

Interaction of Peptides Corresponding to Mitochondrial Presequences with Membranes*

(Received for publication, May 6, 1991)

David W. Hoyt§||, Douglas M. Cyr‡¶, Lila M. Gierasch§, and Michael G. Douglas‡**

From the §Departments of Biochemistry and Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235 and the ‡Department of Biochemistry and Biophysics, University of North Carolina Medical School, Chapel Hill, North Carolina 27599-7260

The transport of the F_1 -ATPase β -subunit precursor into mitochondria is dependent upon a presequence at its amino terminus. Within the mitochondrial membrane translocation site the potential amphiphilic character of the presequence region may be necessary to stabilize binding to the mitochondrial inner membrane. To better understand its role in protein import, the interaction of the $F_1\beta$ -presequence with lipid membranes was measured using circular dichroism and surface tensiometry. These studies reveal that a 20-residue peptide containing the $F_1\beta$ -presequence binds to phospholipid vesicles ($K_d = 4.5\text{--}6.0 \times 10^{-8}$ M) and adopts a predominantly α -helical structure. Although the presequence peptide binds avidly to lipids, it does not appear to penetrate deeply into the bilayer to perturb a reporter probe in the membrane interior. Compared with the effect of the peptides with demonstrated membrane insertion and lytic properties, the $F_1\beta$ -presequence appears to displace phospholipid head groups but not insert deeply into the bilayer. High concentrations ($>50 \mu\text{M}$) of presequence peptides are required to noticeably perturb import of the full length $F_1\alpha$ - or $F_1\beta$ -subunit precursors. Thus, the $F_1\beta$ -presequence alone is not sufficient to efficiently compete for import but may require a protein context or a minimal length to assist insertion into the transport site. These observations are discussed in light of the different requirements for import of various presequence containing precursors into mitochondria.

Import of most proteins from the cytoplasm to mitochondria requires sequences located at the extreme amino terminus of the nascent polypeptide chain (Douglas *et al.*, 1986, 1991; Hurt and van Loon, 1986; Roise and Schatz, 1988; Hartl *et al.*, 1989). Gene fusion studies have shown that these sequences contain information that can function independent of the passenger protein in directing the protein to mitochondria and in promoting its translocation through the membrane (Verner and Lemire, 1989). At the present time, data support that these presequences direct the correct delivery of cyto-

plasmic precursors to receptors on mitochondria *in vivo*.

Insight into the mechanism of presequence selectivity and function is obscured to some extent by the observation that essentially any sequence of approximately 20 residues which is capable of forming a basic amphiphilic structure is capable of promoting intercellular delivery and transport of a mitochondrial precursor (Allison and Schatz, 1986; Baker and Schatz, 1987; Lemire *et al.*, 1989). These studies, among others (reviewed in Hartl *et al.*, 1989), have shown that the determinants for protein import consist of sequences with the potential to form amphiphilic structures at the amino-terminal end (Von Heijne, 1986; Roise and Schatz, 1986; Lemire, *et al.*, 1989).

Analysis of the F_1 -ATPase¹ β -subunit precursor indicates that it also should conform to an amphiphilic helical structure (Bedwell *et al.*, 1989). The first 15 residues of the $F_1\beta$ -precursor are sufficient to direct this subunit (Vassarotti *et al.*, 1987) or dihydrofolate reductase into mitochondria (Walker *et al.*, 1990). The $F_1\beta$ -precursor lacking a presequence fails to localize into mitochondria *in vivo* and *in vitro* (Vassarotti *et al.*, 1987; Bedwell *et al.*, 1989). Furthermore, the precursor lacking its import signal will mutate to adopt a basic amphiphilic structure at the amino terminus under growth conditions which demand its transport into the organelle (Vassarotti *et al.*, 1987b; Bilbus *et al.*, 1988).

Transport of some protein precursors into mitochondria requires their association with cytosolic protein factors (Ohta and Schatz, 1984; Chen and Douglas, 1987a, Deshaies *et al.*, 1988, Murakami *et al.*, 1988, Sheffield *et al.*, 1990; Murakami and Mori, 1990) and the hydrolysis of ATP (Pfanner *et al.*, 1987; Eilers *et al.*, 1987, Chen and Douglas, 1987a). This, however, is not the case for all mitochondrial precursors some of which can be efficiently imported as purified refolded precursors independent of associated factors. Purified $F_1\beta$ -subunit precursor fails to import into mitochondria unless cytosolic components are present (Ohta and Schatz, 1984; Chen and Douglas, 1987b). Thus the $F_1\beta$ -subunit precursor apparently requires more than a presequence to interact productively with the membrane.

Recent studies have defined components present on the mitochondrial surface which appear to mediate the efficient delivery of mitochondrial precursors to the translocation site

* This research was supported in part by Grants GM26713 and GM36537 (to M. G. D.) and GM34962 (to L. M. G.) from the National Institutes of Health and grants (to M. G. D. and L. M. G.) from The Robert A. Welch Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by American Heart Association Postdoctoral Fellowship NC-90-F4.

|| Submitted in partial fulfillment for completion of the PhD degree.

** To whom correspondence should be addressed.

¹ The abbreviations used are: F_1 -ATPase, soluble portion of the mitochondrial inner membrane-bound ATPase complex; CCCP, carbonyl cyanide *m*-chlorophenylhydrozone; TFE, trifluoroacetic acid; SDS, sodium dodecyl sulfate; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; LUV, large unilamellar vesicle; DPH, 1,6-diphenyl-1,3,5-hexatriene; 3,3'-Di-S-C3(5), 3,3'-dipropylthiadicarbocyanine iodine; HPLC, high performance liquid chromatography; SUV, small unilamellar vesicle; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(Sollner *et al.*, 1989; Hartl and Neupert, 1990; Hines *et al.*, 1990; Baker and Schatz, 1991). Movement of the precursor through the translocation site essentially as an extended polypeptide molecule requires the participation of membrane components within the outer membrane (Vestveber *et al.*, 1989; Baker and Schatz, 1991; Kiebler *et al.*, 1990) and mitochondrial proteins of the Hsp70 family on the matrix face of the mitochondrial inner membrane (Kang *et al.*, 1990; Scherer *et al.*, 1990).

After insertion into the proteinaceous components of the contact site, little is known about how the mitochondrial presequence interacts with and inserts into the inner membrane. Earlier studies have shown that synthetic peptides containing mitochondrial presequences block protein import (Ito *et al.*, 1985; Roise *et al.*, 1986; Roise and Schatz, 1988; Gillespie *et al.*, 1985; Glasser and Cumsky, 1990). The interpretation of these experiments is obscured by the observation that in some cases these peptides may coincidentally influence the integrity of the bilayer and the ability of the membrane to maintain the electrochemical potentials necessary for import.

All these studies indicate that at some point the association of the mitochondrial presequence with membrane phospholipid is a necessary step for the transport of the precursor at some point after binding to the mitochondrial surface (Endo and Schatz, 1988). The ability of reagents which interact preferentially with inner membrane phospholipids to block protein import suggests that this membrane may directly interact with the presequence during translocation (Eilers *et al.*, 1989).

In the present study we have examined the biophysical properties of the F_1 -ATPase β -subunit presequence which result from its interaction with biological membranes. These data reveal that the $F_1\beta$ -presequence associates strongly with phospholipid membranes; however, the peptide does not penetrate deeply into the bilayer. These observations and additional studies (Cyr and Douglas, 1991) indicate insertion into the transport site to reach the committed step for translocation may also require the action of components at the point of contact between the inner and outer membrane.

EXPERIMENTAL PROCEDURES

Strains and Media—The *Escherichia coli* strain MC1066 (F^- lac X74, galU galK rpsL hsdR trpC9830 leuB600 pyrF74::Tn5) (Casadaban and Cohen, 1980) was grown in LB medium (Miller, 1972). Ampicillin-resistant transformants were selected in the medium supplemented with 50 μ M ampicillin (Maniatis *et al.*, 1982). The yeast strain D273-10B (ATCC 25657, MAT α) was used for mitochondrial preparations. The cells were grown in the semisynthetic medium containing per liter: 3.0 g of yeast extract, 0.4 g of CaCl₂ 2H₂O, 0.5 g of NaCl, 1.0 g of KH₂PO₄, 1.2 g of (NH₄)₂SO₄, 0.7 g of MgSO₄ 7H₂O, 0.5 ml of 1% FeCl₃, 2 ml of 50% glucose and 40 ml of 50% lactate, pH 5.5, and harvested at OD₆₀₀ = 1.2.

DNA Techniques—Restriction enzyme digestions and ligations with T4 ligase were performed according to the instructions from the commercial suppliers. Transformation of *E. coli* strain MC1066 and preparation of small scale plasmid DNA from transformants were as described (Maniatis *et al.*, 1982). DNA templates for *in vitro* transcription were isolated from low gelling temperature agarose (Saltz-gaber *et al.*, 1983).

Transcription and Translation—The pT7-ATP1 and pT7-ATP2 transcription vectors were described previously (Takeda *et al.*, 1986; Chen and Douglas, 1987a). The plasmids pT7-ATP1 and pT7-ATP2 were linearized with *Hind*III and *Xba*I, respectively. The *in vitro* translation reactions contained 35 μ l of nuclease-treated reticulocyte lysate (Promega) which had been supplemented with creatine phosphate, creatine phosphokinase, hemin, calf liver tRNA, 140 mM potassium acetate, and 1 mM magnesium acetate, 19 amino acids, 50 μ Ci [³⁵S]methionine and 5 μ l of gel filtered mRNA in a total volume of 50 μ l. After a 60-min incubation at 30 °C, 1 μ l of the translation

was spotted on a filter for counting. The filters were washed with 5% cold trichloroacetic acid for 10 min, followed by boiling in 5% trichloroacetic acid for 3 min. After rinsing with ethanol, the dried filters were counted in toluene-based scintillation fluid. We estimate that 10 pmol of radiolabeled proteins were synthesized from 2 μ g of DNA.

In Vitro Import Experiments—Cells grown in lactate medium were harvested and mitochondria were isolated as described (Gasser *et al.*, 1982). *In vitro* import of the labeled precursor was performed in 100- μ l reactions containing 20 mM HEPES-KOH, pH 7.4, 0.6 M mannitol, 1 mM dithiothreitol, 1 mM MgCl₂, 100 mM KCl, 7.5 ml of lysate, and 50–70 μ g of mitochondria. Supplements to the import reaction were as indicated previously (Chen and Douglas, 1987a). Import efficiency was assessed by proteinase K digestion (250 μ g/ml) for 30 min at 0–4 °C.

Peptide Synthesis—The peptides were synthesized by a modified solid-phase peptide synthesis method described by Merrifield (Stewart and Young, 1984), and completion of the coupling was monitored by ninhydrin assay (Sarin *et al.*, 1981). The completed peptides were deprotected and released from the resin by hydrogen fluoride cleavage. After evaporation of the hydrogen fluoride, the peptides were precipitated with diethyl ether and separated from the resin by dissolving the precipitated peptides in 5% acetic acid followed by filtration. The lyophilized peptides were purified by reverse-phase HPLC on Vydac phenyl columns and then analyzed by Edman degradation to determine sequence and purity.

Surface Tensiometry Experiments—Surface tensiometry measurements were obtained on a Fisher Autotensiomat, equipped with a platinum-iridium ring, employing the du Noüy ring method (Adams, 1976) at 25 °C. Concentrated presequence peptide samples were dissolved in 25 mM dithiothreitol, 5.0 mM Tris, pH 7.3. A monolayer of POPC, POPE, and POPG (40:24:36) (Avanti, Birmingham, AL) was spread from a chloroform solution onto 5.0 mM Tris, pH 7.3. to a final surface pressure of 20 mN/m after evaporation of the chloroform. Concentrated peptide solutions were added by injecting beneath the lipid-water interface. Peptide suspension buffer alone had no effect on surface pressure.

Lipid Vesicle Preparation—Stock solutions of lipid, dissolved in chloroform, were dispensed in amounts to form a 40:24:36 (mol %) mixture of POPC/POPE/POPG. Small unilamellar vesicles (SUVs) were prepared according to the method of Huang (Huang, 1969). Large unilamellar vesicles (LUVs) were made according to the freeze-thaw/extrusion method of Mayer (Mayer *et al.*, 1986). The suspension was freeze-thawed (five times) using liquid nitrogen bath and then extruded through two stacked 100-nm pore polycarbonate filters under nitrogen pressure (10 times) using an Extruder (Lipex Biomembranes, Inc.). LUVs containing carboxyfluorescein (CBXF) were made by including 0.1 M carboxyfluorescein in the hydration buffer and passing the vesicles over a Sephadex G-10 column to remove extravesicular carboxyfluorescein. Iso-osmotic conditions were preserved by including 0.1 M NaCl in the eluting 5 mM Tris, pH 7.3, buffer. Phospholipid concentrations of all vesicle samples were determined using an ashing/phosphate assay (Ames and Dubin, 1960).

Circular Dichroism—All CD spectra were obtained on a Landis Associates Jasco CDSP Spectropolarimeter at 25 °C. The amplitude of the CD signal was calibrated using a 0.1% (w/v) solution of D-(+)-camphorsulfonic acid (Aldrich), and the wavelength of the CD signal was set using standard absorbance peaks of benzene vapor (Aldrich, spectral grade). Peptide concentrations were determined by quantitative amino acid analysis. CD spectra were obtained on peptide samples of 5 μ M concentration in a 5-mm cell for bulk solvents and 2-mm cell for SUVs unless stated otherwise. Estimates of peptide secondary conformation were made by analyzing CD spectra using a curve-fitting algorithm which calculates a nonrestrained least squares fit to Greenfield and Fasman (1969) polylysine reference data. Helical content was also calculated using CD signal intensity according to the method of Chen (Chen *et al.*, 1974).

Carboxyfluorescein Leakage Assay—Peptide-induced leakage of the vesicle-entrapped carboxyfluorescein dye from LUVs was measured by monitoring the emission at 520 nm (excitation wavelength 480 nm) as a function of time. Fluorescence measurements were made on an ISS Inc. steady-state spectrofluorometer (model Greg PC slit widths of 0.5 mm) using constant stirring at 25 °C.

Steady-state Fluorescence Anisotropy—LUVs (65:35, POPE/POPG) prepared in either 5 mM Tris, pH 7.3, or 0.1 M NaCl 5 mM Tris, pH 7.3) were diluted to a 1 mM lipid concentration (1200 μ l of final volume). To the sample cell 2 μ l of 2 mM 1,6-diphenyl-1,3,5-hexatriene (DPH) (dissolved in tetrahydrofuran) was added to give a ratio of 1:300, DPH/lipid. DPH fluorescence was measured with an

ISS steady-state spectrofluorometer model Greg PC at 25 °C unless stated otherwise. Wavelengths were set at 360 and 430 nm, respectively (L format). When DPH anisotropy was recorded as a function of temperature, samples were equilibrated at each point for 45 min.

Miscellaneous—Calculation and classification of the properties of different peptides used in this study were performed according to Segrest *et al.*, 1990. SDS-gel electrophoresis was performed as described (Laemmli, 1970). After electrophoresis, the gel was treated with sodium salicylate (Chamberlain, 1979) and exposed to x-ray film at -80 °C.

RESULTS

Structure of Presequence Peptides—The F₁-ATPase β -subunit presequence is required for the import of the precursor protein into mitochondria (Vassarotti *et al.*, 1987; Bedwell *et al.*, 1987; Walker *et al.*, 1990). Although the β -subunit precursor contains redundant import information (Bedwell *et al.*, 1987), as little as the first 15 residues alone is sufficient to promote import of this precursor or other passenger proteins into mitochondria both *in vivo* and *in vitro* (Vassarotti *et al.*, 1987; Walker *et al.*, 1990). In order to examine the influence of the F₁ β -presequence on the association and import of mitochondrial precursors, the 19-residue presequence was chemically synthesized using all D or all L amino acids. A control peptide was also prepared which contains residues 33 through 49 from a mature region of the F₁ β -subunit. This peptide also contains the peptide sequence GGGYCG at its carboxyl terminus for use in other studies and will be referred to as the "control peptide" (Fig. 1). Except by chirality of their peptide backbones these L and D peptides are identical by peptide sequencing, coelution from HPLC, and identical one-dimensional NMR spectra (not shown).

To examine the structures which these peptides form upon association with membranes, circular dichroism measurements were first performed on the peptides in neutral aqueous buffer as a function of increasing of trifluoroethanol (TFE) and SDS. These conditions have been shown in earlier studies to influence the conformation properties of peptides under conditions which mimic association with the membrane (Briggs *et al.*, 1984; Hoyt *et al.*, 1987). The observed CD spectra (Fig. 2) were fit to combinations of α -helix, β -structure, and random coil standards (Greenfield and Fasman, 1969). Both the D and L sequence peptides and the control

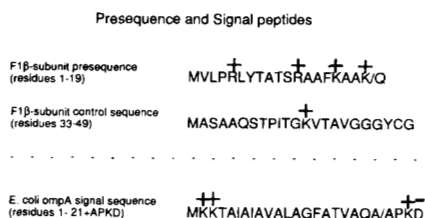


FIG. 1. Peptides used in this study. Peptides were synthesized by a modified solid-phase method as described under "Experimental Procedures" and purified to a single peak on Vydac phenyl columns. The protein concentration of stock solutions was performed by amino acid analysis. In each case, peptides were sequenced to confirm sequence.

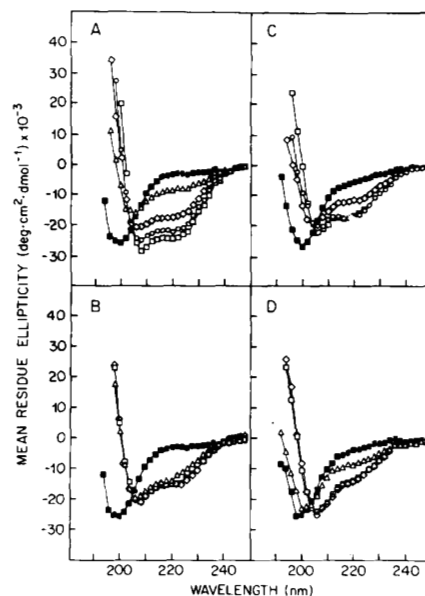


FIG. 2. CD spectra of all L presequence and control peptides at several concentrations of TFE and SDS. Spectra were obtained on our Landis Associates Jasco CDSP Spectropolarimeter in a 1-mm cell at ambient temperature (see "Experimental Procedures"). A and B, presequence peptide concentrations were 5×10^{-5} M in 5.0 mM Tris, pH 7.3, for each spectrum. C and D, control peptide concentrations were 2.5×10^{-5} M in 5.0 mM Tris, pH 7.3, for each spectrum. A and C, the TFE concentration was adjusted to 0% (closed squares), 20% (triangles), 40% (diamonds), 60% (circles), and 80% (open squares). B and D, the SDS concentration was adjusted to 0 mM (closed squares), 10 mM (triangles), 20 mM (diamonds), and 40 mM (open squares).

TABLE I
Mitochondrial peptide conformation from CD Spectra

F ₁ β -ATPase presequence ^a			
M-V-L-P-R-L-Y-T-A-T-S-R-A-A-F-K-A-A-K-Q			
Solvent	% Helix	% β	% Random
Buffer ^b	15	5	80
80% TFE	75	5	20
40 mM SDS	45	10	45
F ₁ β -ATPase sequence 33-49			
M-A-S-A-A-Q-S-T-P-I-T-G-K-V-T-A-V-G-G-G-Y-C-G			
Buffer ^b	20	5	75
50% TFE	45	10	45
40 mM SDS	45	0	55

^a Based on reference spectra from Greenfield and Fasman (1969).

^b 5 mM Tris-HCl, pH 7.3, peptide conc. ~ 50 μ M. The relative content of α -helix, β -structure, and random coil under different conditions were determined as described in Fig. 2 and "Experimental Procedures."

peptide exhibit essentially random structure in neutral aqueous buffer. However, the chiral peptides both have a higher propensity to adopt α -helical content in a bulk hydrophobic environment (80% trifluoroethanol). Association with detergent micelles (40 mM SDS) yields peptides with predominantly α -helical content (Table I). Upon titration with TFE (not shown), the conformation of the all D presequence was of equal magnitude but opposite sign to that for the L presequence.

When structure of the peptides was monitored upon association with phospholipid vesicles, the D presequence yielded reproducibly greater α -helical content than the L presequence at the same protein to lipid ratios (Fig. 3, A and B). This difference due solely to the chirality of the peptide is not apparent in titrations of enantiomeric LamB signals with

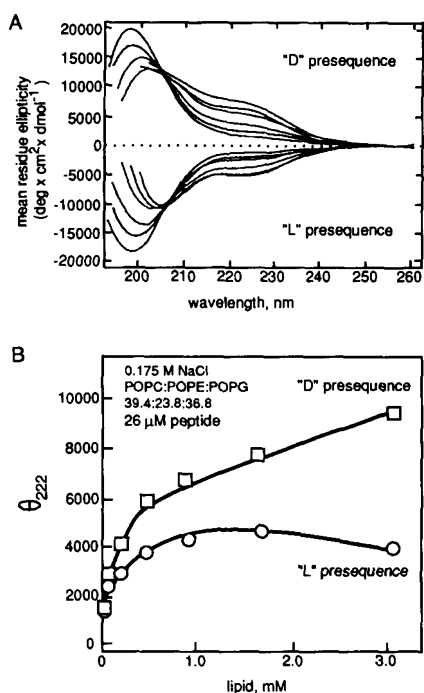


FIG. 3. Spectra of all D versus all L presequence peptides upon binding to small unilamellar vesicles. CD spectra were measured as described in the legend to Fig. 2 (see also "Experimental Procedures") A, all D or all L $F_{1\beta}$ -presequence peptide (26 μ M) was equilibrated at ambient temperature with increasing SUV (POPC/POPE/POPG, 19.4:23.8:36.8). B, the ellipticity at 222 nm was plotted as a function of the concentration of SUV present.

phospholipid vesicles.² This differential in the extent of α -helical structure implies that perhaps the peptide structures are being influenced by the chirality of the lipid headgroups. Although additional studies are needed to unambiguously prove that the lipid headgroup chirality is the difference, the most likely explanation is that the enantiomeric glycerol moiety of the phospholipid (the second carbon) directly influences the tendency of the associating presequence peptides to adopt α -helical structure.

Membrane Binding of Presequence Peptides—Previously a family of LamB protein export signals has been shown to exhibit membrane binding properties that correlated with their ability to function *in vivo* (Briggs *et al.*, 1985). The lipid-dependent formation of secondary structure by D versus L presequence indicated that they strongly associated with the model membrane. The interaction of the L- and D-peptides with the membrane was measured using surface tensiometry (see "Experimental Procedures"). In each case, the phospholipid monolayer consisting of POPC/POPE/POPG in a 40:24:36 ratio was used. This mixture approximates the composition of the yeast mitochondrial membrane phospholipid content both in charged and zwitterionic headgroups (POPG substituted for cardiolipin) and in the unsaturation of fatty acyl chains (Tzagoloff, 1982; Sperka-Gottlieb *et al.*, 1989). Injection of synthetic peptides into the subphase beneath the phospholipid monolayer caused an increase in the packing pressure of the monolayer. The phospholipid monolayers were spread at an initial pressure of 20 mN/m which was greater than the equilibrium surface pressure measured for the peptides alone. This reduced the possibility of the formation of pure phospholipid patches at the air-water interface. The L and D presequences and the control peptide exhibited a max-

imum surface pressure ($\Delta\pi_{\max}$) of 12.5 mN/m at concentrations greater than 10 μ M in an air-water surface experiment (Fig. 4B).

Both $F_{1\beta}$ -presequence peptides associated strongly with the phospholipid monolayer which was formed at an initial surface pressure of 20 mN/m. The surface pressure increased due to interaction of the L- and D-peptides with the lipid to yield a $\pi\Delta_{\max}$ of 8.2 mN/meter and 6.68 mN/meter, respectively. The half-maximal concentration determined for insertion was 45 and 60 nM for the L- and D-peptides, respectively (Fig. 4A, Table II). The control peptide, on the other hand, caused no detectable increase in surface pressure under these conditions until the subphase concentration was greater than 10 μ M (Fig. 4A). The maximal increase in surface pressure was not determined for the control peptide because of the prohibitively high quantities of peptide required.

Peptides Fail to Insert into the Bilayer—The strength of association of the presequence with the monolayer was similar to that observed with presequence peptides of the COX4 leader at the same initial monolayer surface pressure (Roise *et al.*, 1986). This association was, however, less than that measured for insertion of bacterial presecretory signals (~12–15 mN/m) which insert deeply into the bilayer. To determine whether the $F_{1\beta}$ -presequence would insert deeply into the bilayer as described for the bacterial OmpA presecretory signal, two experiments were performed to directly compare the affect of the peptides upon interaction with the membrane. Earlier studies of the membrane binding using fluorescence

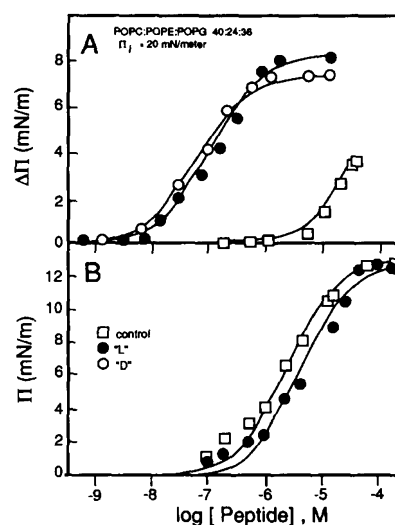


FIG. 4. Surface interactions of D versus L presequence and control peptides at lipid-water and air-water interfaces. A, insertion of the mitochondrial peptides into POPC, POPE, and POPG (40:24:36) lipid monolayers with an initial surface pressure of 20 mN/m. Concentrated solutions of peptide were injected beneath the lipid monolayer into a subphase of 5.0 mM Tris, pH 7.3. B, migration of the mitochondrial peptides at an air-water interface. Concentrated peptide solutions were injected into 5.0 mM Tris, pH 7.3. The L presequence peptide and D presequence peptide data are represented by filled- and open circles, respectively. The open squares represent data obtained from the control peptide.

TABLE II
Mitochondrial peptide monolayer insertion

	$\Delta\pi_{\max}$ mN/m	K_d nM	K_{assoc} 10^7 M^{-1}
$F_{1\beta}$ presequence			
All L	8.02	60	1.7
All D	6.68	45	2.2
$F_{1\beta}$ -ATPase control 33–49	>3.5	>103	

² D. W. Hoyt, unpublished results.

polarization spectroscopy and calorimetric methods had shown that the ornithine transcarbamylase presequence was unable to penetrate deeply into the hydrophobic core of the bilayer (Myers *et al.*, 1987). Similar observations have been made for the COX4 presequence (Tamm, 1986; Roise *et al.*, 1986). Titration of the bilayer with the OmpA peptide caused an increase in the anisotropy value of the DPH reporter in the membrane (Fig. 5). This change in the anisotropy of DPH (decreased fatty acyl chain mobility, Lentz, 1989) due to deep penetration of the OmpA peptide has been reported previously for other bacterial signal peptides and supports the insertion of this protein deep into the core of the bilayer (see also McKnight *et al.*, 1989). By contrast, DPH anisotropy values for the F₁β-presequence (L) peptide exhibited no change even at high protein to lipid ratios. At concentrations of F₁β peptide approaching 3 orders of magnitude greater than that to half-maximally associate with monolayers (Fig. 4), there was no detectable perturbation of the DPH peptide. No difference could be detected between the F₁β signal and the negative control, poly L-lysine, which binds at the vesicle surface via electrostatic interaction with the lipid head groups. This further supports the association of F₁β-subunit presequence with the phospholipid membrane via a binding to the phospholipid headgroups at the membrane surface.

A sensitive and independent measure of the membrane perturbative properties of added peptides is their effect on the rate of carboxyfluorescein leakage from fluorophore-loaded vesicles (Fig. 6). The lytic bacterial signal sequence peptides promote a rapid release of the fluorophore due to membrane break down (McKnight *et al.*, 1989). Upon addition of peptides to carboxyfluorescein-loaded LUVs, the time course of the fluorescein dequenching was monitored at 524 nm. The OmpA bacterial peptide which inserts deeply into the membrane promoted rapid release of fluorophore due to LUV breakdown (Fig. 6A). Complete dequenching was noted upon addition of Triton X-100. By contrast, there was no measurable release of fluorophore due to D- or L- F₁β-presequence peptide as well as poly L-polylysine (Fig. 6B). These data support monolayer and DPH studies that binding of the presequence peptide with a membrane is primarily via the association with the lipid head groups.

Interaction of Presequence Peptides with Mitochondria—To examine the influence of the presequence on the import properties of isolated mitochondria, experiments were designed to determine the effect of the presequence peptides on the electrochemical gradients required for protein import. Import of labeled full-length precursors prepared in a translation lysate was also examined as a function of presequence

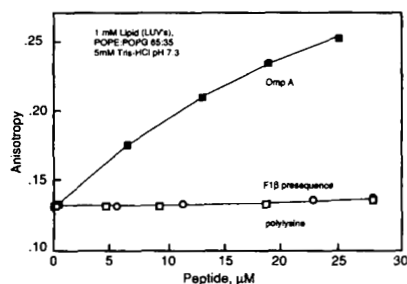


FIG. 5. Steady-state DPH fluorescence anisotropy as a function of peptide concentration. Large unilamellar vesicles (1 mM) of composition POPE/POPG 65:35 (mol %) equilibrated with DPH at 23 °C in 5 mM Tris-HCl, pH 7.3. Fluorescence emission intensity at 430 nm was measured parallel and perpendicular to the plane of excitation (excitation at 360 nm). All anisotropy values were calculated from data obtained immediately after the addition of peptide at the indicated concentration.

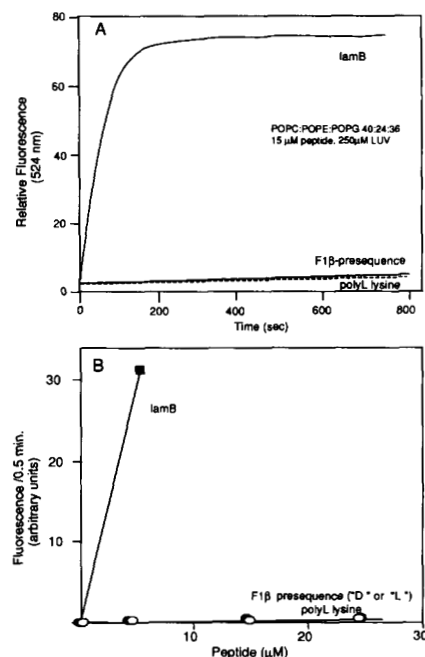


FIG. 6. Effect of peptides on the integrity of small unilamellar vesicles. Vesicles (POPC/POPE/POPG, 39.4:23.8:36.8) loaded with 0.1 M carboxyfluorescein were prepared as described under "Experimental Procedures." The entrapped vesicle volume contained 0.1 M carboxyfluorescein, 5 mM Tris, pH 7.3; the extravesicular buffer was 0.1 M NaCl, 5 mM Tris, pH 7.3. Fluorescein emission at 524 nm was measured (excitation at 480 nm). A, 250 μM SUV treated at a lipid to peptide ratio of 16.6. Dequenching of the fluorophore was measured as an increase in fluorescence yield. B, the initial rate of fluorescein release at 15 μM peptide. lamB, closed squares; D or L presequence peptide, closed circles; poly L-lysine, open circles.

peptide concentration. The influence of the peptides on the coupling properties was determined in two experiments. First, the ability of the peptides to uncouple state 4 mitochondrial respiration was measured (Fig. 7A). Addition of CCCP completely dissipated the membrane potential to yield a maximum stimulation of respiration (4.4-fold). The Omp A secretory signal effectively uncoupled respiratory control with half-maximal stimulation occurring at 5–6 μM peptide. The F₁β-presequence on the other hand at concentrations in vast excess of that required to saturate model membranes yielded no detectable change in state 4 respiration. To increase the sensitivity of the membrane potential measurement, we measured the influence of added peptide on the behavior of the membrane potential-dependent fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide, Di-S-C3(5). Titration of the presequence peptide up to 100 μM caused a gradual but small reduction in the membrane potential, measured as succinate-dependent sequestration of the cationic dye. Relative to complete uncoupling by CCCP or valiniomycin + K⁺, half-maximal reduction in maximal coupling occurred at 50–100 μM. The data in Fig. 7B indicated that at concentrations of the presequence peptides which fail to significantly perturb artificial membranes, there was some effect on ability of mitochondria to maintain a maximal membrane potential.

The influence of the peptides on the import of the F₁-ATPase α- and β-subunit precursors was determined and correlated with the affects measured on the membrane potential (Fig. 8). Surprisingly, import of the labeled precursors was inhibited weakly by the F₁β-presequence alone. Concentrations of L presequence almost 3 orders of magnitude greater than that required to saturate the model membranes exerted

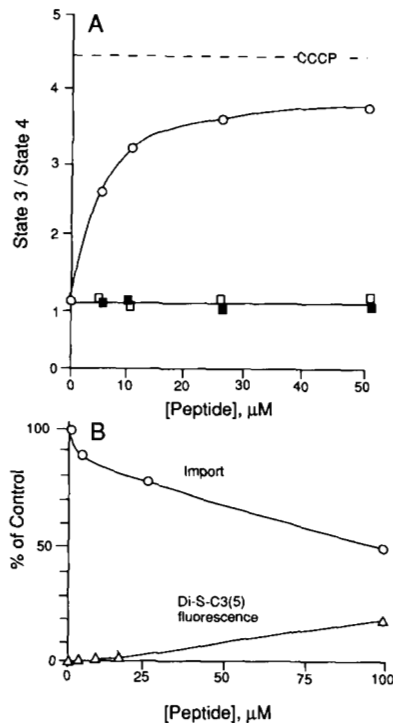


FIG. 7. Influence of the $F_{1\beta}$ -presequence peptide on the respiratory control of mitochondria. *A*, mitochondrial respiration was monitored in a 0.8-ml temperature-controlled chamber containing an oxygen electrode (Yellow Springs International). Mitochondria, 0.4 mg, were suspended in 0.6 M mannitol, 100 mM glycylglycine, 10 mM sodium succinate pH 7.0. For each presequence peptide concentration a linear rate of respiration was recorded as well as for addition of 0.5 μ M CCCP. The respiratory control ratio was calculated as the stimulated rate divided by the initial rate of respiration in the absence of peptide. The maximum respiratory control was established by addition of CCCP after addition of presequence peptide. *Open circles*, addition of 5 μ M ompA peptide; *closed circles*, control peptide, 50 μ M; *open squares*, L presequence peptide, 50 μ M; *closed squares*, D presequence peptide, 50 μ M. *B*, protein import into isolated mitochondria of the $F_{1\beta}$ -subunit precursor is as described under "Experimental Procedures." Control indicates import in the absence of presequence peptide. Mitochondria were stirred in import buffer containing the cationic fluorescent dye Di-S-C3(5) at 2 μ M (see Cyr and Douglas, 1991). Fluorescence increase of Di-S-C3 is compared to a control fluorescence increase measured in the presence of 0.2 μ M valinomycin. Thus, a decrease in the electrochemical potential of protons (uncoupling) is detected as an increase in fluorescence relative to valinomycin-treated mitochondria.

little inhibitory effect. At concentrations of peptide less than 30 μ M, import of the $F_{1\alpha}$ - and $F_{1\beta}$ - subunits was reduced 5 and 20%, respectively. Since the level of competing peptide was in such vast excess over the presequence of the full-length precursor these data indicate that more than the presequence is necessary to block the rate-limiting step in import. Fifty % inhibition was not reached until approximately 100 μ M competitor peptide was added. This gradual inhibition of import by high levels of peptide was paralleled by a reduction in the maximum membrane potential reported by 3,3-Di-S-C3(5). This reduction in $\Delta\psi$ reached about 20% at the highest concentration of peptide tested. The D-peptide exhibited an inhibition profile for $F_{1\alpha}$ - and β -subunit import which was indistinguishable from the L-peptide (Fig. 8). Import of the $F_{1\alpha}$ -subunit precursor was reduced only about 20% at 200 μ M compared to that of the $F_{1\beta}$ -subunit which was inhibited up to 60% by both peptides.

DISCUSSION

Analysis of the membrane binding properties of the β -subunit precursor presequence reveals a peptide behavior like

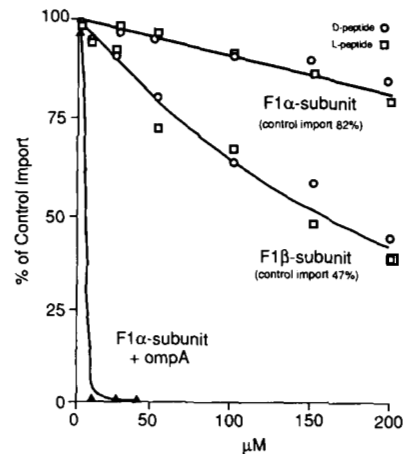


FIG. 8. Protein import into mitochondria. *In vitro* import into yeast mitochondria was measured as previously described (Chen and Douglas, 1987b). Mitochondria were incubated for 5 min with the indicated concentration of peptide prior to the initiation of import at 22 °C by addition of translation lysate harboring either the $F_{1\alpha}$ -ATPase α - or β -subunit precursor (see "Experimental Procedures" for details). In each case following import for 30 min, samples were split and half were treated with proteinase K prior to resolution on a gel. Gels from transport experiments were quantitated by laser scanning densitometry. Import efficiency was calculated as the amount of subunit protected from proteinase K divided by the total amount of precursor plus mature subunit present at the end of the import assay.

that of the polypeptide hormones. One common mechanism of all the polypeptide hormones is that they all appear to bind to a receptor sequestered in the membrane which requires recognition of amphiphilic helical domain(s) (see Segrist *et al.*, 1990). The surface seeking properties of the mitochondrial presequence peptides described here and elsewhere (Epand *et al.*, 1986; Tamm, 1986; Roise *et al.*, 1986; Glaser and Cumsky, 1990) promote the formation of α -helical structure of high hydrophobic movement. From the combination of surface area measurements and intrinsic fluorescence data, these peptides appear to adopt helical conformation along an axis parallel to the plane of the bilayer (Tamm, 1986; Meyers *et al.*, 1987; Roise *et al.*, 1986).

The ability of the presequence to insert deeply into the bilayer appears to be closely coupled to the electrochemical potential across the organelle inner membrane. This insertion may require an electrophoretic mechanism (Roise 1986; Epand *et al.*, 1986; Cyr and Douglas, 1991) or may involve the participation of factors which may operate in conjunction with the presequence (Murikomi and Mori, 1990). The $F_{1\beta}$ -presequence peptides interfered with protein import of subunit precursors but at peptide concentrations almost 3 orders of magnitude higher than that which saturate model membrane systems. At the concentrations of presequence peptide which compete for protein import, we observe some modification of steady-state membrane potential across the inner membrane. We were, however, unable to detect any modification to the binding of the $F_{1\alpha}$ - and $F_{1\beta}$ -precursors on the mitochondrial surface under conditions where 50–100 μ M presequence peptide was present to partially inhibit import.

A binding and insertion model for the delivery of the presequence to the committed step may mimic features of peptide hormone binding to the cell surface. First, the inability of the 20-residue presequence peptide to perturb binding of the full-length mitochondrial precursor to the mitochondrial surface indicates that either more than the presequence may be recognized by surface receptors and/or delivery to the receptor site sequestered within the contact site may require

residues in addition to the amphiphilic presequence. In the latter case, we envision additional sequences providing the sites for the binding of factors either cytosolic or membrane associated which may assist insertion of the presequence to the inner membrane. The strong inhibition of the $F_1\beta$ -presequence plus additional residues is consistent with this model (Cyr and Douglas, 1991). Second, the interaction of these amphiphilic helices which perturb import also perturb the electrochemical potential across the inner membrane. Since the presequence peptide exerts a detectable reduction in the maximal membrane potential at concentrations several orders of magnitude above that required to saturate model membranes, competition by presequences most likely occurs at points where import of full-length precursors occurs. One would anticipate that a general uncoupling to block import would occur at presequence concentrations which saturate the bilayer. This appears not to be the case. Earlier studies have suggested the possibility that presequences may directly bind to the bilayer on the outer membrane and scan the phospholipid surface in search of a proteinaceous receptor element (Epan and *et al.*, 1986). The inability of the presequence peptide to influence import efficiency within the range of saturation binding favors an alternative type of mechanism.

All data presented here are compatible with a model that association of the presequence with the mitochondrial inner membrane precedes the membrane potential dependent translocation step. The surface seeking properties of the peptide are compatible with the formation of an amphiphilic α -helical structure which is on its own unable to penetrate deeply into the inner membrane bilayer. Full-length labeled precursor which is synthesized in the translation lysate is able to effectively compete for insertion at concentrations on the order of 4 orders of magnitude below that of the competing peptide. In the companion article we show that the longer synthetic peptides do effectively compete at low concentration; however, this competition can be overcome with additional full-length precursor from the translation lysate (Cyr and Douglas, 1991). These data suggest that additional peptide sequences carboxyl-terminal to the presequence are important in improving the efficiency of binding and import. Since the additional sequences can be any residues fused to the presequence, these residues most likely serve as a site for action of the translocation factors to assist in inserting the presequence to the membrane potential step. This model makes two predictions. First, a presequence fused to additional residues of any kind should more effectively compete for import of the full-length precursor. Second, the membrane potential is reversibly discharged by the presequence peptide during transport in much the same manner as the return to state 4 of mitochondrial respiration follows phosphorylation of ADP. Data in support of both of these predictions are discussed in the accompanying paper (Cyr and Douglas, 1991).

Acknowledgments—The expert technical assistance of Debbie Caudle is gratefully acknowledged. M. G. D. would like to acknowledge the suggestions of Dr. Christie Brouillette, University of Alabama at Birmingham for the classification peptides which used a combination of different computational methods prior to general availability.

REFERENCES

- Adamson, A. W. (1976) *Physical Chemistry of Surfaces*, pp. 176–227, Wiley, New York
- Allison, D. S., and Schatz, G. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 9011–9015
- Ames, B., and Dubin, D. (1960) *J. Biol. Chem.* **235**, 769–775
- Baker, K., and Schatz, G. (1991) *Nature* **349**, 205–208
- Bedwell, D., Klionsky, D., and Emr, S. (1987) *Mol. Cell Biol.* **7**, 4038–4047
- Bedwell, D., Strockel, S., Yun, K., Jungeward, G., and Emr, S. (1989) *Mol. Cell Biol.* **9**, 1014–25
- Billus, C., Lemire, B., Suda, K., and Schatz, G. (1988) *J. Biol. Chem.* **263**, 13097–13102
- Briggs, M. S., and Gierasch, L. M. (1984) *Biochemistry* **23**, 3111–3114
- Briggs, M. S., Gierasch, L. M., Zlotnick, A., Lear, J. D., and Degrado, W. F. (1985) *Science* **228**, 1096–1099
- Briggs, M. S., Cornell, D. G., Dluhy, R. A., and Gierasch, L. M. (1986) *Science* **233**, 206–208
- Casadaban, M., and Cohen, S. (1980) *J. Mol. Biol.* **138**, 179–207
- Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132–135
- Chen, Y.-H., Yang, J., and Chan, K. (1974) *Biochemistry* **13**, 3350–3359
- Chen, W.-J., and Douglas, M. G. (1987a) *Cell* **49**, 651–658
- Chen, W.-J., and Douglas, M. G. (1987b) *J. Biol. Chem.* **262**, 15598–15604
- Cyr, D., and Douglas, M. G. (1991) *J. Biol. Chem.* **266**, 21700–21708
- Deshais, R., Koch, B., Werner-Washburne, M., Craig, E., and Schekman, R. (1988) *Nature* **322**, 800–805
- Douglas, M. G., McCammon, M., and Vassarotti, A. (1986) *Microbiol. Rev.* **50**, 166–178
- Douglas, M. G., Smagula, C., and Chen, W.-J. (1991) in *Intracellular Trafficking of Proteins* (Steer, C., and Hanover, J., eds) pp. 658–696, Cambridge University Press, Cambridge
- Eilers, M., Oppliger, W., and Schatz, G. (1987) *EMBO J.* **6**, 1073–1078
- Eilers, M., Endo, T., and Schatz, G. (1989) *J. Biol. Chem.* **264**, 2945–2950
- Endo, T., and Schatz, G. (1988) *EMBO J.* **7**, 1153–1158
- Gasser, S. M., Daum, G., and Schatz, G. (1982) *J. Biol. Chem.* **257**, 13034–13041
- Gillespie, L., Argan, C., Taneja, A., Hodges, R., Freeman, K., and Shore, G. (1985) *J. Biol. Chem.* **260**, 16045–16048
- Glaser, S., and Cumsy, M. (1990) *J. Biol. Chem.* **265**, 8808–8816
- Greenfield, N., and Fasman, G. D. (1969) *Biochemistry* **13**, 4108–4116
- Hartl, F.-U., and Neupert, W. (1990) *Science* **247**, 930–938
- Hartl, F.-U., Pfanner, N., Nicholson, D., and Neupert, W. (1989) *Biochem. Biophys. Acta* **988**, 1–45
- Hines, V., Brandt, A., Griffiths, G., Hortsman, H., Brustch, H., and Schatz, G. (1990) *EMBO J.* **9**, 3191–3200
- Hoyt, D. W., McKnight, C. J., Briggs, M. S., Stroup, A. N., and Gierasch, L. M. (1987) *Peptides 1986 Proceedings of the 18th European Peptide Symposium* (Theodoropoulos, D., ed) pp. 373–376, Walter de Gruyter and Co., Berlin
- Huang, C. (1969) *Biochemistry* **8**, 344–352
- Hurt, E., and van Loon, A. P. G. M. (1986) *Trends Biochem. Sci.* **11**, 204–207
- Ito, A., Ogishima, T., Ou, W., Omura, T., Aoyagi, H., Lee, S., Mihara, H., and Izumiya, N. (1985) *J. Biochem. (Tokyo)* **98**, 1571–1582
- Kang, P., Ostermann, J., Schelling, J., Neupert, W., Craig, E., and Pfanner, N. (1990) *Nature* **348**, 137–143
- Kiebler, M., Pfaller, R., Sollner, T., Griffiths, G., Horstmann, H., Pfanner, N., and Neupert, W. (1990) *Nature* **348**, 610–616
- Lemire, B., Fankhauser, C., Baker, A., and Schatz, G. (1989) *J. Biol. Chem.* **264**, 20206–20215
- Lentz, B. (1989) *Chem. Physics Lipids* **50**, 171–190
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Mayer, L., Hope, M., and Cullis, P. (1986) *Biochem. Biophys. Acta* **858**, 161–168
- McKnight, C. J., Briggs, M., Gierasch, L. (1989) *J. Biol. Chem.* **264**, 17293–17297
- Miller, J. (1972) *Experiments in Molecular Genetics*, pp. 352–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Murakami, H., Pain, D., and Blobel, G. (1988) *J. Cell Biol.* **107**, 2051–2057
- Murakami, K., and Mori, M. (1990) *EMBO J.* **9**, 3201–3208
- Myers, M., Mayorga, O., Ematage, J., and Freire, E. (1987) *Biochemistry* **26**, 4309–4315
- Ohta, S., and Schatz, G. (1984) *EMBO J.* **4**, 1129–1135
- Pfanner, N., Tropschug, M., and Neupert, W. (1987) *J. Cell Biol.* **49**, 815–823
- Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., and Schatz, G. (1986) *EMBO J.* **5**, 1327–1334
- Roise, D., Theiler, F., Horvath, S., Tomich, J., Richards, J., Allison, D., and Schatz, G. (1988) *EMBO J.* **7**, 649–653
- Saltzgaber, J., Kunapuli, S., and Douglas, M. G. (1983) *J. Biol. Chem.* **258**, 11465–11470
- Sarin, V. K., Kent, S. B. H., Tam, J. P., and Merrifield, R. P. (1981) *Anal. Biochem.* **117**, 147–150
- Scherer, P., Kreig, U., Huang, S., Vestweber, D., and Schatz, G. (1990) *EMBO J.* **9**, 4315–4322
- Sheffield, W., Shore, G., and Randall, S. (1990) *J. Biol. Chem.* **265**, 11069–11076
- Segrist, J., De Loof, H., Dohlman, J., Brouillette, C., and Anantharamaiah, G. (1990) *Proteins Struct. Funct. Genet.* **8**, 108–117
- Sollner, T., Griffiths, G., Pfaller, R., Pfanner, N., and Neupert, W. (1989) *Cell* **59**, 1061–1070
- Sperka-Gotlieb, C., Hermetter, A., Paltauf, F., and Daum, G. (1989) *Arch. Biochem. Biophys.* **271**, 471–478
- Stewart, J. M., and Young, J. D. (1984) *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Rockford, IL
- Tamm, L. (1986) *Biochemistry* **25**, 7470–7476
- Takeda, M., Chen, W.-J., Saltzgaber, J., and Douglas, M. G. (1986) *J. Biol. Chem.* **261**, 1516–15133
- Tzagoloff, A. (1982) *Mitochondria*, pp. 30, Plenum Press, New York
- Vassarotti, A., Chen, W.-J., Smagula, C., and Douglas, M. G. (1987a) *J. Biol. Chem.* **261**, 411–418
- Vassarotti, A., Stroud, R., and Douglas, M. G. (1987b) *EMBO J.* **6**, 705–712
- Vestveber, D., Brunner, J., Baken, A., and Schatz, G. (1989) *Nature* **341**, 205–209
- Verner, K., and Lemire, B. (1989) *EMBO J.* **8**, 1491–1495
- von Heijne, G. (1986) *EMBO J.* **5**, 1335–1342
- Walker, M., Valentine, E., and Ried, G. (1990) *Biochem. J.* **266**, 227–234