior to mitochondrial import: Chen and Douglas (1988) have found that a mutant of the F_1-ATPase ß-subunit precursor which can no longer form tetramers, also does not require ATP for import.

In an attempt to examine precursor unfolding in reticulocyte lysate, the protease sensitivity and enzymatic activity of two artificial precursors, pOCAT and pODHFR, were studied under various conditions (Skerjanc et al., manuscript in preparation; Chapter 4 of this thesis). Treatment of reticulocyte lysate translation products with NEM and depletion of ATP were found to abolish import but had no effect on the enzymatic activity or protease sensitivity of the precursors. These findings argue that bulk unfolding of precursors in solution does not play an obligatory role in the acquisition of import competence. However, we also found that the formation of unfolded

translocation intermediates associated with intermediate-density submitochondrial vesicles requires ATP (as described in section 1.1.5a) and therefore it is possible that cytosolic factors may be involved in unfolding precursors only after the translocation apparatus has been engaged. On the other hand, it is possible that a small undetectable fraction of the precursor population could be unfolded in reticulocyte lysate at a given time.

e) Evidence for Precursor Unfolding on Membrane Surfaces

Eilers et al. (1987) found that a highly purified fusion protein, pCoxIV-DHFR, can be imported in the absence of additional cytosolic factors but still requires ATP. This suggests that for pCOXIV-DHFR, ATP hydrolysis is required at a step other than interaction with cytosolic factors, unless these factors are capable of binding tightly to mitochondria.

Examination of the interaction of pCoxIV-DHFR with mitochondria has shown that the precursor can unfold on the outer membrane in an ATP-independent manner (Eilers et al. 1988). It was found that when the precursor was incubated with mitochondria in the absence of ATP, a putatively unfolded intermediate on the import pathway was formed, which showed enhanced sensitivity to trypsin digestion and could no longer bind methotrexate. Subsequent studies have shown that the precursor may unfold on liposomes containing acidic phospholipids (Endo

and Schatz 1988; Endo et al. 1989) and that adriamycin, a drug which binds acidic phospholipids, can inhibit import (Eilers et al. 1989). Taken together, these results suggest that pCoxIV-DHFR binds to the lipids of the outer membrane and unfolds in an ATP-independent manner on the amphiphilic surface; ATP hydrolysis is required at a subsequent step in the import process.

In contrast to results summarized above for pCoxIV-DHFR, several natural mitochondrial precursors have been found not to exhibit conformational changes upon binding to liposomes (Endo et al. 1989). Consequently, they have suggested that pCoxIV-DHFR requires unfolding on lipid prior to import because it exists in a more tightly folded conformation than the natural precursors. Recently, using methods similar to those described above, the artificial precursors pOCAT and pODHFR were found not to unfold on mitochondrial membranes or on artificial liposomes (Skerjanc et al., manuscript in preparation; Chapter 4 of this thesis). This suggests that unfolding on membranes is not an obligate step in the import process, neither for natural nor for artificial precursors.

1.1.6 Import Requirements for Purified Precursors

The import competence of several purified mitochondrial protein precursors, which have been over-expressed in E. coli, has been examined with varying results. The precursor to rat liver aspartate

aminotransferase was purified in native form and found not to require additional cytosolic components for efficient import (Altieri et al. 1989). It would be interesting to determine whether this precursor bypasses the requirement for cytosolic unfolding factors by unfolding on membranes, as pCoxIV-DHFR seems to do. In contrast to the above result, pOCT, after purification from E. coli in 8 M urea, was found to require reticulocyte lysate components for import (Murakami et al. 1988b). A hybrid protein containing the pOCT signal sequence fused to DHFR, pODHFR, has been purified in the presence of 7 M urea and has been found to exhibit a conformation-dependent requirement for reticulocyte lysate components for import (Sheffield, W.P., Shore, G.C., and Randall, S.K. manuscript in preparation). For example, immediately after purified pODHFR is diluted out of 7M urea, import is efficient and did not require the presence of reticulocyte lysate, suggesting it is already in an import competent conformation. However, if the precursor is diluted out of urea and preincubated at 30°C for several hours prior to addition of mitochondria, the presence of lysate in the preincubation is required for efficient import. These findings suggest that a cytosolic component is involved in maintaining the precursor in an import competent conformation, which may not necessarily be unfolded. The differential requirement of various precursors for

cytosolic factors may simply reflect their different intrinsic three-dimensional structures, some of which require factors in order to be import competent, and some of which do not.

1.1.7 Summary

Amino terminal extensions of precursors, termed signal peptides, have been shown to contain the necessary information required to correctly target mitochondrial proteins. Although no consensus sequence has been determined for signal peptides, all peptides examined to date have been shown to be amphiphilic in nature and thus to have membrane surface-seeking properties. The evidence so far is consistent with a model in which the precursor initially associates with the lipids of the outer membrane and uses this surface to enhance subsequent diffusion to the import apparatus, thus modulating the overall rate of import. Whether or not these signals interact with the hydrophobic core of the lipid bilayer during subsequent translocation is still an open question, although an interaction with proteins is well established.

It seems that precursors must at some stage adopt an unfolded conformation in order to be imported. The mechanism and extent of unfolding remain to be further characterized. Evidence for several precursors is consistent with the requirement for an ATP-dependent "unfoldase" in reticulocyte lysate. Another type of

unfolding has been shown for one artificial precursor, which can unfold on the lipid surface of the outer membrane. Cytosolic factors may be involved in maintaining the precursor in an import-competent form, which, however, may not be unfolded. Finally, it is possible that the precursor may spontaneously unfold when the signal sequence engages the import apparatus and begins translocation. Further studies will be greatly aided by the purification of more components of the translocation apparatus in order to elucidate the exact mechanism of protein translocation into mitochondria.

1.2 Secretion of Proteins from Prokaryotes and Eukaryotes

Protein translocation across any membrane requires the correct targeting of a precursor, penetration of the membrane by both the hydrophobic and hydrophilic groups of the protein, translocation, and cleavage of the signal sequence on the other side of the membrane. One might expect that the basic concepts obtained by studying one system, such as protein secretion from cells, would be useful for understanding mechanisms in another system, such as protein import into mitochondria, and vice-versa. For this reason, I have included a discussion on secretion of proteins from prokaryotes and eukaryotes (for reviews see Briggs and Gierasch 1986; Walter and Lingappa 1986; Robinson and Austen 1987; Verner and Schatz 1988; Randall and Hardy 1989). Import of proteins into another

organelle, chloroplasts, has not been discussed here but has recently been thoroughly reviewed (Keegstra 1989; Keegstra et al. 1989).

Proteins destined to be secreted from both bacteria and eukaryotic cells are synthesized as higher molecular weight precursors with amino-terminal extensions. These signal sequences, which are markedly different from those of mitochondrial precursors, show no recognizable primary sequence homology (Watson 1984), but their structures typically exhibit three structurally similar regions: a positively charged amino-terminal region, a central hydrophobic core, and a polar carboxyl-terminal region ending with the cleavage site (von Heijne 1985; von Heijne 1988).

The finding that signal sequences are almost entirely interchangeable between prokaryotes and eukaryotes (Fraser and Bruce 1978; Talmadge et al. 1980; Watanabe et al. 1986) indicates that certain aspects of the secretory pathway must be highly conserved during evolution. For example, Talmadge et al. (1980) found that the eukaryotic signal sequence for insulin was just as effective as the bacterial signal sequence for penicillinase in targeting the secretion of rat insulin in E. coli. Even the internal signal sequence of chicken ovalbumin can be recognized by E. coli, resulting in secretion (Fraser and Bruce 1978). Further, the bacterial integral membrane

protein, LamB, can be translocated across canine microsomal membranes (Watanabe et al. 1986), although the stop transfer sequence is no longer recognized. The interchangeable nature of signal sequences from different organisms, and the lack of signal sequence homology at the primary sequence level, suggests that some very general aspect of their structure is recognized by the translocation apparatus.

1.2.1 Properties of Synthetic Signal Sequences

As with mitochondrial import, synthetic secretory signal peptides have been used to gain insight into the mechanism of protein translocation in the endoplasmic reticulum. Austen et al. (1984) have created a synthetic signal peptide representing a consensus of known signal sequences and have found it to inhibit the in vitro translocation of nascent preproteins into dog pancreatic microsomes. Similar inhibition of translocation was found using a synthetic signal peptide of parathyroid hormone (Majzoub et al. 1980).

Signal peptides corresponding to the wild type E.coli LamB precursor protein were found to inhibit the in vitro translocation of precursors of both alkaline phosphatase and outer membrane protein A, OmpA, into E. coli membrane vesicles (Chen et al. 1987). Further, using signal peptides derived from mutant and pseudorevertant strains, these workers demonstrated that the inhibition of in vitro

translocation correlated with the *in vivo* capacity to facilitate export. Similarly, the signal peptide of an *E. coli* outer membrane pore protein, PhoE, inhibited *in vitro* translocation of PhoE across inverted *E. coli* inner membrane vesicles (de Vrije *et al*. 1989). Whether the observed inhibitions are due to a specific interaction of the signal peptides with a proteinaceous component of the translocation apparatus or with lipid components of the membrane is still unclear. However, it has been found, recently, that an all-D-amino-acyl LamB wild-type signal peptide is less effective than the all-L-amino-acyl peptide in inhibiting pro-OmpA translocation (Gierasch 1989). This suggests that a protein, which can distinguish the opposite handedness of the all-D-amino-acyl peptide, would be required.

Circular dichroism studies of the signal peptide of parathyroid hormone demonstrated an increase in -helical conformation in nonpolar solvents (Rosenblatt *et al*. 1980), suggesting that the signal peptide undergoes a conformational change in passing from the cytoplasm to the membrane. Further, short fragments of the chicken lysozyme signal peptide were found to bind to small unilamellar lipid vesicles. The analysis of the binding of longer fragments was complicated by extensive aggregation of the sample (Nagaraj 1984).

Bacterial signal peptides appear by circular

dichroism to adopt more α-helical conformation in a hydrophobic environment. This has been demonstrated for the signal peptides of PhoE, LamB, M_{13} coat protein, OmpA, and glucitol and mannitol permeases (Batenburg et al. 1988a; Briggs and Gierasch 1984; Shinnar and Kaiser 1984; Gierasch 1989; Tamm et al. 1989; respectively). Further, spontaneous insertion into lipid monolayers has been shown for the signals of LamB (Briggs et al. 1985), PhoE (Batenburg et al. 1988b), and glucitol and mannitol permeases (Tamm et al. 1989). Examination of the orientation of PhoE and LamB signal peptides by biophysical methods suggests that the peptide inserts into the monolayer in an α-helix perpendicular to the monolayer plane (Batenburg et al. 1988a,b; Cornel et al. 1989).

1.2.2 The Involvement of Lipids in Translocation

The ability to interact with lipids has been correlated with in vivo function for E. coli LamB signal peptides (Briggs et al. 1985). They found that, while wild-type and pseudorevertant LamB signal peptides inserted into monolayers and adopted an α-helical conformation, a peptide corresponding to the signal sequence of a nonfunctional mutant displayed no change in CD spectra in the presence and absence of PE-PG vesicles and did not significantly insert into monolayers. These findings suggest that signal sequences may have some contact with membrane lipids during secretion.

Dierstein and Wickner (1985) have examined the ability of the precursor to maltose binding protein, pre-MBP, and various mutants to bind amphiphiles (Dierstein and Wickner 1985). They found that only functional precursors and mutants could bind to Triton X-100 micelles while the mature protein and non-functional mutants could not.

An involvement of negatively charged phospholipids for bacterial secretion was shown by de Vrije et al. (1988). Using mutants of E.coli defective in the synthesis of phosphatidylglycerol, they found that the rate of in vivo and in vitro translocation of pre-PhoE was decreased by reduced levels of anionic phospholipids. These results are consistent with a role for signal peptide/lipid interactions in protein translocation across the E. coli inner membrane. Procoat, the precursor of the major coat protein of coliphage M_{13}, has been found to be integrated into liposomes reconstituted with bacterial leader peptidase and properly cleaved (Ohno-Iwashita and Wickner 1983). Further, procoat was shown to be degraded by chymotrypsin encapsulated in pure lipid vesicles (Geller and Wickner 1985). These results demonstrate that procoat can cross a membrane without the aid of any other membrane proteins. However, this seems to be restricted to a very few protein precursors, notably procoat protein and apocytochrome c (see section 1.1.3). Further, M13

procoat is different from most bacterial precursors in that it does not require either secA or secY for efficient export (Wolfe et al. 1985; see section 1.2.3c).

Thus, although there is a great deal of intriguing evidence for the interaction of signal sequences with the lipids of the membrane during translocation, the question is still open whether or not this is actually a critical element in protein translocation in vivo.

1.2.3 Membrane Components Involved in Translocation

a) Various Mechanisms of Translocation have been Proposed

The process of protein translocation across membranes has been hypothesized by several groups to occur via a lipid-mediated pathway. Engelman and Steitz (1981) have suggested that the primary translocation event involved the hydrophobically driven partitioning of the signal sequence in a helical hairpin configuration into the membrane bilayer. Similar theoretical calculations by von Heijne and Blomberg (1979) suggested that the energy gained from the binding of an α-helical signal sequence to the core of any membrane, as well as the energy gained from the binding of a ribosome to a binding site on the membrane, will force even strongly hydrophilic residues into the lipophilic phase. Further, Wickner (1979) formulated the membrane trigger hypothesis, in which the interaction of the completed polypeptide chain with the lipid bilayer led to spontaneous membrane insertion of the

precursor, and recently published an updated version of the hypothesis to incorporate cytosolic factors and receptors (Wickner 1989).

Alternatively, the signal hypothesis predicted that translocation occurred in an aqueous environment through a proteinaceous pore (Blobel and Dobberstein 1975; Gilmore and Blobel 1985). Subsequently, Rapoport (1985) proposed the amphiphilic tunnel hypothesis, in which the translocation apparatus consists of an amphipathic tunnel that can bind both hydrophobic and hydrophilic parts of the nascent protein. The tunnel might be formed of a protein(s) with several types of binding sites, or of lipids arranged to provide both polar and apolar regions, or both.

b) Membrane Proteins Involved in Eukaryotic Secretion

Evidence has accumulated consistent with a requirement for membrane proteins during precursor secretion across the endoplasmic reticulum and the existence of an aqueous pore. For example, as precursors are translocated through the ER membrane they remain accessible to hydrophilic protein denaturants such as urea (Gilmore and Blobel 1985). In addition, a synthetic signal peptide has been shown to cross-link to a 45 kDa protein from dog pancreas microsomes (Robinson et al. 1987). Further, an integral, glycosylated protein from dog pancreas microsomes (~35 kDa) has been found to cross-

link to nascent preprolactin (Wiedmann et al. 1987). Recently, this protein has been purified and antibodies against it were found to inhibit translocation across the ER (Hartmann et al. 1989). This integral membrane protein could be involved in forming a protein pore across the bilayer. However, the exact role of these proteins in translocation remains to be elucidated.

Recently, it has been found that chemical alkylation of canine pancreas microsomes had no effect on nascent preprolactin targeting and signal sequence insertion, although translocation of the nascent chain into the lumen of the ER was inhibited (Nicchitta and Blobel 1989). These results suggest that the requirements for protein translocation are distinct from those for signal sequence insertion, and they imply that the activity of a proteinaceous component in the ER membrane is required for the process of protein translocation. Similar results have been obtained using proOmp A and yeast microsomes (Sanz and Meyer 1989).

c) Membrane Proteins Required for Prokaryotic Secretion

Genetic approaches have been used to identify sets of genes whose products are required to promote protein secretion in E. coli, and which probably encode components of the export machinery (for review see Benson et al. 1985). In E. coli at least four different genes have been identified that are essential for precursor proteins to be

translocated across the inner membrane. These include secA (Oliver and Beckwith 1981), secB (Kumamoto and Beckwith 1983), secD (Gardel et al. 1987), and secY, also known as PrlA (Emr and Hanley-Way 1981; Shiba et al. 1984).

The secA gene encodes a 102 kDa peripheral membrane protein and has been shown to be essential for translocation of in vitro translated alkaline phosphatase and proOmpA, as well as purified proOmpA, into E. coli membrane vesicles (Cabelli et al. 1988; Cunningham et al. 1989). Recently, it has been demonstrated that secA is an ATPase, dependent on SecY/PrlA, E. coli inner membrane vesicles, and proOmpA for ATPase activity (Lill et al. 1989). Both the hydrolysis of ATP and an electrochemical potential are essential for prokaryotic translocation, as has been found for import into mitochondria (Geller et al. 1986; Chen and Tai 1985). Thus, secA has a central role in coupling the hydrolysis of ATP to the transfer of precursor across the membrane. Exactly how this coupling occurs is still unclear.

SecY/PrlA is a 49 kDa integral membrane protein (Cerretti et al. 1983; Akiyama and Ito 1985). It is believed to interact with signal sequences, since PrlA mutations suppress mutations in the signal sequence of an envelope protein (Emr and Hanley-Way 1981) and since antibodies against PrlA/SecY inhibit the binding of LamB

and MalE to E. coli inverted vesicles (Watanabe and Blobel 1989). SecY/PrlA is a good candidate for providing a central translocation channel across the membrane, either by itself, or in combination with other, still unidentified proteins.

1.2.4 Proteins Involved in Precursor Targeting

a) The Signal Recognition Particle

Targeting of most mammalian ER proteins is mediated by the signal recognition particle (SRP) and the SRP receptor. SRP has been purified from canine pancreatic microsomes (Walter and Blobel 1980) and found to be an 11S small cytoplasmic ribonucleoprotein consisting of a 300 nucleotide 7SL RNA and six nonidentical polypeptide chains organized into four SRP proteins (Walter and Blobel 1982). SRP has been found to bind to the signal sequence region of the nascent polypeptide as it emerges from the ribosome during translation (Walter et al. 1981). Cross-linking studies have shown that the interaction is via the 54 kDa polypeptide component of SRP (Kurzchalia et al. 1986; Krieg et al. 1986). Recently, this polypeptide has been cloned and found to have a putative GTP-binding domain and an unusually methionine-rich domain (Romisch et al. 1989; Bernstein et al. 1989).

The interaction of SRP with the signal sequence in in vitro translation systems causes an arrest of polypeptide chain elongation (Walter et al. 1981; Meyer et al. 1982a).

A defective SRP, missing the two smallest polypeptides, was found to be unable to arrest elongation but was still active in signal recognition and targeting. Thus, elongation arrest is not a prerequisite for protein translocation across membranes (Siegel and Walter 1985). Meyer (1985) has found that SRP causes elongation arrest of IgG light chain translated in wheat germ lysate but not in reticulocyte or HeLa cell lysates. If elongation arrest occurs *in vivo* at all, it may serve to maintain the precursor in a translocation-competent state by preventing synthesis of the entire polypeptide chain of a translocated precursor in the cytoplasm.

b) The SRP Receptor

In vitro, elongation arrest is released when SRP binds to its receptor (docking protein) on the ER membrane, thus targeting the nascent chain to the ER and displacing SRP from the ribosome (Meyer *et al*. 1982a; Gilmore and Blobel 1983). The SRP receptor consists of two polypeptides, one of 69 kDa (α-subunit) and one of 30 kDa (ß-subunit). The α-subunit was the first to be purified using two independent methods (Gilmore *et al*. 1982a,b; Meyer *et al*. 1982b) and was subsequently cloned (Lauffer *et al*. 1985). The ß-subunit was identified and purified due to its tight association with the α-subunit (Tajima *et al*. 1986). The SRP receptor is unlikely to be part of the translocation apparatus itself, as the

receptor is present in the ER membranes in substoichiometric amounts with respect to membrane-bound ribosomes, and it has been shown not to bind to either ribosomes or polysomes synthesizing preprolactin (Gilmore and Blobel 1983). It probably functions "catalytically" and is recycled once correct targeting has been achieved . Yeast components analogous to SRP or the SRP-receptor have not yet been identified, although Schizosaccharomyces pombe contains an essential 7S RNA that is homologous to the 7S RNA in mammalian SRP (Brennwald et al. 1988).

c) Bacterial Components Involved in Precursor Targeting

Trigger factor has been isolated by exploiting its ability to form a 1:1 stoichiometric complex with proOmpA (Crooke et al. 1988b) and found to be a soluble 63 kDa protein. The finding that trigger factor is a dissociable component of the bacterial ribosome and that it may bind to a specific bacterial membrane receptor (Lill et al. 1988), suggests that trigger factor plays a role in targeting proOmpA to the membrane for secretion. GroEL, a bacterial heat-shock protein, is also a dissociable component of ribosomes and thus may have a similar role (Neidhardt et al. 1981). Both of these proteins are involved in conformational stabilization of precursors, as discussed in section 1.2.7b.

1.2.5 Post- vs Co-Translational Translocation

Protein translation and translocation across the ER was first thought to be obligately linked (Blobel and Dobberstein 1975). This idea was supported by the observed absence of detectable precursors in tissues, *in vivo* and by the finding that cleavage of the signal sequence occurred prior to completion of translation. It was also believed that the energy required for protein translocation was derived from extrusion of the polypeptide chain during translation (von Heijne and Blomberg 1979). Finally, the mechanism discussed above for targeting precursors to ER membranes, involving SRP and SRP receptor, also supports the idea of co-translational translocation. Nonetheless, it now appears that translocation and processing of precursor proteins are not necessarily coupled to translation *in vitro* (reviewed in Zimmerman and Meyer 1986). Using a hybrid mRNA which joined the signal sequence of β-lactamase to a portion of the α-globin message lacking a termination codon, completion of translation was not followed by release from the ribosome. This precursor was found to be efficiently translocated across canine pancreatic microsomes (Perara *et al*. 1986). Translocation could be abolished by releasing the nascent chain from the ribosome by artificial termination with puromycin. Similar results were found for a C-terminally truncated form of the human

glucose transporter (Mueckler and Lodish 1986a) and for a hybrid protein of the bovine rhodopsin signal sequence fused to α-globin (Perara et al. 1986). When translation of preplacental lactogen in rabbit reticulocyte lysate was terminated by cycloheximide, the precursor remaining attached to the ribosome could still be post-translationally processed by dog pancreas microsomes (Caulfield et al. 1986). Taken together, it is clear that the energy of translation is not obligately coupled to protein translocation.

In contrast to most precursors tested which, as a minimum, require attachment to the ribosome for translocation, small precursors such as honeybee prepromelittin, frog prepropeptide GLa, and bacteriophage M13 procoat, can post-translationally translocate across dog pancreatic microsomes in a ribosome- and SRP-independent fashion (Watts et al. 1983; Zimmerman and Mollay 1986; Schlenstedt and Zimmerman 1987). Similarly, yeast prepro-α-factor may be translocated across yeast microsomes in a post-translational, ribosome-independent fashion (Hansen et al. 1986; Waters and Blobel 1986; Rothblatt and Meyer 1986). Recently, prepro-carboxypeptidase Y and a truncated form of pre-invertase were also shown to exhibit post-translational translocation (Hansen and Walter 1988).

Although in vivo secretion seems to be co-

translational, it can be concluded that translation of at least some precursors across eukaryotic membranes is not necessarily co-translational *in vitro*. It is possible that the ribosome is required to maintain translocation competence of the precursor as it is translated (discussed in section 1.2.7a). All cases of ribosome-independent translocation have been found to involve small precursors which are almost completely synthesized before their signal sequence has fully emerged from the ribosome.

In general, prokaryotic secretion differs from eukaryotic secretion in that it is not obligately coupled to translation *in vivo* (Josefsson and Randall 1981; Randall 1983) and no analogue of SRP has been found that arrests translation (reviewed in Lee and Beckwith 1986). Secretion of proteins from bacteria requires a characteristic set of proteins that help maintain the import-competence of the precursor, as discussed in section 1.2.7b.

1.2.6 Energy Requirements

The *in vitro* uncoupling of translation from translocation allowed the nucleotide requirement of translocation to be examined. By depleting yeast translation systems of nucleotides after translation, it was shown that post-translational processing of prepro-α-factor is dependent upon nucleotide hydrolysis (Hansen *et al*. 1986; Waters and Blobel 1986). A ribonucleotide

requirement was also shown for post-translational translocation across mammalian microsomes (Perara et al. 1986; Mueckler and Lodish 1986b; Schlenstedt and Zimmerman 1987; Wiech et al. 1987) Membrane integration of the nascent polypeptides of opsin, the G protein of vesicular stomatitis virus, and the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus, were found to require GTP or a non-hydrolyzable GTP analogue (Hoffmand and Gilmore 1988; Wilson et al. 1988). The latter result implies that a GTP-binding protein performs an essential role during insertion of proteins into the endoplasmic reticulum. Recently, the GTP requirement was found to be involved in the SRP receptor-catalyzed displacement of SRP from ribosomes. GTP specific binding was localized to the α-subunit of the receptor (Connolly and Gilmore 1989).

As is the case for mitochondria, both ATP hydrolysis and an electrochemical potential are required for protein secretion in bacteria (Geller et al. 1986; Chen and Tai 1985). However, the polarity of the potential along the direction of translocation is opposite in the two cases- mitochondrial precursors are translocated from the positive to the negative side across the membrane, while bacterial precursors are exported from the negative to the positive side of the bacterial inner membrane. It is thus difficult to formulate a simple common model for the role of the potential in protein translocation in the two

systems.

1.2.7 Conformation of Precursors

a) Eukaryotic Precursors

Why can some eukaryotic precursors be translocated in a ribosome-independent manner whereas others cannot? The answer probably lies in the conformational properties of an individual precursor protein (reviewed by Meyer 1988). Loss of translocation competence may be due to folding or oligomerization of the protein such that interactions with either the translocation or targeting machinery can not occur. A few proteins (such as yeast prepro-α-factor and honeybee mellitin) retain translocation competence even as free, completed polypeptides. For most proteins, however, translocation competence requires binding of the signal sequence to SRP while the carboxyl terminus is attached to the ribosome. In this way, folding is prevented and translocation can be initiated.

In yeast, where a SRP equivalent has not been identified, two independent approaches have led to the finding that the 70 kDa heat shock proteins can stimulate the translocation of prepro-α-factor. Deshaies et al. (1988) mutated the hsp70 genes in yeast and found a cytoplasmic accumulation of secreted proteins like prepro-α-factor (as well as accumulation of a mitochondrial precursor). Chirico et al. (1988) purified hsp70 from yeast post-ribosomal supernatants by monitoring a

stimulatory activity for translocation of prepro-α-factor that was synthesized in wheat germ extracts. It is possible that these heat shock proteins could act as ATP-dependent "unfoldases", as discussed in section 1.1.5d (Rothman and Kornberg 1986; Pelham 1986).

For mammalian systems, purified mammalian hsp70 was found to stimulate the post-translational translocation of M13 phage procoat protein across pancreatic microsomes (Zimmerman et al. 1988). It remains to be seen if hsp70 is needed for co-translational translocation and how this relates to the role of SRP.

Recently, SRP was found to stabilize proOmpA for post-translational translocation across bacterial inner membrane vesicles, yeast microsomes, and pancreatic microsomes (Sanz and Meyer 1988; Crocke et al. 1988b). It was also found to stabilize prepro-α-factor for translocation across yeast microsomes. How these observations relate to the in vivo function of SRP in eukaryotes remains to be elucidated.

b) Prokaryotic Precursors

Since prokaryotic protein secretion is not obligately linked to translation in vivo, one would expect that the organism would develop mechanisms for maintaining the precursor in a translocation competent form (see section 1.1.5). Randall and Hardy (1986) established a correlation between export competence and a lack of stable

tertiary structure for preMBP. Further, it has been shown that the leader peptides of pre-ß-lactamase and pre-MBP slow the rate of folding of these precursors, presumably allowing time for correct interaction with the export apparatus (Park et al. 1988; Laminet and Pluckthun 1989). Subsequently, it was shown that SecB functions as an antifolding factor by interacting with the mature region of pre-MBP to prevent its premature folding into a translocation-incompetent form (Collier et al. 1988). Purification of SecB identified it as a 90 kDa multimeric protein composed of identical 17 kDa subunits (Weiss et al. 1988; Kumamoto et al. 1989) and confirmed its role in stimulating the export of preMBP and proOmpA, via its antifolding activity.

Trigger factor has been found to stabilize proOmpA in a form correctly folded for membrane assembly (Crooke and Wickner 1987; Crooke et al. 1988a). Since trigger factor is also a component of ribosomes, the correct folding of proOmpA is believed to occur co-translationally and is guided by the formation of a complex with trigger factor (Lill et al. 1988). In addition, the heat-shock groEL protein, which also binds ribosomes, was shown to interact with unfolded, newly synthesized pre-ß-lactamase (Bochkareva et al. 1988).

Recently, Lecker et al. (1989) have shown that secB, trigger factor, and GroEL can each bind proOmpA, forming

an isolatable complex and stabilizing an 'open' conformation for translocation.

1.2.8 Summary

The processes of secretion of proteins in prokaryotes and eukaryotes are similar to each other and to mitochondrial protein import in some respects, while in others, each is unique. All three systems have signal sequences capable of interacting with lipid bilayers, require ATP hydrolysis, and require a translocation-competent conformation of precursors, with the involvement of heat shock proteins. Although bacterial signal sequences are very similar in character to eukaryotic secretory sequences, *in vivo* translocation of proteins in prokaryotes seems to be post-translational, as it is for organellar translocation, while ER translocation is co-translational. An electrochemical potential is required for translocation across the mitochondrial and bacterial membranes. With the exception of heat shock proteins, the proteins involved in translocation seem to be different for all three systems. With the current major emphasis on purification and reconstitution of translocation components, detailed mechanisms of translocation should soon be available.

Footnote: The literature cited in the Introduction is included in the references for the General Discussion following Chapter 5.

CHAPTER 2

THE INTERACTION OF A SYNTHETIC MITOCHONDRIAL SIGNAL PEPTIDE WITH LIPID MEMBRANES IS INDEPENDENT OF TRANSBILAYER POTENTIAL

SUMMARY

We have used fluorescence measurements and assays of vesicle disruption (contents leakage) to monitor the interaction between lipid vesicles and a synthetic peptide corresponding to the N-terminal 27 amino acids of rat mitochondrial pre-ornithine carbamyltransferase (pOCT). This peptide and two fluorescent derivatives bind reversibily to vesicles composed of neutral and anionic phospholipids with increasing affinity as the proportion of anionic lipids in the vesicles increases. The affinity of the peptide for lipid vesicles is unaffected by the presence of a transbilayer potential (inside negative) of at least -80 mV across the vesicle membranes. Our results support the proposal that the signal sequence of pOCT may promote an initial association of the precursor protein with mitochondrial membranes prior to binding to a specific receptor. However, we find no evidence that the pOCT signal sequence can subsequently undergo transfer into or across the lipid bilayer, even in the presence of a transmembrane potential of the magnitude found previously to support the import of precursor proteins into mitochondria.

INTRODUCTION

Gene fusion studies have established that signal sequence information located at the N-terminus of mitochondrial precursor proteins is responsible both for targeting proteins to the surfaces of mitochondria and for triggering their subsequent translocation across mitochondrial membranes (Hurt et al., 1984; Horwich et al., 1985; Hurt et al., 1985; Nguyen et al., 1986; Emr et al., 1986; Keng et al., 1986; van Loon et al., 1986). Although the available evidence suggests that targeting is a receptor-mediated process involving a signal sequence recognition component (Hennig et al., 1983; Riezman et al., 1983; Zwizinski et al., 1983; Gillespie et al., 1985), it is not yet known whether subsequent internalization is facilitated by a proteinaceous translocation system (e.g., a pore) or involves a lipid-mediated pathway. In spite of the fact that mitochondrial signal sequences carry a high net positive charge and are characterized by overall polar properties, they also have the potential to form amphiphilic helices with the ability to perturb lipid bilayers (Roise et al., 1986; Epand et al., 1986). Roise et al. (1986) have recently concluded that a synthetic signal peptide of this type senses and responds to a transbilayer potential (inside negative) in artificial liposomes, a situation which may mimic that normally encountered during protein translocation across the mitochondrial inner membrane. These observations have led to the suggestion (Roise et al., 1986; von Heijne et al., 1986) that mitochondrial signal sequences function as bilayer-perturbing, potential-sensing entities that mediate protein translocation into the organelle via a lipid pathway, presumably by a process loosely analogous to transmembrane electrophoresis.

In the present study, we have considered this problem by employing a synthetic peptide (Gillespie et al., 1985; Epand et al., 1986) corresponding to amino acids 1-27 of pre-ornithine carbamyltransferase (pOCT), a precursor targeted to the matrix compartment of mitochondria in liver and intestinal mucosa. This peptide, designated pO-(1-27)-peptide amide, is potentially amphiphilic and has been shown previously to bind to and to disrupt vesicles composed of anionic lipids at high peptide/lipid ratios (Epand et al., 1986). We show here that this peptide can bind even to lipid surfaces containing physiological mole fractions of anionic lipids, but that neither the lipid-binding nor the bilayer-disrupting properties of the peptide are affected by the presence of a transbilayer potential. Taken together with earlier findings (Gillespie et al., 1985) that pO-(1-27)-peptide amide inhibits import of heterologous precursor proteins under conditions where mitochondria remain fully energized, we propose that amphiphilic signal sequences provide membrane surface-seeking properties which function at the cis face of the membrane and which promote subsequent diffusion of mitochondrial precursor proteins to the import receptor and protein translocation machinery, employing principles previously described for other surface-seeking entities (Sargent and Schwyzer, 1986).

Results

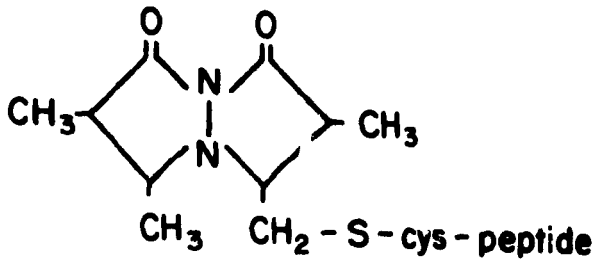
Binding Measurements

In order to maximize the sensitivity of measuring peptide-vesicle interactions, and to eliminate the need to separate physically free from vesicle- associated peptide molecules, we have employed fluorescent probes. A synthetic peptide designated pO-(1-27)-CysNH_2 was

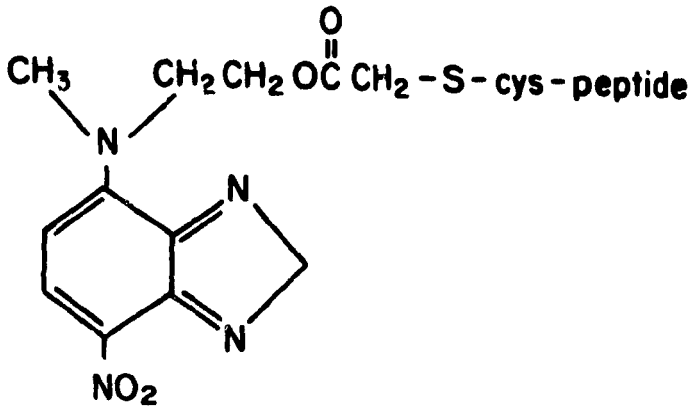
constructed, with a cysteine residue added to the C-terminal end of pO-(1-27) (in place of the glycine-28 residue in natural pOCT [McIntyre et al., 1984], a residue that can be deleted from the precursor without impairing its import into mitochondria [Nguyen et al., 1987]). This cysteine was selectively labeled with either a bimane or an (N-methyl-N-(nitrobenzoxadiazolyl)aminoethoxycarbonyl)-methyl (NBDA) fluorescent group (Figure 1). The binding of these labeled peptides to lipid vesicles could then be detected when we included in the vesicles small proportions of a fluorescent lipid [N-(nitrobenzoxadiazolyl)-phosphatidylethanolamine (NBD-PE) for bimane-labeled peptide, N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (Rho-PE) for NBDA-labeled peptide] that can serve as an energy-transfer acceptor for peptide molecules associated with the vesicle surface. This point is illustrated by the fluorescence trace shown in Figure 2A. When 4:1 phosphatidylcholine (PC)/cardiolipin vesicles (120 μM) containing 2.5 mole % Rho-PE are added to NBDA-labeled pO-(1-27)-$CysNH_2$ (0.3 μM), a rapid quenching of fluorescence is observed. When an excess of unlabeled cardiolipin vesicles, which have a substantially higher affinity for the peptide (see below), is added subsequently, a rapid reversal of the fluorescence quenching is seen. It is apparent that binding of the labeled peptide to the PC/cardiolipin vesicles is both rapid and readily reversible. Similar results were obtained using bimane-labeled peptide and lipid vesicles containing 5 mole % NBD-PE as an energy-transfer acceptor (not shown).

When bimane- and NBDA-labeled pO-(1-27)-$CysNH_2$ are mixed in aqueous solution at sub-micromolar concentrations, the fluorescence of the bimane-labeled species rapidly becomes partly quenched by resonance

Figure 1. Bimane- and NBDIA-labeled pO(1-27) CysNH_2. Details concerning the fluorescence properties of these peptides are provided in Methods.

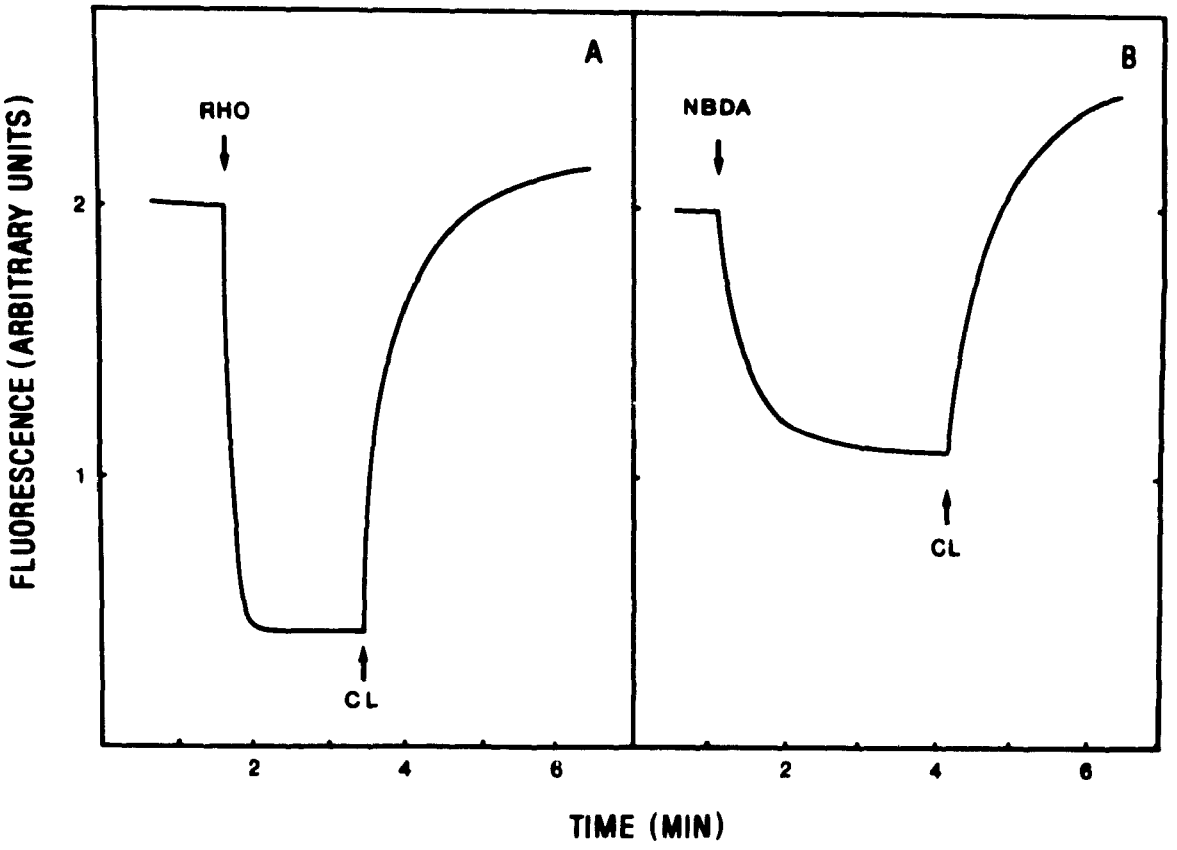


pO (1-27) cys-S-bimane



pO (1-27) cys-S-NBDIA

Figure 2. (A) Time course of fluorescence when NBDA-labeled pO-(1-27)-$CysNH_2$ (0.3 μM) was mixed successively with 120 μM 4:1 PC/cardiolipin vesicles containing 2.5 mole% Rho-PE (first arrow) and with 120 μM unlabeled cardiolipin vesicles (second arrow). (B) Time course of fluorescence when bimane-labeled pO-(1-27)-$CysNH_2$ (0.3 μM) was mixed with 0.3 μM NBDA-labeled pO-(1-27)-$CysNH_2$ (first arrow), then with 120 μM unlabeled cardiolipin vesicles (second arrow). Details of fluorescence measurements are given in Materials and Methods.


A
B
RHO
NBDA
CL
CL
FLUORESCENCE (ARBITRARY UNITS)
2
1
2
4
6
2
4
6
TIME (MIN)

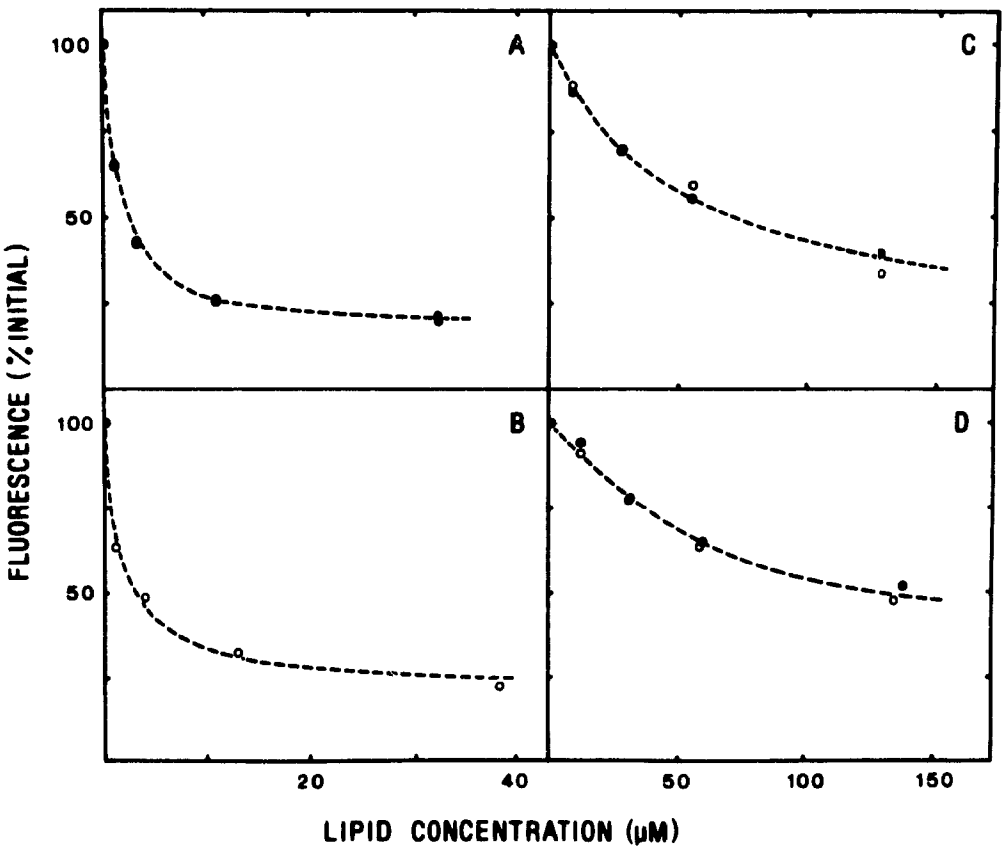
energy transfer to the NBDA-labeled peptide (Figure 2B). This result suggests that at least a portion of the labeled peptides exist in solution as oligomers in the μM concentration range. However, when an excess of cardiolipin vesicles (120 μM) is added to a mixture of the bimane- and NBDA-labeled peptides, the bimane fluorescence rapidly increases to the level observed in a parallel experiment in which no NBDA-labeled peptide is present. Further, we found that addition of NBDA-labeled peptide (0.3 μM) produced no detectable quenching of the fluorescence of bimane-labeled peptide already associated with cardiolipin vesicles (result not shown). These findings suggest that while the labeled peptide may have some tendency to oligomerize in solution, it exists as monomers when associated with phospholipid vesicles at least at high lipid-to-peptide ratios. Similar results were obtained using 4:1 PC/cardiolipin vesicles at high concentrations (≥200 μM).

When bimane- or NBDA-labeled pO-(1-27)-$CysNH_2$ was exposed to increasing concentrations of NBD- or Rho-PE-labeled lipid vesicles, quenching of the peptide fluorescence varied in a hyperbolic manner with increasing lipid concentration, as the data shown in Figure 3 illustrate. Since the extent of quenching of peptide fluorescence by the lipid vesicles provides a measure of the fraction of peptide that is bound to the vesicles, we can use data like those shown in Figure 2 to calculate an effective dissociation constant K_d^{eff} for the equilibrium

$$\text{Peptide (bound)} \rightleftharpoons \text{Peptide (free)}$$

Figure 3. Quenching of fluorescence of bimane- or NBDA-labeled pO-(1-27)-$CysNH_2$ by interaction with lipid vesicles containing membrane-bound energy-transfer acceptors. In each experiment, the labeled peptide (0.3 μM) was mixed with increasing amounts of acceptor-labeled lipid vesicles. The fluorescence readings obtained after successive additions of vesicles, corrected for small fluorescence contributions from the vesicles themselves, are presented as a percentage of the fluorescence initially measured for the peptide sample in the absence of lipid. The dashed curves in each panel represent the best fits to the data points obtained using equation [2]. (A) NBDA-labeled peptide was mixed with cardiolipin vesicles containing 2.5 mole% Rho-PE. (B) Bimane-labeled peptide was mixed with cardiolipin vesicles containing 5 mole% NBD-PE. (C) NBDA-labeled peptide was mixed with 4:1 PC/cardiolipin vesicles containing 2.5 mole% Rho-PE. (D) Bimane-labeled peptide was mixed with 4:1 PC/cardiolipin vesicles containing 5 mole% NBD-PE. Open circles - vesicles without a transbilayer potential were added; closed circles - vesicles with a transbilayer potential were added.

Control experiments using $TPMP^+$ accumulation to monitor the membrane potential demonstrated that 4:1 PC/cardiolipin vesicles exhibited a transmembrane potential of -130±20 mV (mean of three experiments) after 1 hr incubation with pO-(1-27)-peptide amide at peptide and lipid concentrations of 10 μM and 1.5 mM, respectively. The concentrations of lipid and peptide used in the $TPMP^+$ experiments were chosen to give molar ratios of <u>bound</u> peptide to lipid of 1:200 to 1:150, values comparable to those in the fluorescence binding assays. Parallel experiments using di-S-C_3(5) likewise showed that a membrane potential in excess of -80 mV, the limit of linearity of the assay, was maintained in 4:1 PC/cardiolipin vesicles (30 μM) over the time scale of our experiments even at the highest peptide concentrations examined here (4 μM). Similar results were obtained using the di-S-C_3(5) assay with cardiolipin vesicles, using a vesicle concentration of 30 μM and peptide concentrations of 0.4 μM and 1 μM.


A
B
C
D
100
50
100
50
20
40
50
100
150
FLUORESCENCE (% INITIAL)
LIPID CONCENTRATION (μM)

where

$$K_d^{eff} = \frac{([\text{Peptide}]_{free})([\text{Lipid}])}{([\text{Peptide}]_{bound})} \quad [1]$$

This analysis is valid only in cases where, as in the present experiments, the ratio of bound peptide to lipid is low and where the ratio of bound to free peptide is independent of the peptide concentration. As discussed below, these conditions were fulfilled in our experiments. We can then interpret K_d^{eff} operationally as the concentration of lipid vesicles of a particular composition at which one-half of the total peptide in the system is vesicle-bound. In other treatments, it is customary to express the affinity of the peptide for the vesicle surface in terms of a partition coefficient K_p or an affinity constant K_a for binding to well-defined 'sites' on the vesicle surface. Using either of these treatments, we can readily calculate values for the appropriate affinity constants using the relationships K_p = ((surface activity of peptide)/(activity of peptide in solution)) = $(v_L K_d^{eff})^{-1}$, where v_L is the partial molar volume of the lipid, or K_a = (N/K_d^{eff}), where N is the number of phospholipid molecules that constitutes a single 'binding site'.

The dashed curves plotted through the different sets of data points in Figure 3 represent the best fits to the data using the equation

$$(\text{Fluorescence}) = F_b + (F_o - F_b)\left(\frac{K_d^{eff}}{K_d^{eff} + [\text{Lipid}]}\right) \quad [2]$$

where F_o is the fluorescence of a sample of peptide in the absence of any vesicles, and F_b is the residual fluorescence of the peptide when it is entirely bound to the vesicles. For simplicity, the binding measurements were normally carried out at low peptide concentrations (0.3 μM) to ensure that the ratio of bound peptide to lipid was very

low. In some experiments, a peptide concentration of 1.0 μM was also used, giving estimates of binding that were indistinguishable from those obtained using 0.3 μM peptide. Values of K_d^{eff} calculated for binding NBDA- or bimane-labeled pO-(1-27)-$CysNH_2$ to different types of lipid vesicles, evaluated from data like those shown in Figure 2, are given in the first two columns of Table I. It can be seen that the strength of binding of the peptide to PC/cardiolipin vesicles varies substantially with the cardiolipin content of the vesicles. The peptides generally agree reasonably well, although in some cases the NBDA-labeled peptide appears to bind with slightly greater affinity.

Also shown in Table I are the results of experiments in which we examined the importance of vesicle lipid composition, rather than surface charge per se, for the binding of fluorescent derivatives of pO-(1-27)-$CysNH_2$. Replacement of one-half of the PC component in 9:1 PC/cardiolipin vesicles by phosphatidylethanolamine had little effect on the binding of either fluorescent peptide. Vesicles prepared from 2:1 PC/phosphatidylserine (PS) bound the labeled peptides with a roughly twofold lower affinity than did vesicles prepared from 4:1 PC/cardiolipin, which have a comparable surface charge density. It thus appears that the neutral phospholipid composition of the vesicles has little effect on their binding of the labeled peptides, while the nature of the anionic lipid component can have a modest but significant effect on the binding affinity.

To examine the effect of a transmembrane potential (inside negative) on the association of fluorescent pO-(1-27)-$CysNH_2$ derivatives with lipid vesicles, PC/cardiolipin vesicles loaded with K^+-containing

TABLE I

Effective Dissociation Constants Estimated for Binding of pO-(1-27)-Peptide Amide and its Fluorescent Analogues to Lipid Vesicles.

Lipid Composition	K_d^{eff} (μM) Estimated for: NBDA-labeled pO-(1-27)-$CysNH_2$[a] (μM)	Bimane-labeled pO-(1-27)-$CysNH_2$[a] (μM)	pO-(1-27)-Peptide amide[b] (μM)
Cardiolipin	1.5 ± 0.5	1.8 ± 0.6	3.1 ± 0.5
4:1 PC/cardiolipin	44 ± 13	82 ± 20	73 ± 5
2:1 PC/PS	120 ± 14	ND[c]	108 ± 28
9:1 PC/cardiolipin	75 ± 15	67 ± 18	ND
4.5:4.5:1 PC/phosphatidylethanolamine /cardiolipin	77 ± 19	68 ± 17	ND

TABLE FOOTNOTES: [a] Dissociation constants determined by fluorescence energy-transfer measurements, using lipid vesicles labeled with either 2.5 mole% Rho-PE (for NBDA-labeled peptide) or 5 mole% NBD-PE (for bimane-labeled peptide) as the energy-transfer acceptor. Further details of the data analysis are given in the legend to Figure 2.

[b] Dissociation constants determined from calcein-leakage measurements as described in the text, and as shown in Figure 4.

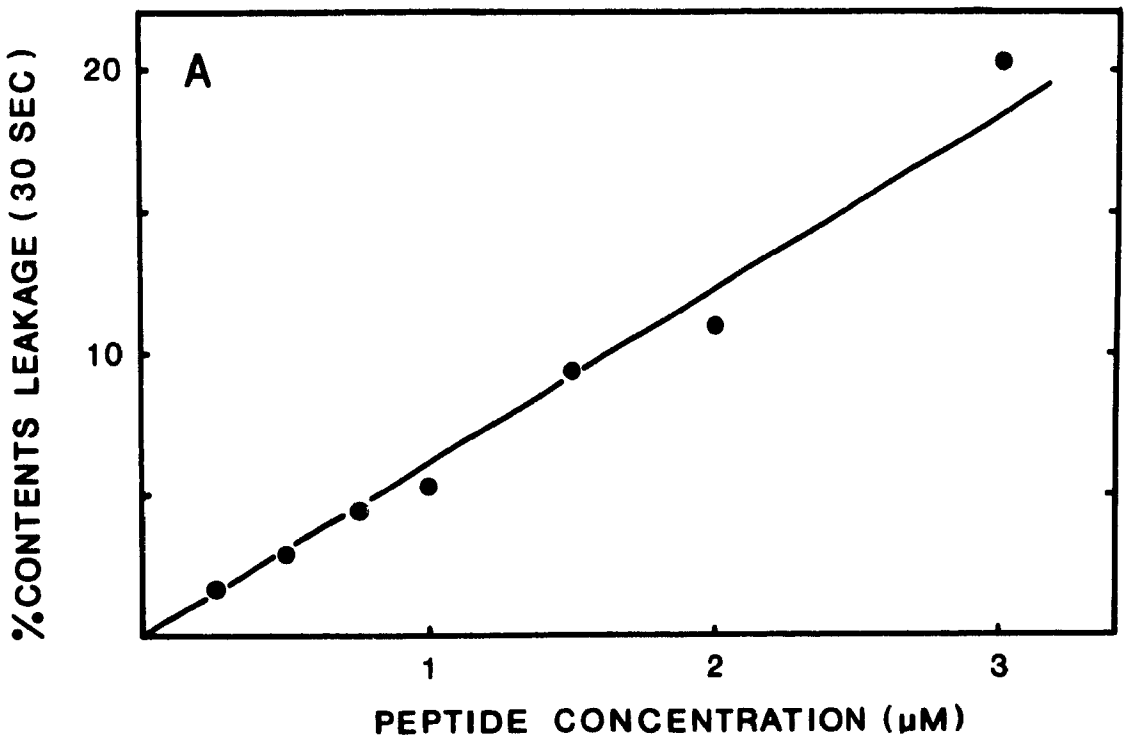
[c] ND, Not determined.

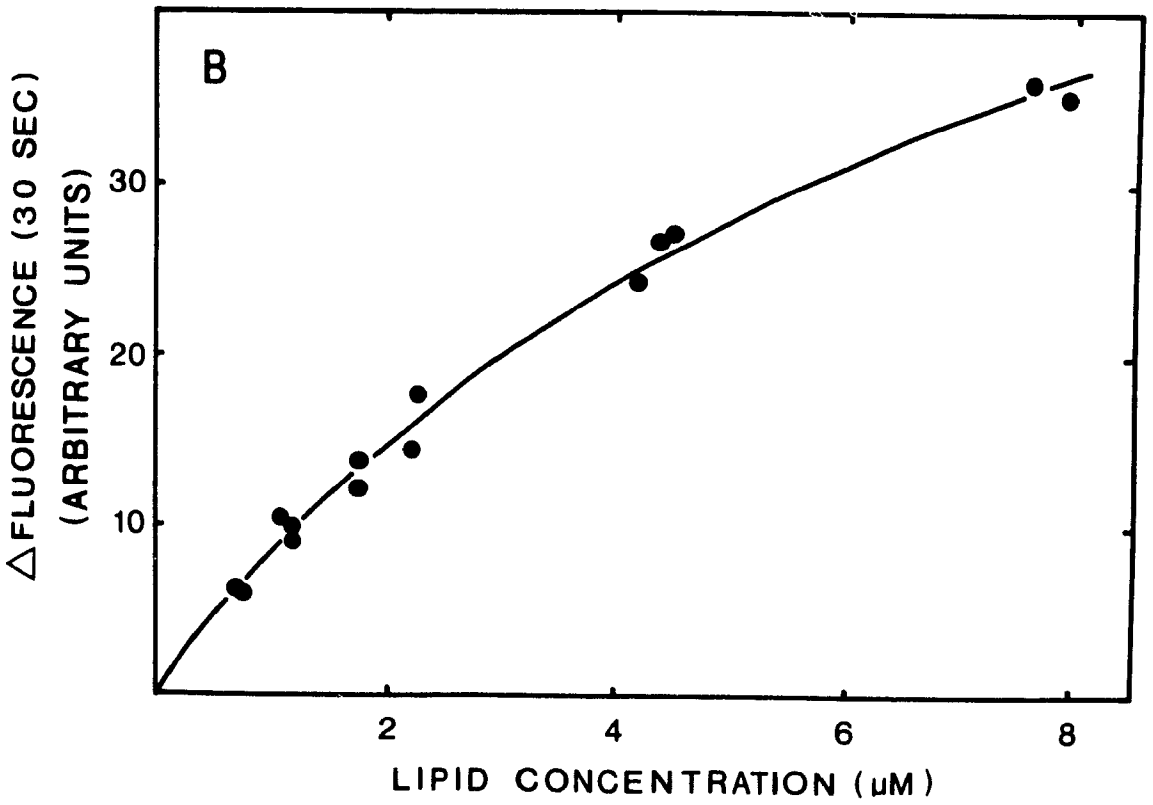
buffer (128 mM KCl, 5 mM histidine, 5 mM Tes, 0.1 mM EDTA, pH 7.4) were suspended in Na^+-containing buffer, and the binding of labeled peptides to the vesicles was assayed in the presence or absence of valinomycin or valinomycin plus gramicidin. In a series of experiments using vesicles containing 10 mole %, 20 mole % or 100 mole % cardiolipin (see Figure 3), we were unable to detect any significant enhancement of peptide binding to the vesicles in the presence of a potential gradient (valinomycin only). Control experiments using radiolabeled triphenylmethylphosphonium iodide ($TPMP^+$) or the fluorescent dye di-S-C_3(5) (Sims et al., 1974) demonstrated that the vesicles maintained a transmembrane potential in excess of -80 mV even in the presence of peptide, as outlined in the legend to Figure 3.

Release of Vesicle Contents by pO-(1-27)-peptide Amide.

To gain further information about the nature and consequences of binding of pO-(1-27)-peptide amide to lipid vesicles, we examined the effects of this peptide on the release of trapped calcein from vesicles of various lipid compositions. The results of a representative experiment, using 100% cardiolipin vesicles, are summarized in Figure 4. As the concentration of the peptide is increased at a fixed concentration of vesicles, the rate of calcein leakage increases in an apparently linear fashion (Figure 4A). By contrast, as the concentration of calcein-loaded vesicles increases at a fixed peptide concentration, the initial rate of calcein release increases in a hyperbolic manner (Figure 4B). These results suggest that the rate of calcein leakage is directly proportional to the amount of peptide bound to the vesicles. Using the data shown in Figure 4, we can then estimate very crudely that binding of pO-(1-27)-peptide amide to cardiolipin vesicles is half-maximal at a cardiolipin concentration of ~7 μM.

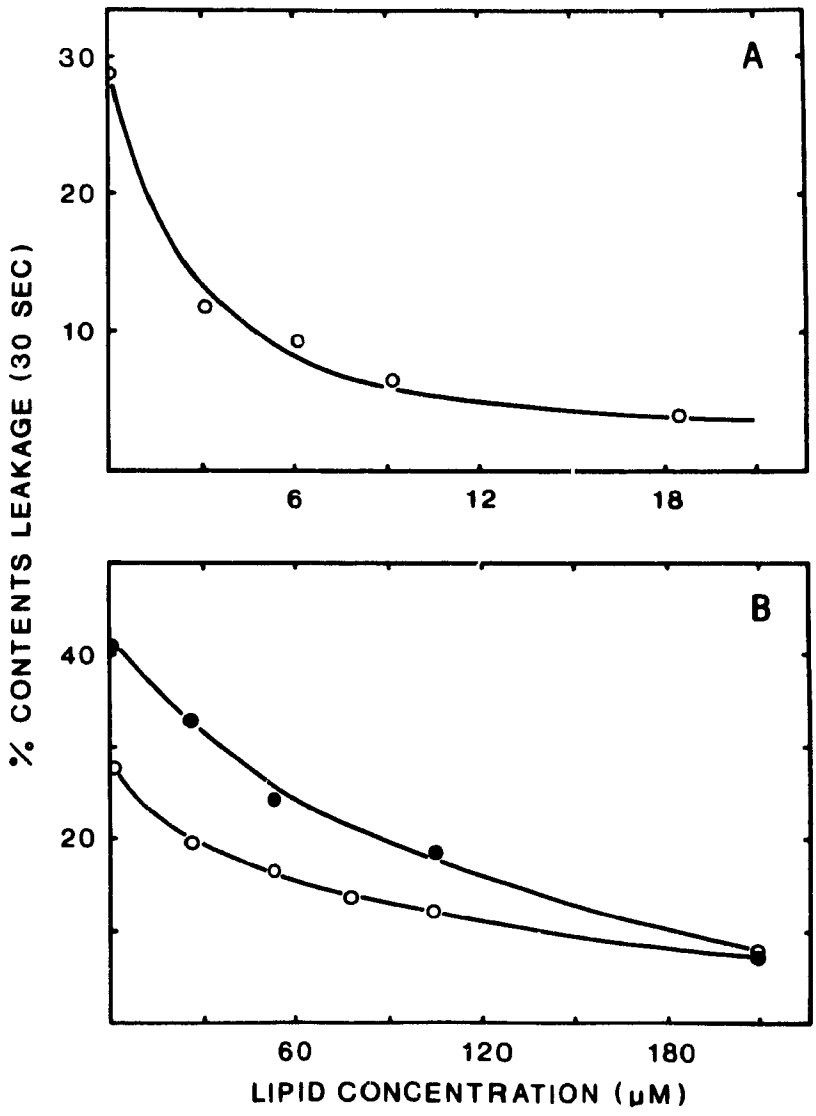
Figure 4. Leakage of trapped calcein from 100% cardiolipin vesicles in the presence of pO-(1-27)-peptide amide. The peptide was added to suspensions of calcein-loaded vesicles, and the initial release of calcein was quantitated as the enhancement of calcein fluorescence 30 sec after the addition of peptide. The fluorescence enhancement corresponding to complete release of calcein was determined by lysing the vesicles with 0.1% Triton X-100. In (A), the concentration of peptide was varied while holding the lipid concentration fixed at 5 μM, and in (B), the concentration of calcein-loaded vesicles was varied at a fixed peptide concentration of 1 μM. In (B), the ordinate axis is calibrated in (arbitrary) fluorescence units, rather than as a percentage of vesicle contents released, because the total amount of releasable calcein is different for each lipid concentration.


A
20
10
%CONTENTS LEAKAGE (30 SEC)
1
2
3
PEPTIDE CONCENTRATION (μM)


B
30
20
10
ΔFLUORESCENCE (30 SEC)
(ARBITRARY UNITS)
2
4
6
8
LIPID CONCENTRATION (μM)

A more precise estimate of the affinity of binding of pO-(1-27)-peptide amide to lipid vesicles can be obtained from experiments in which the leakage of calcein from a fixed (and low) concentration of calcein-loaded vesicles is assayed in the presence of a fixed level of peptide and varying concentrations of 'empty' (buffer-containing) vesicles of the same lipid composition (Figure 5A). Since the 'empty' liposomes compete with the calcein-loaded ones for peptide binding, the rate of leakage from the calcein-loaded vesicles decreases as the concentration of 'empty' vesicles is increased. In practice, an estimate of K_d^{eff} can be obtained from such experiments, as follows: The rate of leakage of contents from a low concentration of calcein-loaded vesicles is determined at some convenient peptide concentration and at one-half this concentration of peptide. The rate of leakage from the calcein-loaded vesicles is then measured, at the higher peptide concentration, in the presence of increasing amounts of 'empty' vesicles of the same lipid composition. At some concentration of 'empty' vesicles, the leakage rate will be decreased to the level measured when the calcein-loaded liposomes alone were treated with the twofold lower concentration of peptide. This concentration of 'empty' vesicles can be shown to be equal to K_d^{eff}. Apparent dissociation constants, estimated from this type of experiment for 4:1 PC/cardiolipin, 2:1 PC/PS and 100% cardiolipin vesicles, are given in Table I and agree well with those obtained from the other experimental approaches discussed above. It thus appears that these vesicles bind pO-(1-27)-peptide amide with affinities very similar to those measured above for the binding of fluorescent derivatives of pO-(1-27)-CysNH_2.

Figure 5. Effects of excess 'empty' (KCl-buffer-containing) lipid vesicles on the leakage of contents induced by pO-(1-27)-peptide amide from a low concentration (0.5 μM) of calcein-loaded vesicles of the same lipid composition. In (A), vesicles of pure cardiolipin were used (peptide concentration: 1 μM), with no transbilayer potential. In (B), 4:1 PC/cardiolipin vesicles were used (peptide concentration : 3 μM), in the presence of valinomycin (1:10,000 lipids, ●) or gramicidin (1:1000 lipids, o). The rates of petide - induced leakage from vesicles in the absence of any ionophores were essentially identical to those shown for vesicles treated with gramicidin. Measurements of the transmembrane potential in these vesicles, using the $TPMP^+$ assay with 1 mM lipid and 10 μM peptide (incubation time 30 min.), estimated the potentials in the valinomycin-treated vesicles as -105 mV and -95 mV in the absence and presence of peptide, respectively, while the potentials in gramicidin-treated vesicles, or in vesicles in the absence of ionophore, were essentially zero (smaller than - 15 mV). Comparable results were obtained with the di-S-C_3(5) assay, using the same lipid and peptide concentrations that were used in the leakage assays.


A
B
30
20
10
6
12
18
40
20
60
120
180
% CONTENTS LEAKAGE (30 SEC)
LIPID CONCENTRATION (μM)

To examine the effects of a transmembrane potential on the leakage of calcein induced from PC/cardiolipin vesicles by pO-(1-27)-peptide amide, vesicles were loaded with calcein solutions containing K^+ as the only alkali cation, and the peptide-induced efflux of calcein into Na^+-containing medium was measured in the presence or absence of valinomycin or valinomycin plus gramicidin. As shown in Figure 5B, 4:1 PC/cardiolipin vesicles with an inside negative potential (valinomycin only present) showed an enhancement of the initial rate of peptide induced calcein release by roughly 40% in comparison with vesicles that were not treated with ionophores or that were treated with gramicidin to dissipate the potential gradient. Conversely, vesicles that were treated with monensin, which confers a modest but significant sodium - selective conductance to the bilayers (Sandeaux et al., 1978), showed a 35% decrease in the rate of peptide - induced calcein leakage, and a significantly more positive internal potential by the di-S-C_3(5) assay, when compared to gramicidin - treated vesicles under the same conditions (not shown). However, when the affinity of the vesicles for the peptide was determined from the data shown in Figure 5B by the analysis described above, we did not find that the presence of an inside negative potential enhanced the affinity of the vesicles for pO-(1-27)-peptide amide.

Parallel experiments to those described above showed that pO-(16-27), a peptide representing amino acids 16 to 27 of the pOCT presequence, caused no significant release of calcein from 4:1 PC/cardiolipin vesicles (30 μM) at peptide concentrations up to 100 μM and incubation times up to 30 min (not shown). While this peptide has a density and distribution of charged residues similar to pO-(1-27)-peptide amide, it has been reported previously not to inhibit

import of pOCT into mitochondria (Gillespie et al., 1985), and deletion-mutagenesis studies have shown that residues 15-19 or 22-30 can be deleted from the pOCT sequence without loss of import function (Lingelbach et al., 1986; Nguyen et al., 1987).

Amphiphilic peptides such as melittin have previously been demonstrated to promote coalescence as well as lysis of lipid vesicles, with concommitant intermixing of lipids between vesicles (Eytan and Almary, 1983; Morgan et al., 1983). To examine whether this process might play a role in the leakage of vesicle contents induced by pO-(1-27)-peptide amide, we assayed the mixing of lipids between 4:1 PC/cardiolipin vesicles in the presence of this peptide (0-10 μM), using the lipid-mixing assay described by Struck et al. (1981). We were unable to detect any significant lipid mixing between these vesicles in the presence of pO-(1-27)-peptide amide, at either 4 μM or 60 μM lipid, on a time scale in which as much as 50% leakage of vesicle contents would be observed. Therefore, we conclude that the peptide-induced leakage of contents from these vesicles is not accompanied by vesicle fusion or similar processes that lead to intermixing of vesicle components.

Discussion

The results of this study extend the previous findings of Epand et al. (1986), who reported that pO-(1-27)-peptide amide can bind to and disrupt negatively charged lipid vesicles at high concentrations of peptide and lipid. It is apparent that this peptide and its fluorescent analogues can bind with moderately high affinity even to lipid surfaces that contain anionic lipids as a minority species (~20 mole%), as is the case for the cytoplasmic surfaces of cellular membranes (Op den Kamp,

1979; Daum, 1985). It should be noted that the vesicles used in this study were of an average diameter large enough (~ 1200 Å) to minimize the perturbations of lipid packing that are associated with highly curved small unilamellar vesicles (diameter < 350 Å). The cytochrome oxidase subunit IV signal peptide has recently been reported to bind with somewhat higher affinity to negatively charged lipid monolayers than we observe here for pO-(1-27)-peptide amide (Tamm, 1986).

One possible complication in the analysis of our binding data, which has not been addressed in previous analyses of signal peptide binding to lipid surfaces (Roise et al., 1986; Epand et al., 1986; von Heijne, 1986), is the possibility that the peptide forms an oligomer in solution, as has been demonstrated for other amphiphilic peptides such as melittin (DeGrado et al., 1982; Bernheimer and Rudy, 1986; Hermetter and Lackowicz, 1986; Vogel and Jähnig, 1986). Although we find that labeled derivatives of pO-(1-27)-CysNH_2 can oligomerize in solution, we also observe that the association of the peptide with lipid surfaces is unaffected by the peptide concentration. This latter result suggests either that the state of oligomerization of the membrane-associated peptide is the same as that in solution, or that the oligomerization of the peptide in solution is relatively weak. The failure of the NBDA-labeled peptide to quench the fluorescence of bimane-labeled peptide when associated with lipid vesicles, although it can do so in solution, suggests that the latter possibility is more likely.

The most important result from both our vesicle-binding studies and our leakage studies is that we find no evidence that the presence of a transmembrane potential (inside negative) can enhance binding of the peptides to vesicles or promote transbilayer movement of the peptides.

Our observation of variations in the rate of peptide - induced calcein leakage from vesicles with different internal potentials (Figure 5B) may appear to contradict this conclusion. However, these effects of the potential on peptide - induced calcein leakage, while they are certainly real, are miniscule in comparison to those expected if even a few of the positively charged residues of pO-(1-27)-peptide amide could penetrate deeply into (or across) the vesicle membrane when a potential is applied. Bilayer membranes containing melittin, for example, show a conductance enhancement of roughly a thousandfold, concommitant with the adoption of a transmembrane arrangement of the melittin molecules, when a membrane potential of > 40 mV is applied (Kempf et al., 1982). Moreover, calcein is an anion at neutral pH, and it is thus to be expected that it will show a somewhat enhanced rate of efflux from vesicles when an internally negative potential is applied, even if the permeability of the membrane in the presence of peptide is potential - independent. Our leakage results thus cannot by themselves be taken to indicate significant penetration of the peptide into the membranes of lipid vesicles carrying an internally negative potential. Experiments with ^{125}I-labeled pO-(1-27)-peptide amide also provided no evidence for sequestration of the peptide in the interior of vesicles possessing an inside negative potential (results not shown). Taken together, our results suggest that pO-(1-27)-peptide amide and its fluorescent analogues bind to lipid membranes in such a fashion that the positively charged amino acid side chains do not sense any significant portion of the transmembrane potential.

Our results indicate that the disruption of lipid vesicles by pO-(1-27)-peptide amide is a slower process than is peptide binding to vesicles, is not correlated with vesicle fusion and is a relatively

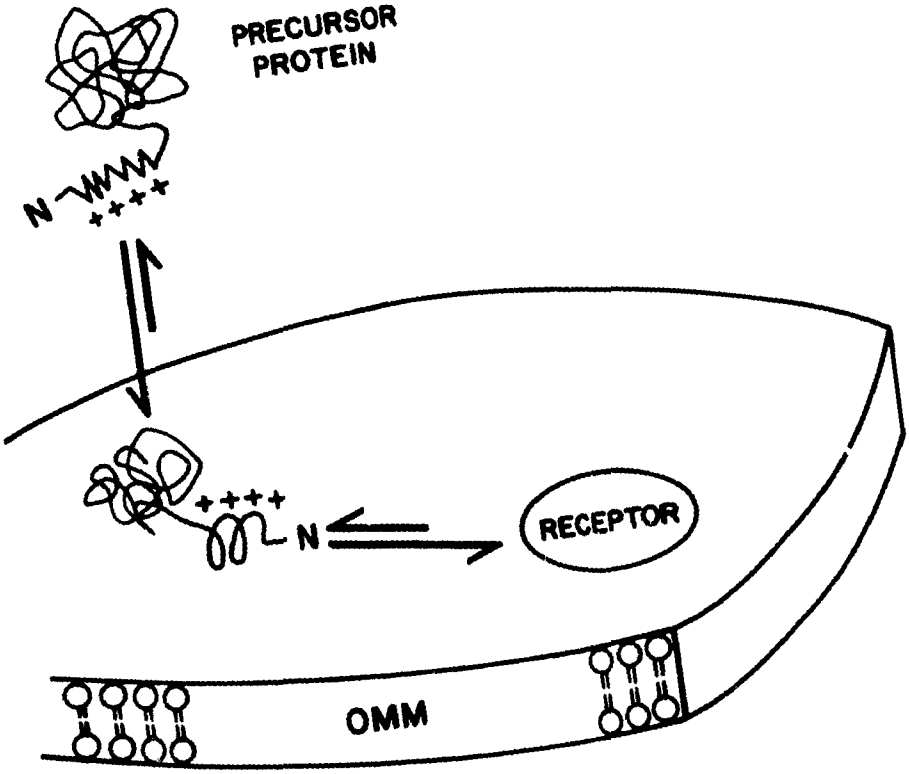
inefficient process: lipid vesicles with a 1:100 molar ratio of bound peptide to lipid (which carry some 1000 peptide molecules per vesicle) lose their contents at a rate of only ~ 7% per minute. A number of amphipathic peptides have been shown to cause similar disruption of lipid vesicles, or natural membranes, often with considerably greater efficiency than we observe for the pOCT signal peptide (Kanellis et al., 1980; Gillespie et al., 1985; Ito et al., 1985; Roise et al., 1986). Unfortunately, the mechanism of this effect is not yet understood in detail even for well-studied cytolytic peptides such as mellitin (DeGrado et al., 1982; Yianni et al., 1986; Bernheimer and Rudy, 1986; Hermetter and Lackowicz, 1986; Vogel and Jähnig, 1986).

In conclusion, and as outlined in Figure 6, we suggest that in vivo the signal sequence of pOCT can mediate an initial reversible interaction of pOCT with the lipid component of cellular membranes. As demonstrated for other ligands, such an interaction can enhance considerably the rate at which the precursor protein associates subsequently with an appropriate membrane receptor (Sargent and Schnyzer, 1986), in this case a putative import receptor located in the mitochondrial outer membrane. We have obtained no evidence, however, that the signal sequence could serve to initiate the insertion of the precursor protein directly into the lipid bilayer, even in the presence of a transmembrane potential of the magnitude of that found in mitochondria (Pfanner and Neupert, 1985).

Materials and Methods

Materials - pO-(1-27)-peptide amide was synthesized as described previously (Gillespie et al., 1985). pO-(1-27)-CysNH_2, synthesized by similar procedures, was obtained from the Alberta Peptide Institute

Figure 6. Schematic representation of a model in which the membrane surface-seeking properties of amphiphilic mitochondrial signal peptides mediate binding of precursor proteins to the surfaces of mitochondria and promote subsequent diffusion to the import receptor and protein translocation apparatus of the organelle.


PRECURSOR
PROTEIN
N
++++
++++
N
RECEPTOR
OMM

(Edmonton, Alberta, Canada). Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids, and phosphatidylethanolamine was synthesized from PC by enzymic transphosphatidylation as described previously (Comfurius and Zwaal, 1977; Silvius and Gagné, 1984). Dioleoyl phosphatidylserine (PS) was synthesized as described elsewhere (Silvius and Gagné, 1984). Cardiolipin (Sigma, from bovine heart) was routinely Folch-washed as described by Papahadjopoulos and Miller (1967) to remove possible divalent cation contaminants. A minor contaminant (5%) present in this material was removed by column chromatography prior to use in some experiments, without any discernible effect on the results obtained. Lipids were stored in chloroform or hexane solutions at -70°C under nitrogen. Valinomycin and gramicidin were obtained from Sigma, as was calcein, which was purified by chromatography on Sephadex LH-20 by a procedure similar to that employed previously for purification of carboxyfluorescein (Ralston et al., 1981). Monobromobimane was obtained from Calbiochem, and di-S-C_3(5) and NBDIA were products of Molecular Probes (Junction City, Ore.). All other common chemicals were of reagent grade or better; all organic solvents were redistilled before use.

Methods

Preparation of lipid vesicles - Lipids were dried down from stock solutions, first under N_2 and then under high vacuum for at least 2 hr. The dried lipid was normally dispersed under N_2 in either 128 mM KCl, 5 mM histidine, 5 mM Tes, 0.1 mM EDTA, pH 7.4 or 100 mM calcein (potassium salt), 5 mM histidine, 5 mM Tes, 0.1 mM EDTA, pH 7.4 by brief vortexing, then bath-sonicated for 5 min and three times freeze- thawed (Pick, 1981). The resultant vesicles, which gave measured trapped volumes of ~ 20 µl/µmole lipid (corresponding to an average vesicle diameter of ~0.6

μm) were filtered through 0.2 μm pore size Nucleopore membranes, then passed through a column of Sephadex G-75 that was equilibrated with 128 mM NaCl, 5 mM histidine, 5 mM Tes, 0.1 mM EDTA, pH 7.4. The final vesicle preparations gave measured trapped volumes of 4-5.5 μl/ μmole lipid phosphorus, corresponding to average vesicle diameters of 1200-1600 Å. Lipid concentrations were determined as described previously (Silvius and Gagné, 1984). Peptide stocks of known concentration were prepared from accurately weighed peptide samples after desalting and lyophilization. Transmembrane potentials (inside negative), generated by adding valinomycin (1 per 100,000 lipids for cardiolipin vesicles, 1 per 10,000 or 1 per 1000 lipids for other vesicles) to vesicles prepared as just described, were monitored by measuring the fluorescence of the potential-sensitive dye di-S-C_3(5) (Sims et al., 1974) or the accumulation of [^{3}H]-triphenylmethylphosphonium iodide ($TPMP^+$). For calculations of membrane potential based on $TPMP^+$ accumulation, vesicle trapped volumes were estimated using carboxyfluorescein or calcein as a marker of the internal aqueous space.

Peptide labeling - pO-(1-27)-$CysNH_2$ was labeled with either monobromobimane or 4-(N-(iodoacetoxy)ethyl-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (1.2 eq/mole peptide) in 1:2 acetonitrile/ 50 mM KH_2PO_4, 1 mM EDTA, pH 7.4 for 2 hr. at 20°C or 6 hr. at 4°C, respectively before quenching the reactions with excess 2-mercaptoethanol. The labeled peptides were repeatedly dissolved in minimum quantities of phosphate buffer, then precipitated by mixing with 20 volumes of acetone and gradually cooling to -15°C. This procedure was shown in control experiments to remove quantitatively low- molecular-weight adducts of the fluorescent labels

from the peptides after two cycles of precipitation; in practice, 3-4 such precipitations were carried out.

Assays of vesicle leakage and peptide binding - Leakage of vesicle contents was assayed using the calcein-release assay described by Allen and Cleland (1980). To assay binding of bimane- or NBDA-labeled pO-(1-27)-$CysNH_2$ to lipid vesicles, vesicles were prepared as above with either 5 mole% NBD-PE or 2.5 mole% Rho-PE, respectively, incorporated into the lipid bilayer. In a typical experiment, the fluorescence of the labeled peptide was measured before and after the addition of varying amounts of vesicles containing the appropriate "quencher" phospholipid. The degree of quenching of the peptide fluorescence in the presence of the vesicles was calculated after correcting for small contributions of the quencher-labeled vesicles themselves to the measured fluorescence. All assays of vesicle leakage and peptide binding were carried out in 128 mM KCl or NaCl, 5 mM histidine, 5 mM Tes, 0.1 mM EDTA, pH 7.4 and at 37°C.

Fluorescence measurements were carried out using a Perkin-Elmer LS-5 spectrofluorimeter, with the following excitation and emission wavelength settings: for calcein, λ_{ex} = 495 nm, λ_{em} =525 nm (slitwidths 3 nm/5 nm); for NBDA-labeled pO-(1-27)-$CysNH_2$, λ_{ex} =485 nm, λ_{em} =540 nm, (slitwidths 15 nm/20 nm); and for bimane-labeled pO-(1-27)-$CysNH_2$, λ_{ex} =395 nm, λ_{em} = 475 nm (slitwidths 15 nm/20 nm); and for di-S-C_3(5), λ_{ex} = 622 nm, λ_{em} = 670 nm (slitwidths 15 nm/20 nm).

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CHAPTER 3

IDENTIFICATION OF HYDROPHOBIC RESIDUES IN THE SIGNAL SEQUENCE OF MITOCHONDRIAL PRE-ORNITHINE CARBAMYL TRANSFERASE THAT ENHANCE THE RATE OF PRECURSOR IMPORT

SUMMARY

Previous studies employing circular dichroism and resonance energy transfer techniques have demonstrated that the signal peptide of mitochondrial pre-ornithine carbamyl transferase (pOCT) has the potential to interact with the surface of an anionic phospholipid membrane via a short amphiphilic helical domain. Here, we have used predictive secondary structure computations as a guide to localize the putative membrane binding region in the pOCT signal sequence, and demonstrate that replacement of leucine residues at positions 5,8, and 9 with the less hydrophobic residue, alanine, significantly reduces the rate of precursor import (4- to 5-fold compared to wild-type); the amino acid substitutions had little effect, however, on the ability of a mitochondrial matrix extract to process the mutant precursor polypeptide. The mutant precursor bound to anionic liposomes with a lower affinity compared to wild-type pOCT, and was inhibited to a lesser extent than pOCT during import into mitochondria in the presence of varying concentrations of liposomes. Taken together, the results suggest that this small region of the pOCT signal sequence, containing a limited number of critical hydrophobic residues, contributes to the optimal rate of precursor import, perhaps by functioning as a membrane surface-seeking entity.

INTRODUCTION

A number of studies, employing either synthetic peptides or intact precursor proteins, have shown that positively-charged mitochondrial signal sequences have the potential to form amphiphilic structures with the ability to bind to anionic phospholipid bilayers and, at high concentrations, perturb and lyse the bilayer structure (1-9). Although the exact mechanism of membrane perturbation by the signal peptide is not well understood, it is unlikely to signify that mitochondrial signal sequences can mediate transfer of precursor proteins into or across membranes by a lipid-mediated route, at least insofar as such a route involves a typical bilayer structure. We have recently shown, for example, that a signal peptide corresponding to amino acids 1-27 of rat liver pre-ornithine carbamyl transferase (pOCT) [1], though potentially membrane-lytic at high concentrations, binds reversibly to the surface of lipid vesicles but is incapable of significant penetration into the membrane, even in the presence of a transbilayer electrochemical potential (inside negative) of at least 80 mV (7). Although these findings (7) do not rule out the idea that the pOCT signal is capable of electrophoresis through a hydrophobic environment, they do exclude the possibility that this can occur in the absence of other membrane proteins. One possible consequence of the membrane surface-seeking properties of this and other mitochondrial signal sequences, however, may be to enhance the diffusion of precursor proteins to the import receptor and protein translocation machinery of the organelle (7). In the present study, we present evidence which is consistent with this interpretation, and identify a short amphiphilic domain within the pOCT signal sequence (residues 5-9) whose net hydrophobicity influences both

the rate of precursor import and, correspondingly, the ability of pOCT to bind to lipid membranes.

EXPERIMENTAL PROCEDURES

General - Earlier publications give details of the procedures for *in vitro* transcription of pSP64 derivatives, translation in the rabbit reticulocyte lysate system, purification of rat heart mitochondria, import and processing of precursor proteins by mitochondria *in vitro*, and preparation of mitochondrial matrix extracts (see refs 10 and 16). Additional details, as well as the procedures employed for the construction of recombinant plasmids, are given in the figure legends.

Preparation of Liposomes - Lipids (obtained as described in ref. 7) were dried down from stock solutions under N_2 and dispersed in 80 mM KCl, 20 mM Hepes, and 2 mM Mg acetate, at pH 7.4, by brief vortexing followed by bath sonication for 5 minutes. Lipid concentrations were determined as described previously (11). Biotinated-PE was prepared by incubating NHS-LC-biotin (Pierce Chemical Co.) with egg PE (3.7 equivalents/mole egg PE) in 2:4:1 $CHCl_3/CH_3OH/H_2O$, overnight at 20°C. It was purified by preparative silica gel thin layer chromatography in 80:25:2 $CHCl_3/CH_3OH/H_2O$.

RESULTS AND DISCUSSION

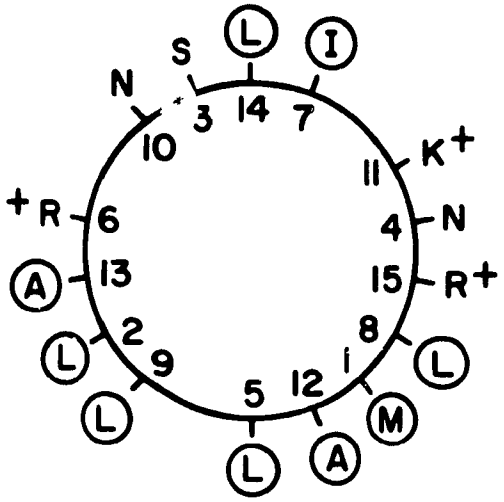
The pOCT signal sequence is comprised of a positively-charged, 32 amino acid segment located at the amino terminus of the precursor polypeptide (12,13) whose function and domain structure have been intensively investigated by both genetic and biophysical procedures (2,4,7,10,14-17). As is the case for other mitochondrial signal sequences (reviewed in ref. 18), the two structural features of the pOCT signal most likely to be important for efficient import of the precursor are charge and amphiphilicity. Mutagenesis of the rat and human pOCT signal sequence (14-17) has revealed that import (membrane translocation) is specified by the amino-terminal one-half to two-thirds

of the signal, whereas residues 22-30 contribute to a critical processing-site recognition domain that specifies correct cleavage of pOCT between amino acids 32 and 33 (16).

With regard to the amphiphilic domain within the pOCT signal sequence, both circular dichroism analysis (4) and helical wheel projections (2) (Fig. 1) predict that the greatest probability for such a structure resides within the first 15 amino acids of the signal peptide. In contrast to helical wheel projections which suggest that an amphiphilic helix can exist throughout this entire region (Fig. 1), however, actual quantitation of the induced helical content of a synthetic pOCT signal peptide bound to lipid membranes revealed a rather short domain of ~ 5 amino acids (4), or about one and a half turns of an α helix; presumably, stable formation of such a short helix is dependent upon its interaction with lipid. Thus, by re-analyzing the helical wheel projection of pOCT residues 1-15 employing a 5 amino acid 'window' to predict the most likely helical structure capable of stable interaction with an anionic lipid surface, residues 5-9 appeared to represent a likely candidate (Fig. 1). This short region satisfies the criteria both for a core α-helical domain (19) and for a strong hydrophobic moment (20,21); it contains 4 contiguous hydrophobic residues on the helical wheel to provide interaction with lipid and an arginine to provide a polar face and selectivity for anionic phospholipid headgroups (Fig. 1). This region, either autonomously or in conjunction with other areas of the pOCT signal, therefore, might be expected to exhibit membrane surface seeking properties.

To examine the functional significance of this putative lipid-binding domain, the leucine residues in pOCT at positions 5,8, and 9 were converted to alanine; the latter is equal to leucine in its

Fig. 1. Helical wheel projections of the potential amphiphilic region in the amino-terminal half of the signal sequence of pOCT. The one-letter amino acid code has been used; hydrophobic residues are circled. Asterisks denote leucine residues at positions 5,8, and 9 of pOCT which were replaced with alanine.


L
I
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3
14
7
10
K+
11
+R
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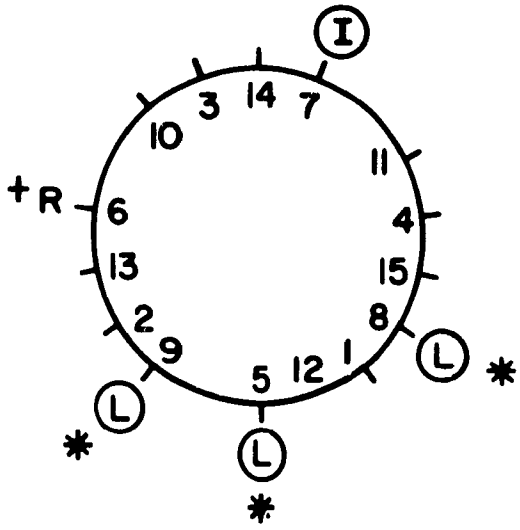
A

1-15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L
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L
*

5-9

compatibility with an α-helix (19) but reduces the hydropathic index of each of the three positions from 3.8 to 1.8 on the Kyte-Doolittle scale (22). For comparison, we analyzed the situation where all of the hydrophobic amino acids contained within pOCT residues 1-15 were also converted to alanine.

Construction of the two mutant derivatives of pOCT, designated pO-A6 and pO-A5,8,9, is described in Fig. 2; cassette mutagenesis was used, employing synthetic double-stranded oligonucleotides in which codons for alanine (GCA and GCT) replaced those for leucine and isoleucine in a XbaI-NcoI fragment of pOCT cDNA encoding amino acids 1-21 (Fig. 1). Efficient transcription - translation of the mutant cDNAs was achieved, yielding polypeptide products whose mobilities following SDS-PAGE were indistinguishable from that of pOCT.

Import of pOCT, pO-A6, and pO-A5,8,9 - The effects of alanine substitutions on the ability of the mutant precursors to be imported by mitochondria *in vitro* were assessed by comparing their initial rates of import to that of pOCT. The appearance of mature, processed product was used as a measure of translocation to the matrix compartment where the pOCT processing enzyme is located (23). Following a five-minute incubation with mitochondria, import and processing of pO-A6 was undetectable (not shown), whereas the apparent import of pO-A5,8,9 was reduced by 4- to 5-fold compared to wild-type pOCT, based on equivalent inputs of precursor polypeptide (Fig. 3, lower right panel). Quantitation of processed products was performed by laser densitometry of fluorograms. Even after 30 minutes of import incubation, uptake and processing of pO-A6 did not take place and, consequently, subsequent treatment with exogenous protease resulted in complete degradation of input precursor (Fig. 3, lower left panel, lanes 5 and 6). Presumably,

Fig. 2. Construction of pO-A6 and pO-A5,8,9. pSPO19, a pSP64 plasmid carrying a full-length copy of pOCT cDNA (10), was cut with XbaI and NcoI, and the deleted fragment replaced with either of two double-stranded synthetic oligonucleotides which converted the wild-type pOCT signal sequence to derivatives in which amino acid residues at positions 2,5,7,8,9, and 14 were replaced with alanine (pO-A6 mutant) or residues at positions 5,8, and 9 were replaced with alanine (pO-A5,8,9 mutant). The codons used for alanine were GCA and GCT. The plasmids were linearized with EcoRI prior to transcription-translation, as previously described (10). Dashed lines, wild-type and mutant signal sequences; arrow, signal peptide cleavage site; wide and narrow boxes, translated and untranslated cDNA sequences, respectively.

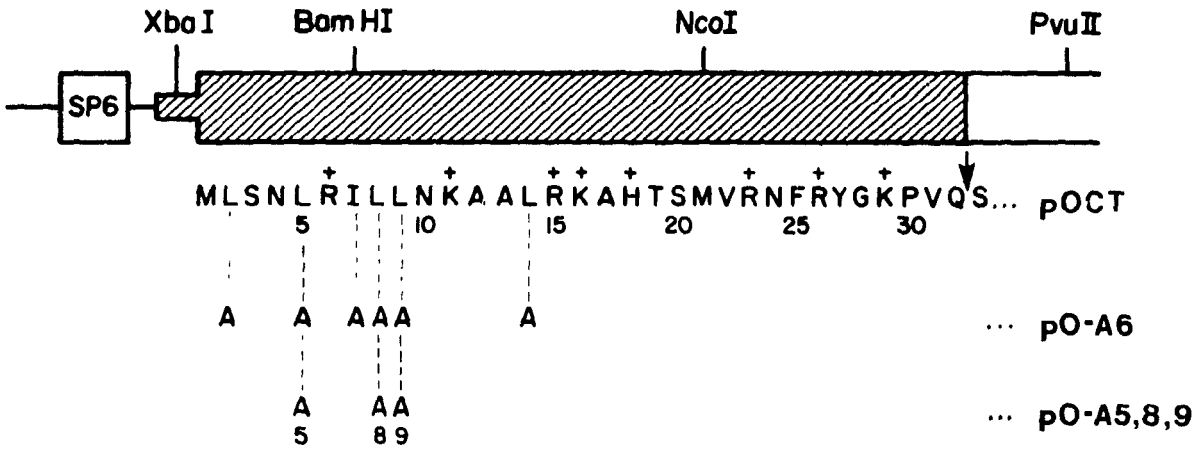
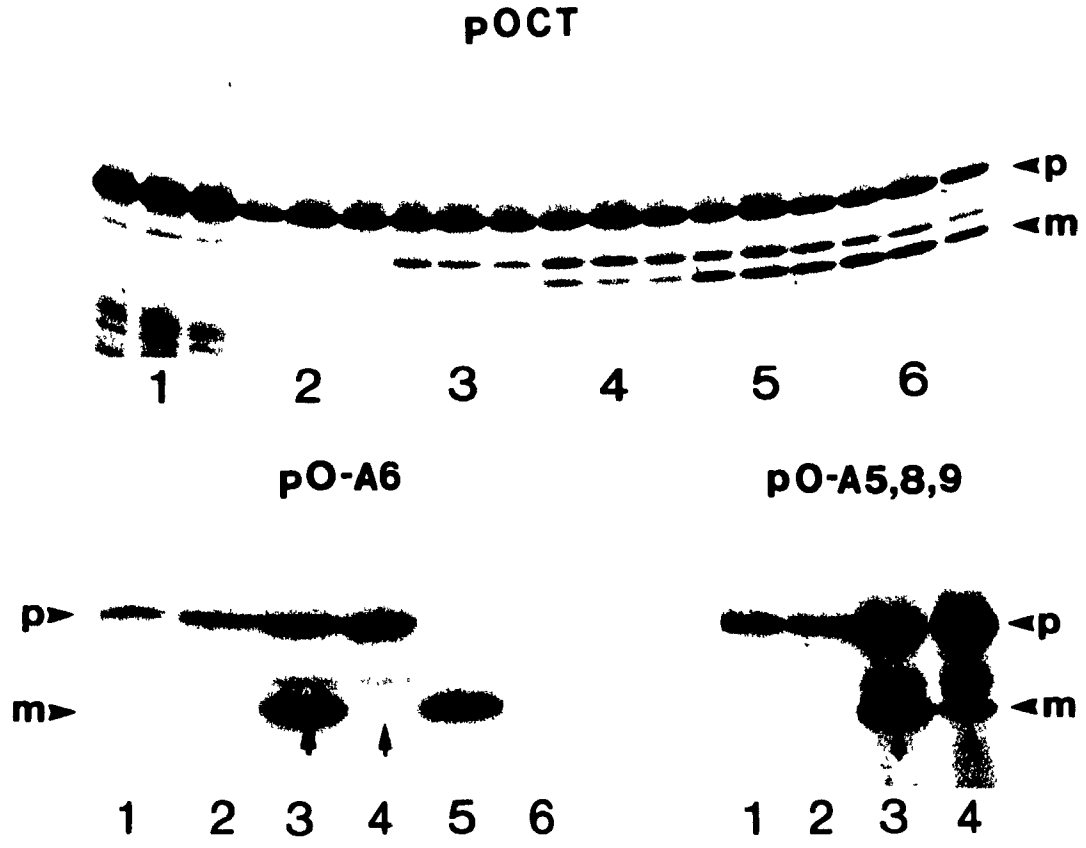

Xba I
Bam HI
Nco I
Pvu II
SP6
M L S N L R I L L N K A A L R K A H T S M V R N F R Y G K P V Q S...
pOCT
5
10
15
20
25
30
A A A A A A
... pO-A6
A A A
5 8 9
... pO-A5,8,9

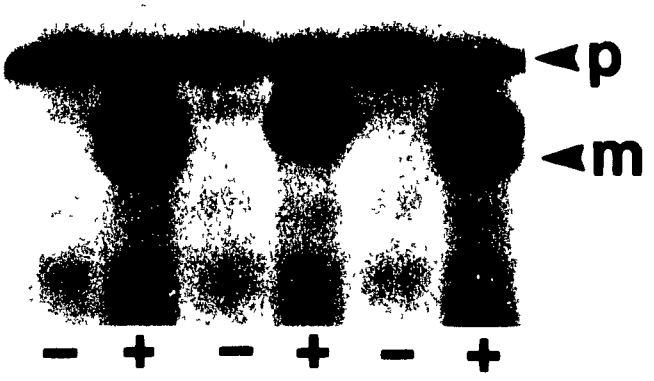
Fig. 3. Comparison of pOCT, pO-A6, and pO-A5,8,9 import into mitochondria. [^{35}S]Methionine-labeled reticulocyte lysate translation products (5 µl) were incubated with rat heart mitochondria (50 µg of protein; final volume=100 µl) at 30°C for varying periods of time, at which time further import was inhibited with carbonyl cyanide m-chlorophenyl hydrazone as described previously (10,16). Following centrifugation at 12,000 xg for 5 minutes at 4°, the mitochondrial pellets were visualized by 10% SDS-PAGE and fluorography. Top panel: Time course of pOCT import, in triplicate. Translation products were incubated without mitochondria (lane 1) or with mitochondria for 1 minute (lane 2), 2 min (lane 3), 3 min (lane 4), 4 min (lane 5), or 5 min (lane 6). Bottom left panel: Comparison of pOCT (lanes 1,3, and 5) and pO-A6 (lanes 2,4, and 6) import into mitochondria after 30 minutes. Translation products were incubated without mitochondria (lanes 1 and 2; 10% of input) or with mitochondria (lanes 3,4,5, and 6). Lanes 5 and 6: after import, the samples were digested with 150 µg/ml proteinase K for 20 minutes at 4°C, followed by the addition of phenylmethylsulfonylfluoride (2 mM final concentration). Bottom right panel: Comparison of pOCT (lanes 1 and 3) and pO-A5,8,9 (lanes 2 and 4) import after 5 minutes. Translation products were incubated without (lane 1 and 2; 10% of input) or with mitochondria (lane 3 and 4). The positions of precursor (p) and mature (m) ornithine carbamyl transferase are indicated.


pOCT
◄p
◄m
1
2
3
4
5
6
pO-A6
pO-A5,8,9
p►
m►
1 2 3 4 5 6
◄p
◄m
1 2 3 4

the pO-A6 mutations interfered with the ability of the pOCT signal to interact with one or more components of the import apparatus. In contrast, treatments with exogenous protease following import degraded completely the precursor forms of both pOCT and pO-A5,8,9 whereas mature products were resistant (not shown). The reduced rate of import of pO-A5,8,9, therefore, was likely due to a lower extent of transmembrane uptake rather than due to a reduced ability of pO-A5,8,9 to be processed within the matrix compartment.

In vitro processing - Among a large number of matrix proteins whose import and processing has been analyzed in vitro, rat liver pOCT is unusual in that two products are observed (24-26, and see fig. 3): a 36 kDa polypeptide representing the mature enzyme derived from correct processing of the precursor between amino acids 32 and 33 (16), and an intermediate size 37 kDa product resulting from cleavage between amino acids 24 and 25 (27). It was recently concluded that the 37 kDa polypeptide represents an intermediate product of 2-step processing (27). When pOCT, pO-A6, and pO-A5,8,9 were incubated with a mitochondrial matrix extract supplemented with Zn^{++}, processing to the 37 kDa and 36 kDa products was observed for pOCT and pO-A5,8,9 whereas pO-A6 was processed to the 37 kDa product, but not to bona fide mature enzyme (Fig. 4). The results with pO-A6 are difficult to reconcile with a 2-step processing mechanism; it assumes, for example, that the pO-A6 mutations can impair the second processing step even though the mutated region of the signal has been removed upon generation of the 37 kDa "intermediate". Alternatively, the results could be reconciled with a two-step processing mechanism if both steps are catalyzed by a single processing complex. Whatever the case, however, the results of Fig. 4 indicate that the overall conformation of the pO-A6 signal sequence is

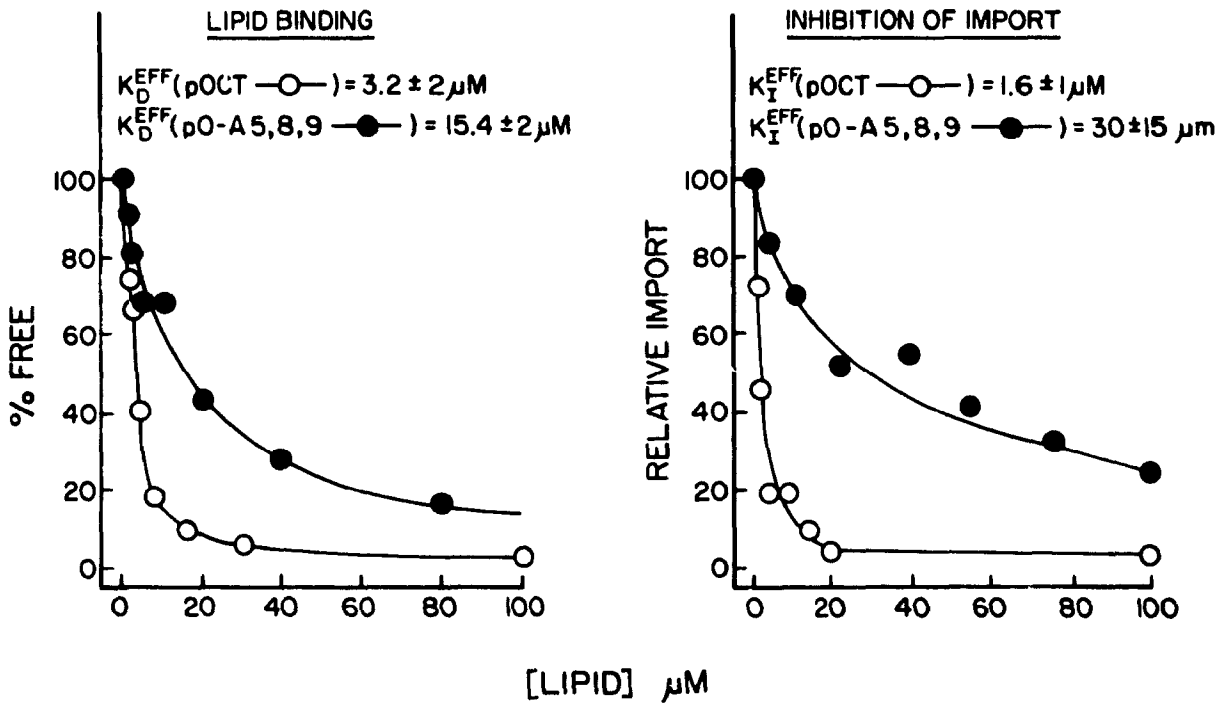
Fig. 4. Processing or pOCT (W), pO-A6 (A6), and pO-A5,8,9 (A5,8,9) by a mitochondrial matrix extract in vitro. Translation products (5 µl) were incubated for 30 min at 30° without (-) or with (+) a mitochondrial matrix extract supplemented with $ZnCl_2$ (0.1 mM final concentration) in a total volume of 30 µl, as described (23). Equivalent aliquots were resolved by 10% SDS-PAGE and radioactive products were visualized by fluorography. The positions of precursor (p) and mature (m) ornithine carbamyl transferase are indicated. The significance of the intermediate-size product is discussed in the text.


W
A6
A5,8,9
p
m
- + - + - +

sufficiently different from that of pOCT to impair correct processing, whereas the pO-A5,8,9 mutations alter the pOCT signal only to a degree that interferes with efficient import. In a number of separate experiments, no significant differences in the extent of in vitro processing of pOCT and pO-A5,8,9 to mature product were observed.

Lipid Affinities of pOCT and pO-A5,8,9 - Results shown in Fig. 5 (left panel) provide a direct demonstration that the predicted reduction in hydrophobicity as a consequence of substituting alanine for leucine at position 5,8, and 9 of pOCT lowers the affinity of the mutant precursor for anionic lipid vesicles. The vesicles employed for this assay contained PC and CL(9:1), as well as 2 mol% biotinated PE. The latter was included to facilitate rapid clustering of liposomes following the addition of avidin and, consequently, easy separation of the free and vesicle-bound populations of input precursor. Both pOCT and pO-A5,8,9 were stable under the conditions of incubation with liposomes (not shown), so that binding was conveniently assayed by the depletion by liposomes of the soluble pool of free precursor polypeptide (Fig. 5). Effective dissociation constants (K_D^{EFF}), calculated as the concentration of liposomes required to bind 50% of input precursor, were determined and gave average values of 3.2 μM for pOCT and 15.4 μM for pO-A5,8,9 (Fig. 5, left panel). Furthermore, as predicted from the dissociation constants, it was found that a lower concentration of liposome was required to inhibit import of pOCT compared to pO-A5,8,9. Inhibition was presumably caused by the liposomes binding the precursor and sequestering it away from the import apparatus. While import of wild-type pOCT was on average 50% inhibited by 1.6 μM liposomes, a vesicle concentration of 30 μM was required to inhibit the import of

Fig. 5. Comparison of liposome binding and liposome inhibition of import of pOCT (—o—) and pO-A5,8,9 (—●—). Translation products were incubated for 10 min at 30°C with varying concentrations of liposomes, prepared as described in Experimental Procedures (0-200 μM; 9:1=PC:CL, 2 mole% biotinated PE, in a total volume of 100 μl), in a buffer containing 80 mM KCl, 2 mM Mg acetate, and 20 mM Hepes, pH 7.4. The mixtures were then divided in half and incubated a further 10 min with either mitochondria in mitochondrial resuspension medium (10) (0.5 mg protein/ml, 100 μl final volume; standard import conditions), for analysis of import, or with mitochondrial resuspension medium alone, for the analysis of precursor binding to lipid. For lipid binding (left panel), liposomes were aggregated by the addition of avidin (1.6 mg avidin/μmole lipid) and subsequent incubation for 20 min at 4°C; liposomes and bound precursor were removed by centrifugation (12,000 g for 10 min), and the free precursor in the supernatant was recovered by precipitation in 12% trichloroacetic acid. For inhibition of import (right panel), mitochondria were recovered by centrifugation. In both cases, products were resolved by 10% SDS-PAGE followed by fluorography, and the amount of mature ornithine carbamyl transferase in the import assays or of free precursor remaining in the supernatant in the liposome binding assays was quantitated by laser densitometry of the fluorograms. Results were plotted versus lipid concentration, with values obtained in the absence of lipid given a relative value of 100. Effective dissociation constants (K_DEFF) for liposome binding and inhibition constants (K_IEFF) for import were calculated as the concentration of liposomes required to bind 50% of the precursor and inhibit import by 50%, respectively. Data points represent an average of 1 to 4 experiments.


LIPID BINDING
K_D^{EFF}(pOCT —O—) = 3.2 ± 2 μM
K_D^{EFF}(pO-A5,8,9 —●—) = 15.4 ± 2 μM
% FREE
100
80
60
40
20
0
0
20
40
60
80
100
INHIBITION OF IMPORT
K_I^{EFF}(pOCT —O—) = 1.6 ± 1 μM
K_I^{EFF}(pO-A5,8,9 —●—) = 30 ± 15 μm
RELATIVE IMPORT
[LIPID] μM

pO-A5,8,9 to the same extent (Fig. 5, right panel). The results for both liposome binding and liposome inhibition of import of pOCT and pO-A5,8,9 are consistent with the correlation between a reduced rate of import of pO-A5,8,9 (Fig. 3) and the decrease in the hydrophobic moment of its signal peptide in the region of amino acids 5-9 (Fig. 1), suggesting that the lipid affinity conferred by this domain in pOCT enhances the import efficiency of the precursor polypeptide.

CONCLUSIONS

Taken together, the findings of this study suggest that a short lipid-binding domain located toward the amino-terminus of rat liver pOCT serves to enhance the rate of precursor import into mitochondria in vitro. The results are consistent with our earlier suggestion (7) that such membrane surface-seeking domains within the signal sequence might contribute to efficient import by enhancing the diffusion of the precursor polypeptide to the import receptor and protein translocation apparatus of the organelle, as has been suggested for other surface-seeking entities (28). Additionally, of course, the amphiphilic domain might facilitate direct interactions between pOCT and one or more protein components of the import apparatus, or it might function to destabilize the tertiary structure of pOCT and consequently stimulate import as a consequence of aiding polypeptide unfolding (29). In view of the recent findings (30,31) that hsp70, a cytosolic protein that binds to exposed hydrophobic surfaces of polypeptides, stimulates protein translocation across membranes, including those of mitochondria, we are currently examining the possibility that the affinity of the pOCT signal sequence for lipid surfaces is modulated by cytosolic factors.

ACKNOWLEDGEMENT - We are grateful to Dr. Stephen Randall for discussions and comments on the manuscript.

[1]**ABBREVIATIONS** - pOCT, pre-ornithine carbamyl transferase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PC, phosphatidylcholine; CL, cardiolipin; PE, phosphatidylethanolamine.

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CHAPTER 4

Import of Proteins into Mitochondria: The Site of Polypeptide Unfolding

ABSTRACT

To examine the conformation of precursor proteins at various points on the mitochondrial import pathway, measurements were made of the sensitivity to exogenous proteases of two hybrid proteins, comprised of the signal sequence of preornithine carbamyltransferase fused either to mouse dihydrofolate reductase (pODHFR) or to bacterial chloramphenicol acetyltransferase (pOCAT). As well, the intrinsic activity of pOCAT was easily measurable from amounts of the precursor obtained by reticulocyte lysate translation. Using these two independent assays for protein conformation, we have found that pOCAT and pODHFR exhibit a near-native structure after synthesis in reticulocyte lysate, and retain this folded conformation upon binding to liposomes containing negatively charged phospholipids, and upon binding to the surface of mitochondria lacking an electrochemical potential. A translocation intermediate of pOCAT has been detected in association with a submitochondrial fraction exhibiting a density intermediate between the outer and the inner mitochondrial membrane. Formation of this intermediate requires an intact signal sequence, a protease-sensitive component of the outer mitochondrial membrane, and ATP. It was found to have a negligible intrinsic activity and an increased susceptibility to trypsin compared to

cytosolic precursor, suggesting that it had at least partially unfolded. Taken together, the results are consistent with a model in which the precursor protein unfolds as a consequence of engaging the mitochondrial translocation apparatus.

INTRODUCTION

The concept that precursor proteins must at least partially unfold in order to be imported into mitochondria has arisen mainly from the findings that precursors whose tertiary structure had been stabilized either by the binding of high-affinity ligands (Eilers and Schatz, 1986; Chen and Douglas, 1987a; Kaput et al., 1989) or by intrapolypeptide disulfide bridges (Vestweber and Schatz, 1988b) were inefficiently translocated into the organelle and, conversely, from the observation that destabilization of tertiary structure, either by chemical denaturation (Eilers et al. 1988) or by internal mutations (Vestweber and Schatz, 1988c), led to enhanced rates of import. Such conclusions are consistent with the detection of a precursor polypeptide that was trapped as a mitochondrial translocation intermediate spanning both the outer and inner membranes simultaneously, originally shown by Schleyer and Neupert (1985). The mechanism and possible locations on the import pathway of polypeptide unfolding, however, remain unclear. The demonstration that ATP is required for import (Chen and Douglas, 1987b; Eilers et al., 1987; Pfanner et al., 1987) and that heat-shock proteins stimulate the import of mitochondrial proteins (Murakami et al., 1988; Deshaies et al., 1988; Randall and

Shore 1989), is consistent with the existence of an ATP-dependent 'unfoldase' in the cytosol, originally postulated by Rothman and Kornberg (1986). On the other hand, Endo et al. (1989) found that a hybrid protein of cytochrome oxidase subunit IV signal sequence fused to dihydrofolate reductase (CoxIV-DHFR) dramatically increased its sensitivity to trypsin in an ATP-dependent manner upon binding to liposomes or to the surface of intact mitochondria.

In this study we have used two independent assays to examine the conformation of hybrid precursors at various steps on the import pathway. One assay measures the intrinsic activity associated with a precursor polypeptide, the other measures the intrinsic sensitivity of the precursor to exogenous protease. Unfolding of the precursor would be expected to be accompanied by enhanced protease-sensitivity (Randall and Hardy, 1986; Endo et al. 1989) and by a loss in intrinsic activity. To this end, we have employed two marker proteins: mouse dihydrofolate reductase (DHFR) and bacterial chloramphenicol acetyl transferase (CAT). Both contain a core structure that is highly resistant to protease, unless the polypeptides are extensively denatured, and the intrinsic activity of CAT is easily detected employing even the exceedingly low levels of enzyme generated by in vitro translation. These properties are retained when the proteins are fused to a

mitochondrial signal sequence, in this case the matrix-targeting domain of pre-ornithine carbamyl transferase. DHFR is monomeric in structure, whereas the type I CAT employed must be a trimer to exhibit enzyme activity (Leslie et al. 1988); the trimer structure of CAT is very stable, to the extent that monomerization of the complex, by various methods, is always accompanied by unfolding of the constituent polypeptides (Shaw 1983).

Here, we present evidence that the bulk population of these hybrid precursors remains in an essentially native conformation prior to precursor engagement of the import apparatus. Unfolded polypeptide translocation intermediates, the formation of which requires ATP, an intact signal sequence, and a protease-sensitive component of the outer mitochondrial membrane, have been detected in association with submitochondrial fractions containing putative contact sites.

EXPERIMENTAL PROCEDURES

Materials:

Egg phosphatidylcholine and bovine heart cardiolipin were obtained from Avanti Polar Lipids. Cardiolipin was routinely Folch-washed, as described by Papahadjopoulos and Miller (1967), to remove possible divalent cation contaminants. Lipids were stored in chloroform solutions at -70°C under nitrogen. Rat heart and liver were obtained from male Sprague-Dawley rats. Potato apyrase (Grade VIII) was obtained from Sigma Chemical Company (St. Louis, MO). Chloramphenicol acetyl transferase (E. coli) was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden).

General:

Earlier publications describe the isolation of rat heart and liver mitochondria, *in vitro* transcription of pSP64 derivatives, translation in rabbit reticulocyte lysate, import and processing of precursor proteins by mitochondria, SDS-PAGE, and fluorography (Argan *et al.* 1983; Shore *et al.* 1983; and Nguyen *et al.* 1986). Additional details are given in the figure legends.

Construction of plasmids:

pSV_2CAT (Gorman et al. 1982) was linearized at its unique HindIII site, and restricted with BamHI to yield a 1032 bp fragment that was inserted between these sites in pSP64 to create pSPCAT. This plasmid was linearized with HindIII, blunt-ended by treatment with Klenow fragment, and restricted with SphI. A 548 bp SphI - PvuII restriction fragment, providing pOCT amino acids 1-36, was then ligated between the SphI and blunted HindIII sites of pSPCAT to generate pSP(pOCAT). Prior to in vitro transcription, pSP(pOCAT) was linearized with SstI.

A 650 bp TaqI - BglII partial restriction fragment from pSV_2DHFR (Subramani et al. 1981) was inserted between the AccI and BamHI sites of pSP64. PstI linearization of this plasmid at a site just 5' to the AccI insertion site, followed by T_4-polymerase-mediated blunt-ending, and restriction at the unique SphI site yielded a major fragment, which, when ligated to the 548 bp SphI - PvuII fragment described above, formed pSP(pODHFR). Prior to in vitro transcription, pSP(pODHFR) was linearized with Eco RI.

Preparation of Liposomes:

Lipids were dried down from stock solutions under nitrogen and dispersed in 80 mM KCl, 20 mM Hepes, and 2 mM Mg acetate, at pH 7.4, by brief vortexing followed by bath

sonication for 5 min. Lipid concentrations were determined as described previously (Silvius and Gagné, 1984).

Preparation of Submitochondrial Vesicles:

Submitochondrial vesicles were prepared according to Ohlendieck et al. (1986). Translation products (300 ul) were incubated with mitochondria (1 mg mitochondrial protein; final volume 2 mls) under various conditions for 15 min (see Figure Legends). After centrifugation at 12,000 x g for 5 min, the mitochondrial pellets were resuspended and, together with 9 mg excess mitochondria, were incubated in a final volume of 2 mls swelling buffer (10 mM phosphate, pH 7.4). After incubating for 20 min at 4°C, 655 ul of 50 % wt/wt sucrose in 10 mM phosphate buffer, pH 7.4, were added followed by another 20 min incubation at 4°C. The mitochondria were then sonicated for 6 x 15 s with a Sonic Dismembrator at power setting 60 (Artek systems corporation) and centrifuged at 9,000 rpm for 15 min in a Sorvall SS-34 rotor. The supernatant was layered over a 34 ml linear sucrose density gradient (15-50 % wt/wt sucrose in 10 mM phosphate, pH 7.4) and centrifuged for 17 h at 27,000 rpm in a SW-28 Beckman rotor. Subsequently, the gradient was divided into about 30 fractions of 1.2 mls.

RESULTS

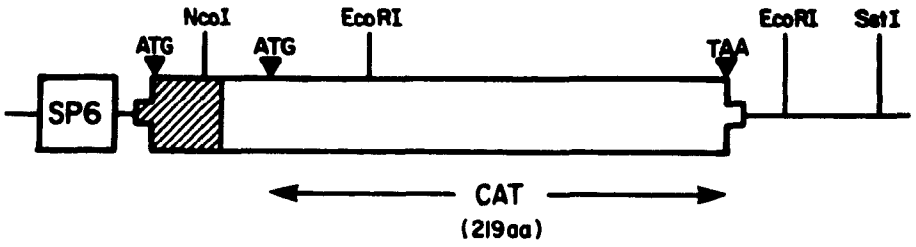
Fusion Proteins and Assays of Polypeptide Unfolding:

Two fusion proteins, pOCAT and pODHFR, were constructed by fusing the amino terminus of pOCT (the 32-amino acid signal sequence plus 4 amino acids of the mature protein) to either mouse DHFR or bacterial CAT (Fig. 1 and see Experimental procedures). The relevant cDNAs were inserted into the pSP64 vector and expressed by in vitro transcription-translation. In both cases, a single translation product was produced which, upon incubation with purified mitochondria from rat heart, was imported and processed in a time- and ATP-dependent manner (Fig. 2 and data not shown). Subsequent treatment of import reaction mixtures with exogenous protease resulted in the conversion of unincorporated, full-length precursor to a protease-resistant core fragment; in the case of pOCAT, a portion of this core product co-sedimented with mitochondria upon recovery of the organelle by centrifugation (Fig. 2, Panel B), while for pODHFR the core remained soluble (not shown).

The protease digestion of pODHFR and pOCAT to protease-resistant cores is the basis of an assay that measures the loosening of polypeptide tertiary structure (i.e., 'unfolding'), allowing the protease to gain access to otherwise buried sites in the protein (Randall and

Fig. 1: Construction of pOCAT and pODHFR. pOCAT consists of pOCT amino acids 1-36 fused to the entire 219 amino acid chloramphenicol acetyltransferase reading frame with an additional twelve codons upstream of the CAT initiator methionine, not normally expressed, resulting in a total of 267 amino acids. pODHFR consists of pOCT amino acids 1-36 fused to amino acids 3-186 of mouse dihydrofolate reductase, with a single glycine residue introduced between the two regions in the manipulations. Details of the construction can be found in Materials and Methods.

pO-CAT



pO-DHFR

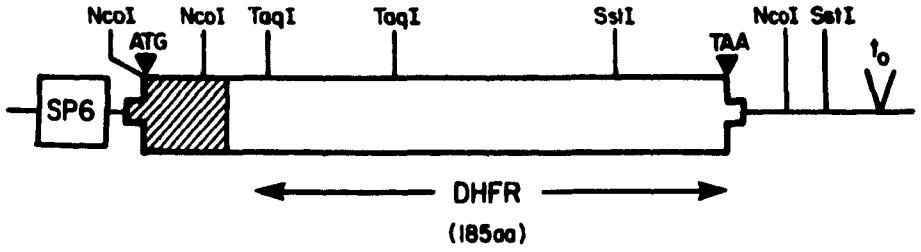
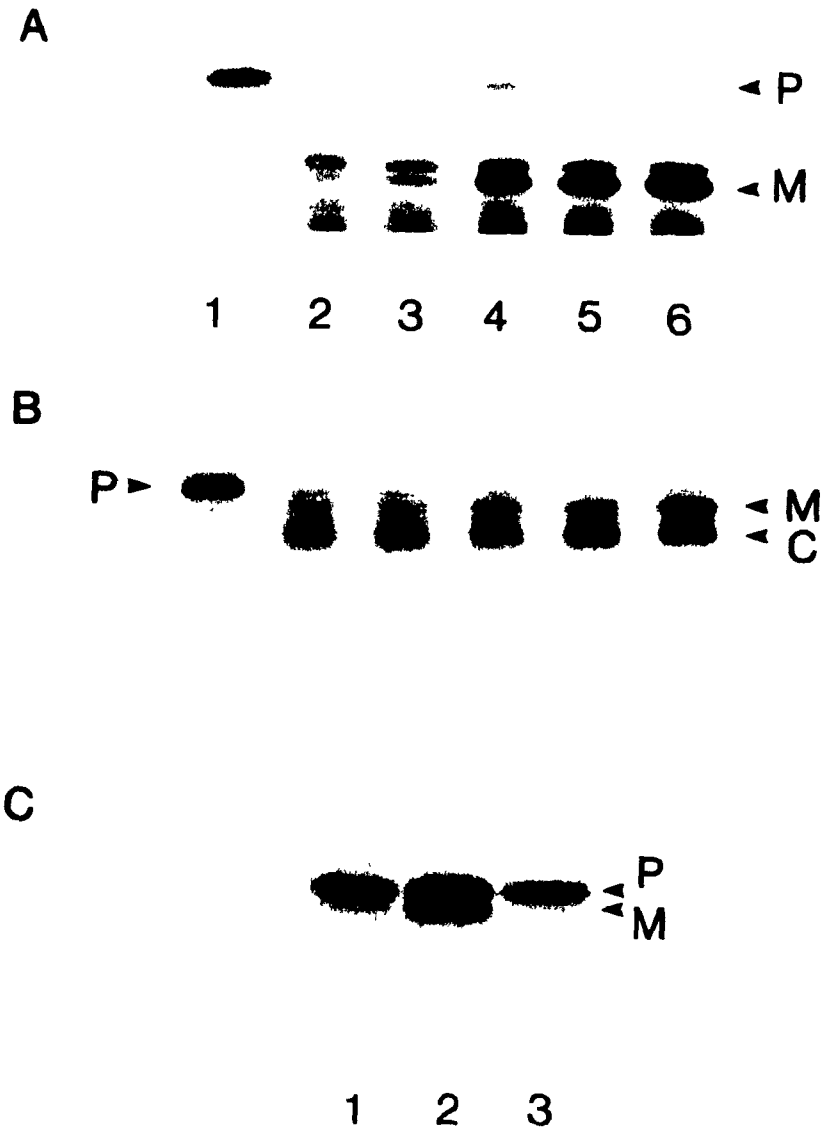


Fig. 2: Import of pOCAT and pODHFR: time course and ATP requirement. [^{35}S]Methionine-labeled reticulocyte lysate translation products (4 ul) were incubated with rat heart mitochondria (50 ug of protein; final volume 100 ul) at 30°C for varying periods of time, and import was terminated by the addition of carbonyl cyanide m-chlorophenylhydrazone, as described previously (Nguyen et al. 1986). Following centrifugation at 12,000 x g for 3 min at 4°C, the mitochondrial pellets were visualized by 12% SDS-PAGE and fluorography. In Panels A (pODHFR) and B (pOCAT), precursors were incubated without mitochondria (lanes 1, 10% input) or with mitochondria for 1 min (lanes 2), 2 min (lanes 3), 4 min (lanes 4), 8 min (lanes 5), or 16 min (lanes 6). After import, the samples were digested with 150 ug/ml proteinase K for 15 min at 4°C, followed by the addition of phenylmethylsulfonyl fluoride (2 mM final concentration). In Panel C, pOCAT was incubated without mitochondria (lane 1, 10% input), with mitochondria (lane 2), or with mitochondria depleted of ATP by preincubation with 4 U/ml apyrase for 10 min at 4°C (lane 3). The positions of precursor (p), mature (m), and core (c) proteins are indicated.


A
P
M
1 2 3 4 5 6
B
P
M
C
C
P
M
1 2 3

Hardy, 1986). In addition to the protease-sensitivity as a probe for 'folded' states, an independent measure of polypeptide unfolding is afforded by pOCAT due to the extremely high sensitivity of the CAT enzyme activity assay. As shown in Figs. 3 and 4, intrinsic activities associated with the in vitro translation products of pOCAT and CAT were readily detectable. Furthermore, both products exhibited K_m values, with respect to chloramphenicol, that are similar to those reported for the native enzyme obtained from bacteria (Shaw 1983), indicating that the presence of a signal sequence has little effect on the ability of CAT to bind chloramphenicol. Alterations in the native structure (e.g. unfolding) of pOCAT during import of the polypeptide into mitochondria, therefore, would be expected to be accompanied by a loss of intrinsic activity.

Importantly, the concentration of substrates employed in these enzyme assays (1.2 mM acetylCoA and up to 38 uM chloramphenicol) did not inhibit import of pOCAT into mitochondria in vitro (not shown). This is in contrast to the situation for pODHFR (not shown) and pCoxIV-DHFR (Eilers and Schatz, 1986) in which the high affinity ligand, methotrexate, is a potent inhibitor of precursor import as a consequence of its ability to stabilize the tertiary structure of the DHFR moiety. The relatively weak affinity of pOCAT for its substrates, therefore, is

Fig. 3: Lineweaver-Burke plots for pOCAT and CAT intrinsic enzyme activity. Reticulocyte lysate (1 ul), containing equivalent amounts of freshly synthesized pOCAT (•) and CAT (▫) were incubated for 5 minutes at 37°C with 1.2 mM acetyl CoA and 0-38 uM [^{14}C]chloramphenicol in standard import buffer (final volume 100 ul)(Argan and Shore, 1985). The reaction was stopped by ethyl acetate extraction and products were separated by thin layer chromatography in chloroform:methanol (95:5). The amount of mono-acetylated [^{14}C]chloramphenicol formed was quantitated by liquid scintillation counting and the velocity of enzyme activity was calculated as pmoles chloramphenicol acetylated/min. The data represents a typical experiment and has been replicated within 5% error.

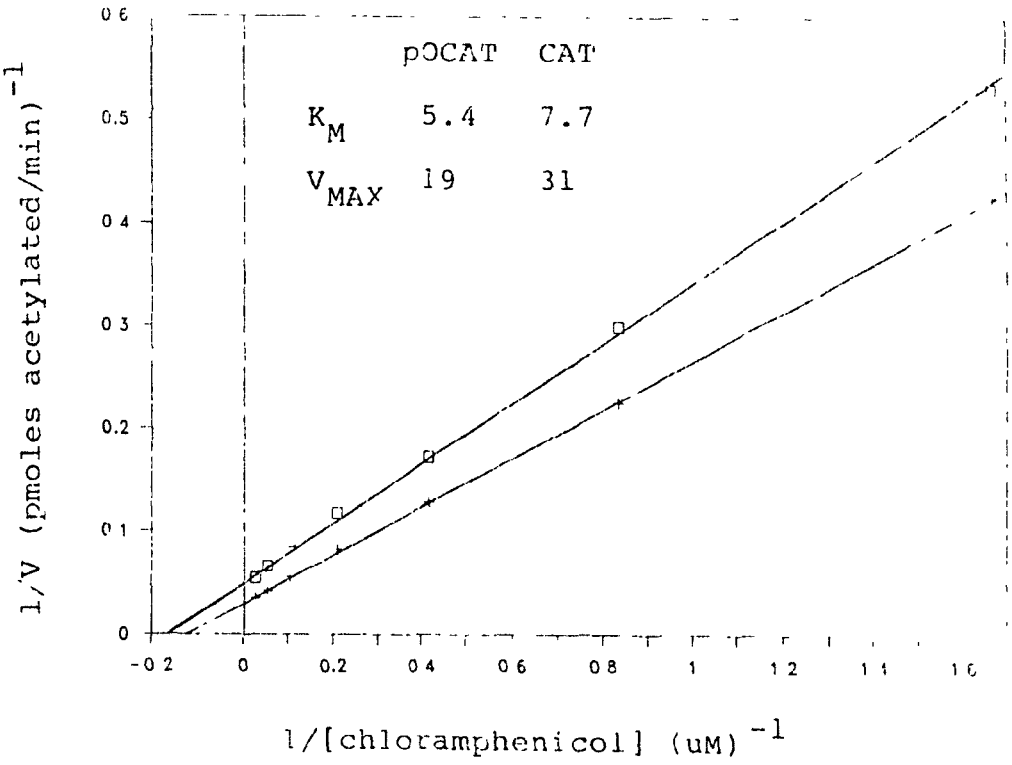
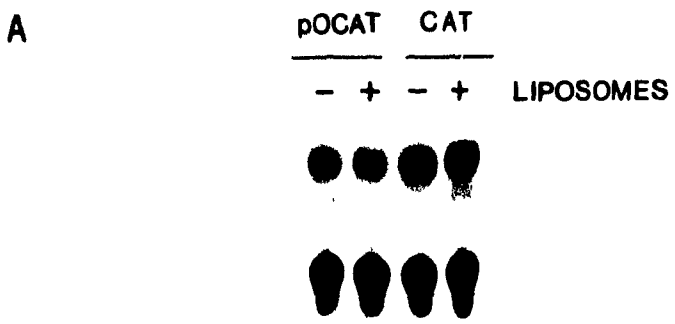
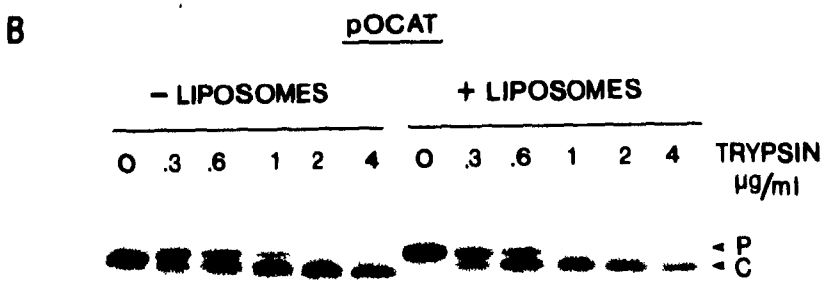
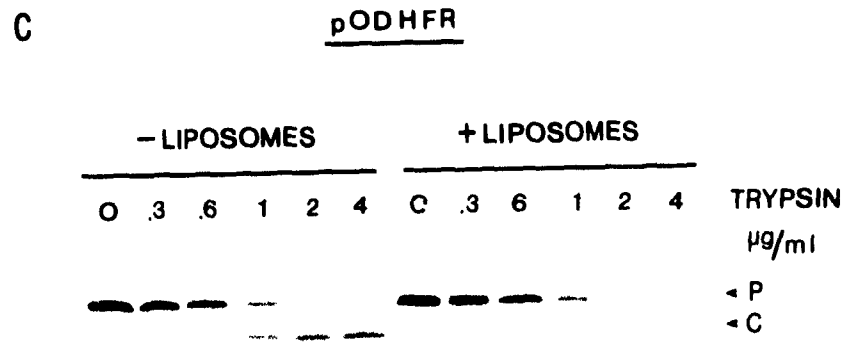

pOCAT CAT
K_M 5.4 7.7
V_{MAX} 19 31
1/V (pmoles acetylated/min)$^{-1}$
1/[chloramphenicol] (uM)$^{-1}$
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6

Fig. 4: Intrinsic activity and trypsin sensitivity of precursor proteins upon binding to liposomes. Translation products (1 ul) were incubated with or without liposomes, prepared as described in Materials and Methods (20 uM; 9:1=phosphatidylcholine:cardiolipin; final volume 100 ul), for 10 min at 30°C, under standard import conditions, and then assayed for enzyme activity or for protease sensitivity. In Panel A, 4.6 uM [^{14}C]chloramphenicol and 1 mM acetyl CoA were added to incubations of CAT and pOCAT and the enzyme assay was carried out as described in Fig. 3. In Panels B and C, pOCAT and pODHFR, respectively, were assayed for protease sensitivity by incubating with varying concentrations of trypsin (0-4 ug/ml) for 10 min at 4°C, followed by the addition of 2 mM phenylmethylsulfonylfluoride. The products were precipitated with 12% trichloroacetic acid and visualized by 12% SDS-PAGE and fluorography. The positions of precursor (p) and core (c) proteins are indicated. These results were replicated at least three times.


A
pOCAT
CAT
– + – +
LIPOSOMES


B
pOCAT
– LIPOSOMES
+ LIPOSOMES
0 .3 .6 1 2 4 0 .3 .6 1 2 4
TRYPSIN
µg/ml
P
C


C
pODHFR
– LIPOSOMES
+ LIPOSOMES
0 .3 .6 1 2 4 0 .3 6 1 2 4
TRYPSIN
µg/ml
P
C

insufficient to 'freeze' the protein into its native structure thereby preventing a potential unfolding machinery from effectively acting on the protein.

Conformation of Soluble pOCAT and pODHFR in Reticulocyte Lysate:

In order to probe the conformations of pOCAT and pODHFR immediately following their release from the ribosome, the newly-synthesized precursors were obtained by translation in reticulocyte lysate and examined for protease-sensitivity (pOCAT and pODHFR) and enzyme activity (pOCAT). As illustrated in Fig. 2, both precursors were competent for import, yet both also exhibited protease-resistant cores (Fig. 4) and pOCAT was enzymatically active (Figs. 3 and 4). In Fig. 4, the concentrations of exogenous protease that were employed to generate core products were from 0.3 to 4 ug trypsin/ ml. The core generated for both pOCAT and CAT was resistant up to at least 60 ug trypsin/ ml and 200 ug proteinase K/ ml (not shown). These concentrations were sufficient to degrade the pOCT signal sequence attached to either protein, but did not degrade the intrinsic protease-resistant cores of the mature region. Furthermore, both pOCAT and CAT, which exhibited relatively similar intrinsic activities in reticulocyte lysate, exhibited similar resistance to digestion by either trypsin or

proteinase K (not shown). We conclude, therefore, that the bulk population of pODHFR and pOCAT molecules in reticulocyte lysate were retained in a tightly-folded state, with the mature portion of the molecule similar to the structure of the native protein while the signal sequence remains at the surface of the molecule, readily accessible to low concentrations of trypsin. Interestingly, depleting lysate of ATP (to the extent that import was inhibited, Fig. 2, panel c), or increasing ATP concentrations up to 10 mM, had no effect on the protease-susceptibilities (not shown) or intrinsic activities (Table 1) measured here. Similarly, no decrease in intrinsic activity of pOCAT was observed when the concentration of reticulocyte lysate in these assays was increased 10-fold (not shown). Thus, while it cannot be ruled out that a proportion of the total precursor population was unfolded, and was too small to be detected by the present assays, the data suggest that the steady-state condition for pOCAT and pODHFR in reticulocyte lysate favours a native conformation.

Conformation of Membrane-bound pOCAT and pODHFR in Reticulocyte Lysate:

If the first interaction of the signal sequence with mitochondria is via the lipids of the outer membrane (Skerjanc et al. 1987; Skerjanc et al. 1988), it is

Table 1 Effect of ATP on CAT and pOCAT Intrinsic CAT Activity

	Intrinsic Activity (% control)		
		Translated	Purified
Condition	pOCAT	CAT	CAT
Control (+apyrase)	100	100	100
5 mM ATP	82	76	72
10 mM ATP	73	65	-

The effect of ATP was examined by incubating translation products with either ATP or 3 U/ml apyrase, for the control, for 30 min at 30°C followed by addition of enzyme substrates and incubation for 10 min at 37°C. The intrinsic activity of the ATP-depleted pOCAT and CAT controls were 49 and 108 pmoles chloramphenicol acetylated/ min/ arbitrary densitometry unit pOCAT and CAT, respectively. CAT from E. coli, was treated similarly to the translated enzyme and the control was found to have an activity of 83 pmoles chloramphenicol acetylated/ min / 0.2 ul enzyme. Enzymatic activity was quantitated as described in Fig 3. Aliquots of each assay were subjected to SDS-PAGE and the amount of enzyme was quantitated by laser densitometry.

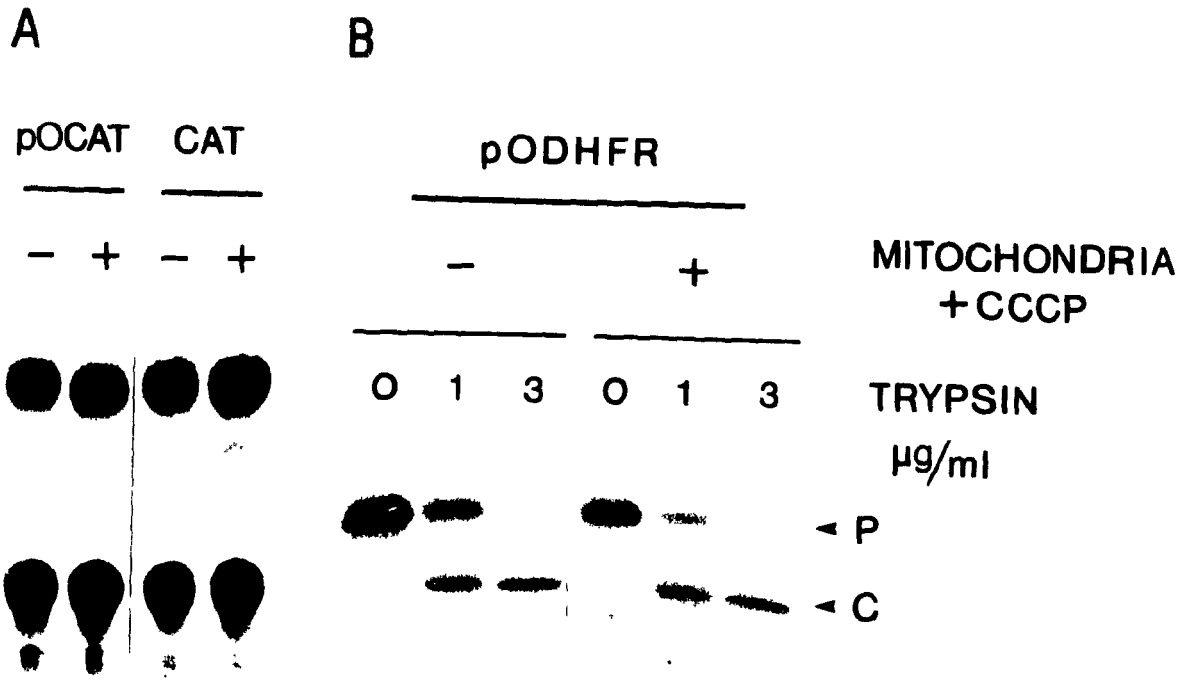
possible that such an amphiphilic surface might unfold the precursors, as has been demonstrated for pCOXIV-DHFR (Endo et al. 1989). POCAT and pODHFR were found to bind liposomes (9 : 1 = phosphatidylcholine : cardiolipin) with an effective dissociation constant (Skerjanc et al. 1988) of about 4 uM, while CAT exhibited negligible binding. Under conditions where the precursors were effectively all bound to liposomes (20 uM lipid), no significant changes were observed in the intrinsic activity of pOCAT and CAT (Fig. 4A), in the trypsin sensitivity of pOCAT (Fig. 4B) and pODHFR (Fig. 4C), and in the sensitivity to proteinase K of pOCAT and pODHFR (data not shown). The trypsin concentrations used in Fig. 4 were up to 4 ug/ml, but the resistance of core products in all cases extended at least up to 60 ug/ml (the highest concentration examined) (not shown). Thus, the lipid surface of vesicles alone was not sufficient to unfold pOCAT and pODHFR.

The results with artificial liposomes were extended as well to include the membrane surface of intact mitochondria. It may be, for example, that the composition of lipid employed for liposomes is deficient in an essential lipid component required for membrane-dependent polypeptide unfolding. Precursors were incubated with rat heart mitochondria treated with carbonyl cyanide m-chlorophenylhydrazone, to dissipate the potential across the inner membrane. Under these

conditions import does not occur, but the precursor still binds to the outer membrane. Under conditions where at least half of the precursor was bound to the mitochondrial surface in the presence of reticulocyte lysate, we again found no indication of unfolding, either by intrinsic activity measurements (Fig. 5A) or by examination of protease sensitivity (Fig 5B; pOCAT protease sensitivity not shown). Further, incubating the precursor bound to mitochondria in the presence of high salt (1.0 M KCl) and pelleting through a sucrose cushion did not release precursor (not shown), indicating that the precursor, even though it was folded, also exhibited relatively tight binding. In the case of pODHFR and pOCAT, therefore, such binding was presumably mediated by the signal sequence rather than by hydrophobic regions of the rest of the molecule that had been exposed as a consequence of polypeptide unfolding.

While the majority of the pOCAT and pODHFR population binds to the surface of mitochondria, in a potential-independent manner, in vitro, only a small amount of the bound precursor is actually imported. Thus, most of the bound precursor is probably interacting with non-specific sites. If unfolding of only a small proportion of bound precursor was mediated via its interaction with a specific mitochondrial protein, therefore, such an unfolded intermediate would be detectable only following

Fig. 5: Trypsin sensitivity of pODHFR and intrinsic activity of pOCAT and CAT upon binding to the surface of mitochondria. Translation products were incubated with 0.5 mg/ml mitochondria pretreated with 1 uM carbonyl cyanide m-chlorophenyl hydrazone, for 10 min at 30°C, under standard import conditions. In Panel A, the samples containing CAT and pOCAT were then assayed for enzyme activity as described in Figs. 3 and 4. In Panel B, samples containing pODHFR were assayed for protease sensitivity as described in Fig. 4. The positions of precursor (p) and core (c) proteins are indicated.

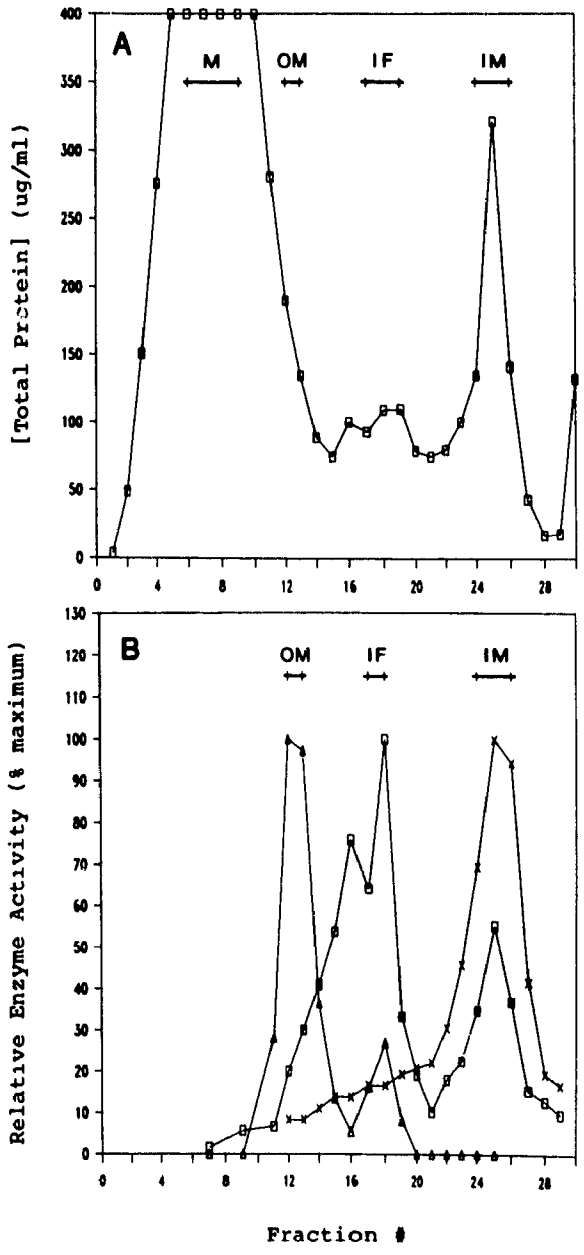

A
B
pOCAT
CAT
pODHFR
– + – +
–
+
MITOCHONDRIA
+CCCP
0 1 3 0 1 3
TRYPSIN
µg/ml
P
C

subfractionation of the organelle.

Identification of an Unfolded Translocation Intermediate:

In view of the fact that the bulk population of pOCAT and pODHFR located either in the cytosol (reticulocyte lysate) or bound to non-specific sites on the surface of mitochondria was in a folded, near-native conformation, unfolding during import, if it occurs, is likely a consequence of the precursor engaging either the receptor or some subsequent component of the import apparatus. Such an unfolded intermediate would be expected to exhibit three characteristics: association with contact points between the outer and inner mitochondrial membrane (Schleyer and Neupert, 1985), dependence for its formation on a trypsin-sensitive component (i.e., receptor) on the surface of the organelle (Gasser et al., 1982, Argan et al., 1983; Zwizinski et al., 1984), and a requirement for ATP (Chen and Douglas, 1987b; Eilers et al., 1987; Pfanner et al., 1987). Submitochondrial vesicles were prepared, following the method of Ohlendieck et al. (1986), and separated by sucrose velocity gradient centrifugation. Characterization of the protein profile and marker enzymes in the sucrose gradient is shown in Fig. 6. A major fraction of the total mitochondrial protein was soluble, presumably deriving from the matrix compartment, and was recovered at the top of the gradient (Fig. 6A). The

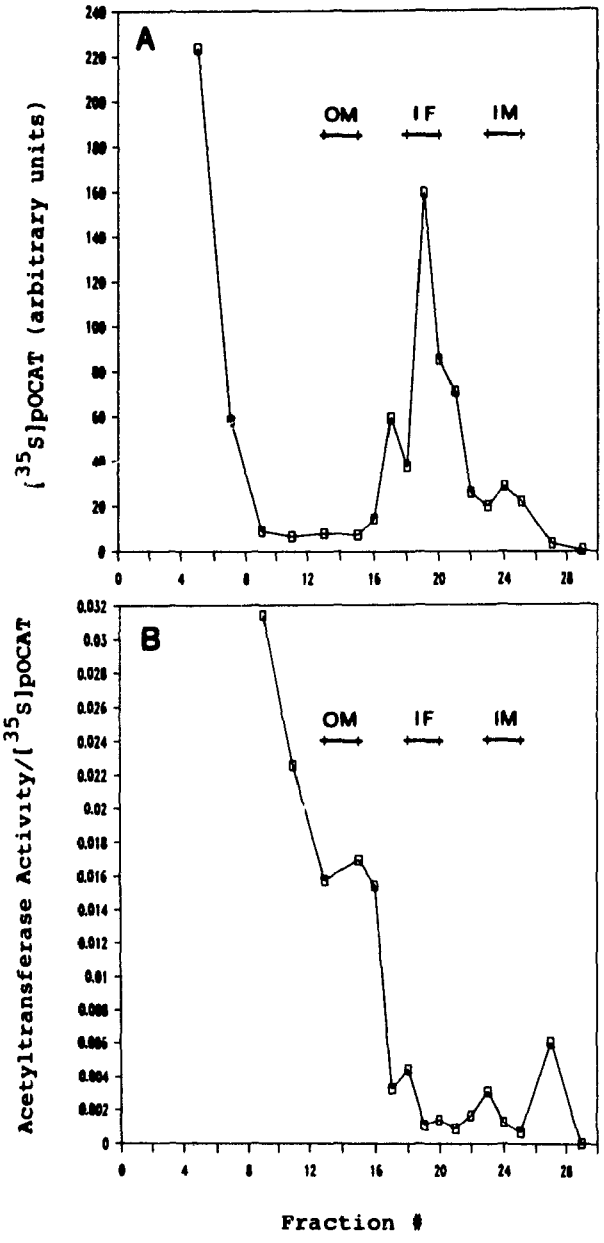
Fig. 6: Sucrose gradient fractionation of submitochondrial vesicles. Submitochondrial vesicles were prepared as described in Materials and Methods and layered onto a 15-50 % wt/wt linear sucrose gradient in 10 mM phosphate buffer, pH 7.4. After ultra-centrifugation for 17 h at 27,000 rpm in a SW-28 rotor at 4°C, fractions of 1.2 ml were collected from top to bottom of the gradient. In Panel A, 50 ul of each fraction were analyzed for total protein by the Bio-Rad protein assay (Bio-Rad Chemical Division, Richmond, California). In Panel B, fractions of the sucrose gradient were assayed for azide-sensitive ATPase activity (×) by a pyruvate kinase/lactic dehydrogenase coupled assay (Josephson and Cantley, 1977), for cytochrome c oxidase activity (□) following the method of Smith and Stotz (1949) and for monoamine oxidase activity (△) by the method of Weissbach et al. (1960). The fraction with the highest activity for each enzyme was arbitrarily given a value of 100. This corresponds to 520 nmoles cytochrome c oxidized/min/ml fraction, 80 nmoles kynuramine oxidized/min/ml fraction, and 200 nmoles NADH dehydrogenated/min/ml fraction for cytochrome c oxidase, monoamine oxidase, and ATPase, respectively. The identified fractions are marked as soluble protein (M), outer membrane (OM), intermediate density (IF), and inner membrane (IM) fractions.


A
M
OM
IF
IM
[Total Protein] (ug/ml)
B
OM
IF
IM
Relative Enzyme Activity (% maximum)
Fraction #

marker enzymes migrated as distinct peaks, with monoamine oxidase marking predominantly the outer membrane (OM), azide-sensitive ATPase marking the inner membrane (IM), and cytochrome c oxidase migrating both with the inner membrane and at an intermediate density, designated the intermediate fraction (IF) (Fig. 6B). The latter fraction also contained a small, but measurable amount of outer membrane (Fig. 6B). The formation of an intermediate density fraction has been widely interpreted to contain contact points (Schleyer and Neupert, 1985; Ohlendieck et al., 1986) and has been shown to co-migrate with translocation intermediates under various conditions (Schleyer and Neupert, 1985).

When pOCAT was imported for 15 min at 30°C prior to the isolation of mitochondria and subsequent formation of submitochondrial vesicles, it was found that most of the precursor protein that had associated with the organelle remained at the top of the sucrose gradient (Fig. 7A). This precursor fraction was degraded to a protease-insensitive core but was presumably derived from precursor that had been imported and processed in the matrix, as well as precursor that was dislodged from the surface of the organelle as a consequence of sonication (not shown). However, a small percentage of precursor, that was full-length pOCAT (as judged by SDS-PAGE, not shown), entered the gradient and co-migrated mostly with the intermediate

Fig. 7: Distribution and intrinsic activity of pOCAT. [^{35}S]methionine-labeled pOCAT (150 ul translation products) was incubated with liver mitochondria (1 mg mitochondrial protein; final volume 2 ml) at 30°C for 15 min, under standard import conditions. After centrifugation at 12,000 g for 3 min, the mitochondrial pellets were resuspended with 9 mg of carrier mitochondria and submitochondrial vesicles were prepared and fractionated on a linear sucrose gradient (see Materials and Methods). In Panel A, 100 ul of each fraction were precipitated with 12 % trichloroacetic acid and visualized by 12 % SDS-PAGE and fluorography. Full- length precursor was found from fractions 13 to 29 and no processed form was evident in the gradient. POCAT was found to be degraded to core protein from the top of the gradient to Fraction 11. The amount of translation product present was quantitated by laser densitometry. In Panel B, 400 ul of each fraction were assayed for chloramphenicol acetyl transferase activity, as described in Fig. 3, but incubated at 10°C for 1.5 h. The specific activity was expressed as % monoacetylated chloramphenicol/ [^{35}S]methionine-labeled pOCAT (from densitometry). Marker enzyme activities were performed as in Fig. 6 and the identified fractions are indicated. These results were replicated at least three times.

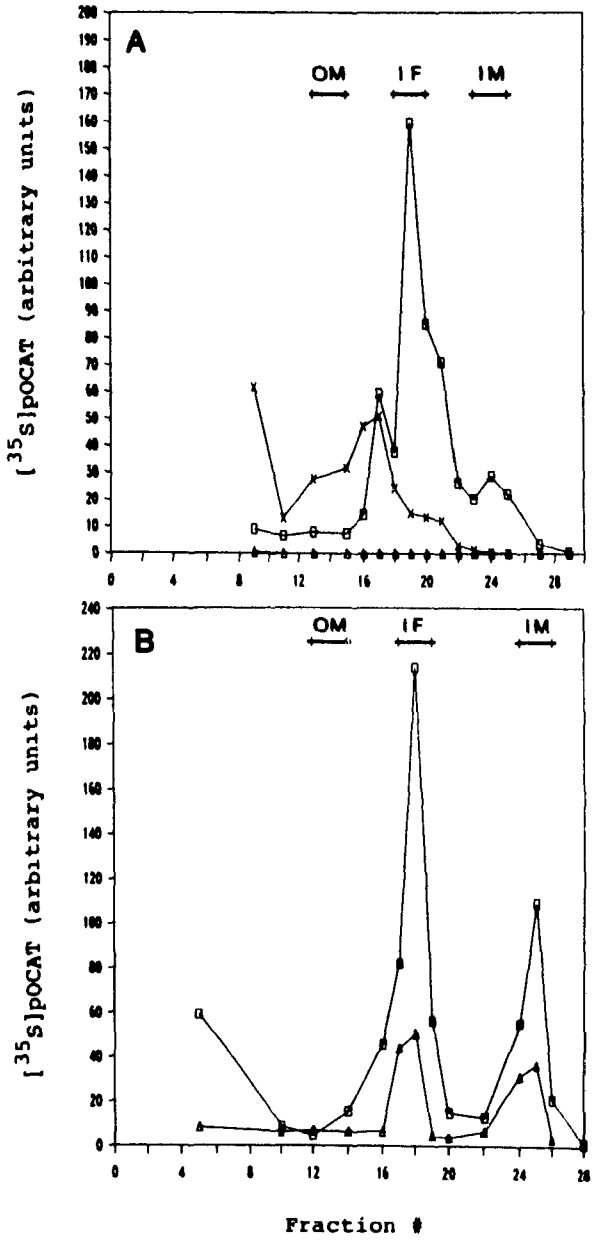

A
OM
IF
IM
[35S]pOCAT (arbitrary units)
B
OM
IF
IM
Acetyltransferase Activity/[35S]pOCAT
Fraction #

density fraction (Fig. 7A). We did not detect any processed precursor associated with any of the mitochondrial membrane fractions, whether import occurred at 10°C or 30°C (data not shown). This suggests that once the signal is cleaved, the mature protein is immediately released from the translocation apparatus (at least it cannot survive sonication and sucrose gradient fractionation).

POCAT that was associated with the intermediate (Fig. 7B, fractions 19-21) and inner membrane (Fig. 7B, fractions 23-26) regions of the gradient was found to exhibit a dramatic 16-fold loss of intrinsic activity when compared to full length precursor in the outer membrane fraction (Fig. 7B, gradient fractions 12-16) or when compared to freshly translated precursor (data not shown). Similar results were also found if the initial import had been performed at 10°C (data not shown). This loss of intrinsic activity exhibited by precursor associated with the intermediate and inner membrane fractions suggests that the precursor is in a partially denatured (i.e., unfolded) conformation.

As illustrated in Fig. 8A, pretreatment of mitochondria with trypsin decreased the amount of pOCAT that subsequently associated with the intermediate fraction by greater than 75%, with a corresponding increase in the amount associated with the outer membrane

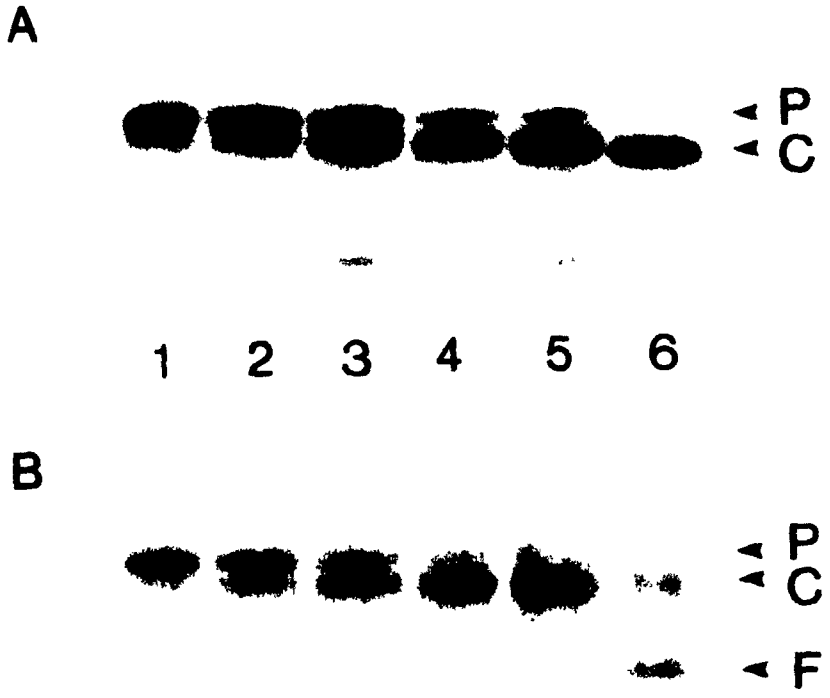
Fig. 8: The effect of mitochondrial pretreatment with trypsin and ATP-depletion on the formation of precursor associated with submitochondrial vesicles. In Panel A, CAT (△) was incubated with mitochondria as described in Fig. 7, and pOCAT was incubated under similar conditions with mitochondria which had (×) and had not (□) been pretreated with trypsin (5 mg/ml mitochondrial protein incubated with 40 ug/ml trypsin for 30 min at 4°C). Submitochondrial vesicles were prepared, the distribution of pOCAT was quantitated as described in Fig. 7, and the amount of precursor associated with each fraction was normalized to the amount of input precursor. In Panel B, pOCAT was incubated with mitochondria for 15 min at 30°C with (△) and without (□) ATP depletion by apyrase (4U/ml) before preparation of submitochondrial vesicles. Marker enzymes designated the positions of outer membrane (OM), intermediate density (IF), and inner membrane (IM) fractions.


A
OM
IF
IM
B
OM
IF
IM
[35S]pOCAT (arbitrary units)
[35S]pOCAT (arbitrary units)
Fraction #

fraction. Association of precursor with the intermediate fraction also depended on the presence of a signal sequence in the precursor, since CAT alone did not show any binding to this, or other, submitochondrial fractions (Fig. 8A). Finally, since ATP is required for import (Pfanner et al., 1987; Eilers et al., 1987; Chen and Douglas, 1987b) and has been implicated in the unfolding step (Verner and Schatz, 1987; Pfanner et al., 1987; Pfanner et al., 1988), we depleted the import incubation of ATP with apyrase, prepared submitochondrial vesicles, and found, again, a 75% loss of the precursor in the intermediate fraction (Fig. 8B). Taken together, these results suggest that the precursor unfolds only after interaction with the translocation apparatus in an ATP-dependent manner, requiring a trypsin-sensitive component.

pOCAT located in the outer membrane and in the intermediate fraction were also examined for protease sensitivity (Fig. 9). The precursor associated with the intermediate fraction was found to have at least one additional exposed tryptic site, resulting in conversion of the core protein to a lower molecular weight product (Fig. 9B), compared to pOCAT associated with the outer membrane (Fig. 9A). This implies that pOCAT is partially, but not extensively, unfolded when located in the intermediate fraction. Protease-sensitivity of the precursor in this fraction may yield a minimal estimate of

Fig. 9: Protease sensitivity of pOCAT associated with outer membrane (Panel A) and intermediate (Panel B) fractions. Equivalent amounts of pOCAT from outer membrane and intermediate fractions (corresponding to fraction # 12 and 18, respectively, Fig. 6) were digested with 0 (lanes 1), 1 (lanes 2), 3 (lanes 3), 10 (lanes 4), 30 (lanes 5), and 100 (lanes 6) ug/ml of trypsin (final volume 100 ul) for 15 min at 25°C. Products were visualized as described in Fig. 4.


A
P
C
1 2 3 4 5 6
B
P
C
F

unfolding if the precursor is in fact shielded from the protease by the translocation complex.

DISCUSSION

This study provides direct evidence for the existence of an unfolded translocation intermediate associated with a submitochondrial fraction, the formation of which requires an intact signal sequence, ATP, and a protease-sensitive component on the mitochondrial surface. Our results indicate that in the initial steps of *in vitro* import, which include diffusion of the soluble precursor protein in reticulocyte lysate and binding to the outer membrane, the bulk of the precursor population exists in a near-native conformation.

The finding that freshly synthesized pOCAT has a similar intrinsic activity and protease sensitivity to those of CAT (Fig. 3 and data not shown) demonstrates that the addition of a signal sequence to the amino-terminus of a protein does not label the bulk population of that protein for unfolding in reticulocyte lysate, although it does target it for import into mitochondria (Fig. 2). These results are in contrast to those obtained for bacterial secretory systems, where *secB*, trigger factor, and GroEL, have been shown to stabilize an 'open' conformation for the precursor (Crooke and Wickner 1987;

Lecker et al. 1989; Randall and Hardy 1989; Wickner 1989). This finding may represent an intrinsic difference between the precursors in mitochondrial import and bacterial secretion. For example, many hybrid proteins containing bacterial signal sequences fused to nonsecretory proteins are inefficiently exported from E. coli, if at all (Kadonaga et al., 1984; Tommassen et al., 1985; Freudl et al., 1988). Thus, while the precursors for bacterial secretion may depend on the intrinsic information of their mature region, this appears to be far less important for mitochondrial import (Vestweber and Schatz 1988a; Vestweber and Schatz 1989).

The demonstration that pOCAT and pODHFR do not change their conformation on membrane surfaces (Fig. 4 and Fig. 5) is in contrast to the situation for pCoxIV-DHFR (Endo et al., 1989). This type of unfolding, therefore, appears not to be an obligate step on the import pathway for all precursors. Interestingly, however, targeting of the precursor for this type of unfolding appears to reside with the type of signal sequence that is present on the precursor since it is only this region that is different between pODHFR and pCoxIV-DHFR. The affinity of a synthetic CoxIV signal peptide for anionic lipid is significantly higher than that for the pOCT peptide (Tamm 1986; Skerjanc et al. 1987). It may be, therefore, that the pCoxIV signal brings the precursor in closer proximity

to the amphiphilic surface of the bilayer than does the pOCT signal, resulting in more efficient membrane-dependent unfolding.

POCAT was found to have a more 'loose' conformation when associated with a submitochondrial fraction containing putative contact points (Fig. 7). Precursor associated with this fraction is probably an import intermediate because its formation requires an intact signal sequence, ATP, and a proteinaceous component of the outer membrane (Fig. 8). It constituted a relatively small percentage of the total input precursor. The possibility exists, therefore, that this fraction was already unfolded at an earlier point on the import pathway and simply was masked by the bulk population of other folded precursors. We consider this unlikely, however, since addition of excess reticulocyte lysate and ATP had no effect on the conformation of pOCAT and pODHFR translation products (Table 1 and not shown). Further, it has been shown that the amount of reticulocyte lysate components required for import is in vast excess under the standard import conditions employed here (W.P. Sheffield et al., manuscript in preparation). However, the idea of a component of reticulocyte lysate, e.g., hsp70, unfolding the precursor after it has engaged the import apparatus is completely consistent with the data described in this paper (Murakami et al., 1988; Deshaies et al., 1988).

In conclusion, this work has examined the conformation of two hybrid precursors at various steps on the import pathway. We found no evidence for the bulk unfolding of the precursor population, either immediately following synthesis in reticulocyte lysate or when bound to the surface of membranes. However, we detected an unfolded intermediate associated with a submitochondrial fraction; delivery of the precursor to this component of the putative translocation site depended on the presence of a signal sequence on the precursor, a protein component of the outer membrane, and ATP.

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CHAPTER 5

GENERAL DISCUSSION

The main focus of this thesis has been to investigate the mechanism(s) of protein translocation into the mitochondrial matrix by studying properties of translocated precursor proteins. A model has been developed which is consistent with all the results observed to date. In this model, the bulk of the precursor molecules in the cytoplasm remains in its native, folded state. The first interaction of the precursor with the mitochondria occurs via the lipids of the outer membrane and does not involve changes in the conformation of the bulk of the precursor population. The rate of subsequent interaction of the precursor with the import receptor, which is as yet unidentified, is enhanced by the reversible binding of the precursor to the lipids. After engaging the translocation apparatus, but before being cleaved by the signal peptidase in the matrix, the precursor undergoes a change in conformation resulting in at least partial unfolding. Finally, translocation across the inner membrane cannot occur through a pure lipid phase, even utilising the energy of the transbilayer potential across the inner membrane, and thus must require the presence of membrane proteins, which remain to be identified.

5.1.1 Precursor Conformation in the Cytoplasm

The most straightforward method to look for a loss of native precursor conformation is by measuring either changes in specific enzymatic activity or changes in sensitivity to proteases. Although this does not give a detailed picture of how much or in what way the precursor has changed conformation, it is a simple diagnostic method for measuring a loss of the native state of the precursor. Using this type of assay, we found no differences in conformation between CAT and pOCAT in reticulocyte lysate at varying concentrations of ATP (Chapter 4). We thus find no evidence that an ATP-dependent "unfoldase", capable of unfolding any protein with a signal sequence at its N-terminus, affects the conformation of a significant fraction of pOCAT translated in reticulocyte lysate.

Other work in this laboratory (W. Sheffield, unpublished) has addressed the question of import competence (see section 1.1.6) of mitochondrial precursors. It has been found that pODHFR, when purified from *E. coli*, shows no difference in protease sensitivity whether it is competent or incompetent for import. Therefore, we have found no evidence for unfolding of mitochondrial precursors in the cytoplasm, before interacting with mitochondria.

However, in bacterial secretion, several cytosolic

factors contribute to efficient polypeptide secretion and are found to stabilize the unfolded state of precursors. Thus, an unfolded conformation correlates with an ability to be secreted (discussed in section 1.2.7b). One might expect cytosolic factors required for mitochondrial import to function in a similar manner, but, as discussed above, there seems to be no evidence for this.

5.1.2 The Surface-Seeking Model

The observation that synthetic signal peptides bind to liposomes containing physiological concentrations of anionic phospholipids with a moderately high affinity and in a reversible manner (Skerjanc et al. 1987; Chapter 2), led us to propose a model in which the signal sequence initially binds to the lipid component of cellular membranes. In this model, this interaction with the lipid bilayer surface enhances the subsequent diffusion of the precursor to the import receptor and translocation machinery. In Chapter 3, we identified a short lipid-binding domain (residues 5-9) capable of influencing both the rate of precursor import and, correspondingly, the ability of pOCT to bind to lipid membranes (Skerjanc et al. 1988). Decreasing the hydrophobicity of this region, by substituting leucine residues with alanine, led to a decrease in the rate of *in vitro* import and a decrease in precursor affinity for liposomes. This finding is consistent with the proposed surface-seeking model, in

which the precursor uses the two-dimensional lipid surface of the outer membrane to enhance its rate of diffusion to the import apparatus.

This type of relationship, between the affinity for lipid bilayers and the efficiency of import, has also been shown by Roise et al. (1988) who found that the ability of artificial signal sequences to insert into monolayers is proportional to their ability to promote import of a cytosolic protein, DHFR, into mitochondria. A similar type of correlation has been demonstrated by Briggs et al. (1985) for wild-type, pseudorevertant, and mutant signal sequences of LamB in bacterial protein secretion. Further, the ability of preMBP to bind Triton X-100 has been correlated to its ability to be exported in E. coli (Dierstein and Wickner 1985). These findings are all consistent with a model in which the ability of the presequence to bind lipids enhances the rate of precursor diffusion to the translocation apparatus. However, they may also be consistent with a requirement for an amphipathic interaction with the bilayer at a later step of import, or with proteins of the translocation apparatus.

5.1.3 Precursor conformation upon Binding to Membranes

Since precursors can bind to liposomes via their signal sequence, it is interesting to consider whether or not the mature protein also interacts with the amphiphilic

surface, and in so doing changes conformation. We found no change in either specific enzyme activity for pOCAT or in protease sensitivity for both pOCAT and pODHFR, upon binding to liposomes (Chapter 4). This suggests that the bulk of CAT and DHFR cannot change conformation by interacting with the surface of liposomes, even when targeted to the lipid surface by a signal sequence. Since the same result was also found upon binding to the surface of mitochondria, bulk unfolding of these precursors can not occur even on the natural lipids of the outer mitochondrial membrane.

Recently, using similar protease sensitivity experiments, Endo et al. (1989) found that the artificial precursor pCoxIV-DHFR was capable of unfolding on liposomes containing negatively charged phospholipids, while other natural precursors were not. Since a synthetic signal peptide corresponding to the signal sequence of pCoxIV (Tamm 1986) seems to have a higher affinity for lipids than a pOCT synthetic signal peptide (Skerjanc et al. 1987; Chapter 2 of this thesis), it is possible that the pCoxIV signal brings the precursor in closer proximity to the amphiphilic surface of the bilayer than does the pOCT signal. However, it is clear that unfolding on the lipid surface is not an obligate step on the import pathway, either for natural or artificial precursors.

5.1.4 Isolation of an Unfolded Translocation Intermediate

Examination of the interaction of pOCAT with mitochondria, under import conditions, has led to the isolation of a full length import intermediate which has lost its native conformation (Chapter 4). We found that a small percentage of the total precursor in an import assay was associated on a sucrose velocity gradient with a mitochondrial fraction of intermediate density between the inner and outer membrane vesicles. The majority of the precursor was degraded to a protease-insensitive core and located at the top of the gradient. The association of precursor with the intermediate density fraction required the presence of a signal sequence, ATP, and a protease-sensitive component on the surface of mitochondria, and thus it is believed to be an intermediate on the import pathway. Since no mature CAT was found in the gradient it is likely that once the signal is cleaved by the processing peptidase, the precursor is released from the translocation apparatus.

Precursor associated with the intermediate density fraction had about a 16 fold lower specific enzyme activity and was more susceptible to trypsin than precursor associated with the outer membrane vesicles or freshly translated pOCAT (Chapter 4). This demonstrates that the precursor loses its native conformation during the import process and this finding is in agreement with

the more indirect observations of others (Eilers and Schatz 1986; Chen and Douglas 1987b; Schleyer and Neupert 1985).

Since such a small amount of precursor was found to be unfolded in the above experiments, it is difficult to state exactly at which point on the import pathway the unfolding occurred. The requirement for ATP in forming the intermediate suggests that an ATP-dependent unfolding mechanism could exist for a small amount of the precursor, either in the cytoplasm or in the translocation apparatus. One would expect that an interaction of the precursor with the translocation apparatus would be essential for regulation of the unfolding. Alternatively, the ATP could be required for phosphorylation of a protein or for coupling the energy of hydrolysis to translocation, with the unfolding simply occurring spontaneously as the precursor is translocated. The idea that a small, undetectable amount of precursor unfolds on the lipid surface cannot be ruled out entirely.

5.1.5 Membrane Components Involved in Protein Translocation

Given the affinity of signal sequences for lipid bilayers, the question arose as to whether or not signal sequences translocate across the inner mitochondrial membrane in the absence of membrane proteins. To examine this possibility, we measured the binding to liposomes of

a chemically synthesized peptide, corresponding to the first 27 amino acids of pOCT, in the presence and absence of an electrochemical potential. Although we found a moderately high affinity of the peptide for lipid surfaces containing anionic lipids (which led to the proposal of the surface-seeking model) we obtained no evidence that the presence of a transbilayer potential could enhance the binding of the peptides to vesicles or promote transbilayer movement of the peptides (Skerjanc et al. 1987; Chapter 2). From these results, it seems highly likely that the precursor requires an interaction with specific membrane proteins in order to translocate across the inner mitochondrial membrane.

At least two precursors have the ability to cross a lipid bilayer without the aid of proteins: apocytochrome c, a mitochondrial intermembrane space protein (Rietveld and de Kruijff 1984), and procoat, the coat protein of bacteriophage M13 (Geller and Wickner 1985). However, these proteins seem to have very distinctive properties which are not representative of most precursors.

Our finding that signal sequences can not translocate across pure lipid bilayers does not rule out the possibility that the signal sequence may, at some point during import, come in direct contact with the hydrophobic core of the bilayer, as postulated by Wickner (1989) and Rapoport (1985; see section 1.2.3a). However, they are

consistent with many observations that suggest a requirement for membrane proteins in import (see section 1.1.4). These observations include the demonstration of specific precursor binding to a limited number of sites on mitochondrial membranes, as well as inhibition of import by proteolysis of mitochondrial outer membranes and by antibodies raised against the outer membrane proteins. Although there is no analogous evidence for the requirement of membrane proteins for protein translocation across the inner membrane, the findings that translocation intermediates occur at areas of close contact between the outer and inner membranes (Schleyer and Neupert 1985), and that these intermediates can be removed from the membrane by treatment with urea (Pfanner et al. 1987b), suggests the presence of a hydrophilic pore, presumably comprised of proteins.

Although there is presently no purified candidate for a mitochondrial protein-translocating pore, in the ER a signal sequence receptor was identified by cross-linking (Kurzchalia et al. 1986), is now purified (Hartmann et al. 1989), and is a good candidate for an integral membrane protein involved in formation of the translocation apparatus. SecY may be part of a similar pore forming complex in bacterial membranes.

5.1.6 Future Work

Examination of the properties of precursor proteins in this thesis has led to the development of a model for protein translocation into mitochondria. As with all models, further studies will either agree or disagree with the present model and will lead to modifications and adaptations in accordance with the new information.

Further examination of the surface-seeking model, in which the rate of precursor import is enhanced by the binding of the presequence to the lipids of the outer membrane, could involve solubilization and reconstitution of mitochondrial binding sites into liposomes, as has been demonstrated by several workers (Ono and Tuboi 1985; Pfaller and Neupert 1987). With large amounts of purified pODHFR, specific binding sites could be characterized. According to our model, one would expect to find a different rate of association of the precursor to the receptor by maintaining the concentration of receptor constant and varying the lipid surface area. For example, a faster rate of association would be found under conditions of a high lipid/protein ratio (for example 100 liposomes with one receptor each) versus a low lipid/protein ratio (for example 1 liposome with 100 receptors), assuming the liposomes in both cases are similar sizes. Also, the type of lipid used in the reconstitution could be tested for effects on the

subsequent rate of association with the receptor. For example, reconstituting the receptor with a higher content of negatively charged phospholipids should increase the affinity of the presequence for the liposomes and, correspondingly, increase the precursor's rate of association with the import receptor, although an effect of the type of lipid directly on receptor conformation could not be ruled out.

The identification of an unfolded intermediate for pOCAT could be extended to other precursors. Since most precursors do not have a measurable enzyme activity in reticulocyte lysate, protease sensitivity could be examined. pOCAT associated with the intermediate density fraction exhibited an increase in trypsin sensitivity compared to precursor associated with the outer membrane. The increase in sensitivity was not dramatic which could be due to a protection of exposed trypsin sites by the import apparatus. However, since the effect is still evident, it would be possible to see if other precursors, both natural and artificial, also exist in an unfolded conformation at this step in the pathway.

It would be interesting to see what would release the precursor from its unfolded conformation in the intermediate-density membrane fraction. Treatment of samples with low levels of detergent or even repeated freeze/thawing did not seem to allow the precursor to

release from the apparatus and refold (Chapter 4). Perhaps reconstitution of a purified preparation of signal peptidase would allow the signal to be cleaved and result in a release from the apparatus. Recently, Cheng et al. (1989) has shown that hsp60 is involved in the renaturation of proteins after import into mitochondria. Possibly, hsp60 is also required for refolding of this intermediate, once the signal has been cleaved (Walter Neupert, unpublished results).

The identification and isolation of membrane proteins involved in protein translocation is essential for understanding mechanisms of import in detail. For mitochondria, the issue is complicated by the requirement to translocate across two membranes. Recently, Hwang et al. (1989) have shown that pCoxIV-DHFR can translocate across purified yeast inner mitochondrial membrane vesicles, in the absence of the outer membrane. This finding demonstrates that the inner membrane contains an import apparatus which can act independently of the outer membrane, and thus reconstitution of protein import across at least one mitochondrial membrane is possible. A reconstituted import system, involving purified precursors and translocation components, would be ideal for detailed examination of translocation mechanisms.

Cross-linking is a powerful technique for identifying interactions between proteins. Cross-linking

mitochondrial precursors to the translocation apparatus has been difficult due to the presence of a very high background caused by nonspecific binding to various membrane proteins (I.S. Skerjanc and X. Liu, unpublished results). This can now be explained since only a small percentage of precursor is interacting with the translocation apparatus during import and the bulk of precursor seems non-specifically associated with the outer membrane, in such a way that it can not easily be removed. However, the majority of precursor associated with the intermediate density fraction, isolated in Chapter 4, should be located in the translocation apparatus and therefore this translocation intermediate provides an ideal probe which may now be used in cross-linking analysis.

5.1.7 Summary

This work has examined the amphiphilic properties of the signal sequence, with the conclusion that these properties may be required to enhance diffusion of precursors to receptors via binding to the lipids of the outer membrane. Studies of conformational changes in the mature portion of two artificial precursors have shown that translocation intermediates exist in an unfolded conformation, and that this unfolding does not seem to occur with the bulk precursor in solution or bound to the outer membrane of mitochondria. A model for protein

translocation into mitochondria has been proposed which is consistent with the observations described in this thesis, and which will serve as a guide in designing new experiments.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

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

1. The characterization of the binding of pO(1-27) to lipid vesicles as rapid, reversible, dependent on anionic phospholipids, and independent of a transbilayer potential.

2. The conclusion that import of precursor proteins across the inner mitochondrial membrane via a lipid pathway is unlikely (i.e. membrane proteins are required).

3. The establishment of a correlation between a reduced rate of import of pOCT and a decreased affinity of the polypeptide for liposomes, using a mutant in which leucines at positions 5,8, and 9 were changed to alanine. We suggested that this small region (amino acids 5-9) of pOCT normally contributes to the optimal rate of precursor import, perhaps by functioning as a surface-seeking entity.

4. The demonstration that at least two precursor proteins, pOCAT and pODHFR, remain in a tightly-folded

conformation after synthesis in reticulocyte lysate. Their conformation was unaffected following binding to liposomes containing negatively charged phospholipids or to the surface of mitochondria, in the absence of a membrane-potential.

5. The detection of an unfolded polypeptide translocation intermediate for pOCAT, the formation of which requires an intact signal sequence, a protease-sensitive component of the outer mitochondrial membrane, and ATP. From these findings, we suggested that unfolding probably occurs only after the polypeptide has engaged the import apparatus.