The EMBO Journal vol.7 no.3 pp.649 – 653, 1988

Amphiphilicity is essential for mitochondrial presequence function

David Roise[1,4], Franziska Theiler[1], Suzanna J.Horvath[2] John M.Tomich[2], John H.Richards[2], Daniel S.Allison[1,3] and Gottfried Schatz[1]

[1]Biocenter, University of Basel, 4056 Basel, Switzerland and [2]The Braun Laboratories of Molecular Biology, California Institute of Technology, Pasadena, CA 91125, USA

[3]Present address: Department of Biochemistry, University of Washington, Seattle, WA 98195, USA
[4]Present address: Department of Chemistry, D-006, University of California, San Diego, La Jolla, CA 92093, USA

Communicated by G.Schatz

We have shown earlier that a mitochondrial presequence peptide can form an amphiphilic helix. However, the importance of amphiphilicity for mitochondrial presequence function became doubtful when an artificial presequence, designed to be non-amphiphilic, proved to be active as a mitochondrial import signal. We now show experimentally that this 'non-amphiphilic' presequence peptide is, in fact, highly amphiphilic as measured by its ability to insert into phospholipid monolayers and to disrupt phospholipid vesicles. This result, and similar tests on three additional artificial presequences (two functionally active and one inactive), revealed that all active presequences were amphiphilic whereas the inactive presequence was non-amphiphilic. One of the active presequence peptides was non-helical in solution and in the presence of detergent micelles. We conclude that amphiphilicity is necessary for mitochondrial presequence function whereas a helical structure may not be essential.

Key words: amphiphilicity/presequence/mitochondria/protein import/lipid model systems.

Introduction

Nuclear-encoded proteins which are transported into the mitochondria generally contain amino-terminal extensions which function as signals for sorting and transmembrane transport. Structural analysis of these targeting signals has previously been limited to comparison of their primary sequences by analysis of cloned precursor genes (Watson, 1984; von Heijne, 1986), and to characterization of critical functional domains of the signals by gene fusion experiments (reviewed by Hurt and van Loon, 1986). These studies have shown that a presequence can contain sufficient information for directing an attached protein into mitochondria and that targeting information is not contained within an obvious consensus sequence. However, such approaches cannot define the physical properties of the targeting sequences. Based on experiments with a chemically synthesized presequence peptide, we have previously proposed that the common structural feature of sequences targeting proteins to the mitochondrial matrix may be their ability to form positively charged, amphiphilic helices (Roise *et al.*, 1986). Similar physical properties have also been described for another mitochondrial presequence peptide (Epand *et al.*, 1986; Myers *et al.*, 1987). Since such a structure can insert directly into phospholipid bilayers, it may mediate the initial association of precursors with the mitochondrial outer membrane and may also initiate transfer of attached proteins through the phospholipid bilayers by responding to the electrical potential across the inner membrane. The apparent lack of primary structural requirements for presequences was demonstrated by the finding that artificially created presequences composed of only three different types of amino acid functioned as efficient import signals (Allison and Schatz, 1986). However, these artificial presequences behaved differently than had been predicted from their design: a sequence designed to be weakly amphiphilic as an α-helix functioned even better than one expected to be strongly amphiphilic in helical plots. The question remained whether or not these sequences could form amphiphilic, helical structures in solution. We have now compared synthetic peptides corresponding to five presequences which import attached proteins at widely varying efficiencies and show that all of the functional presequences form amphiphilic, but not necessarily helical, structures.

Results

The primary sequences of the mitochondrial import signals analyzed here are shown in Figure 1. The deletion mutant, Δ11,12, was derived from the wild-type yeast cytochrome oxidase subunit IV (CoxIV) presequence by site-directed mutagenesis. This deletion was designed to create the maximum disruption of the amphiphilic helix (Roise *et al.*, 1986) in a region which was thought to be critical for import of CoxIV fusion proteins (Hurt *et al.*, 1985). The other sequences were artificial targeting signals which had been used to show that precursors require no specific primary sequence for correct functioning (Allison and Schatz, 1986). All the sequences were fused to mouse dihydrofolate reductase (DHFR) in order to test their efficiencies in importing the attached protein into isolated yeast mitochondria (Figure 2). The small deletion within the wild-type CoxIV presequence (Δ11,12) significantly decreased the efficiency of this presequence as an import signal, both if attached to DHFR (Figure 2) and if attached to CoxIV (not shown). The artificial sequences SynA2 and SynC were both functional, although less so than the wild-type sequence, whereas SynB2 was completely inactive. These results are qualitatively similar to those obtained with CoxIV fusions (Allison and Schatz, 1986), except that the SynA2 presequence is somewhat more efficient if fused to DHFR. This further supports the idea that the function of a presequence does not require a specific passenger protein (Hurt and van Loon, 1986) and provided the basis for using synthetic prepeptides

WT
H_3N-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Ser-Ser-Arg-Ser-Gly-Ile-DHFR
H_3N-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-CONH2

Δ11,12
H_3N-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Trp-Gly-Ser-Gly-Ile-DHFR
H_3N-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-CONH2

SynA2
H_3N-Met-Leu-Ser-Arg-Leu-Ser-Leu-Arg-Leu-Leu-Ser-Arg-Leu-Ser-Leu-Arg-Leu-Leu-Ser-Arg-Trp-Gly-Ser-Gly-Ile-DHFR
H_3N-Met-Leu-Ser-Arg-Leu-Ser-Leu-Arg-Leu-Leu-Ser-Arg-Leu-Ser-Leu-Arg-Leu-Leu-Ser-Arg-Tyr-Leu-Leu-CONH2

SynB2
H_3N-Met-Leu-Ser-Arg-Gln-Gln-Ser-Gln-Arg-Gln-Ser-Arg-Gln-Gln-Ser-Gln-Arg-Gln-Ser-Arg-Trp-Gly-Ser-Gly-Ile-DHFR
H_3N-Met-Leu-Ser-Arg-Gln-Gln-Ser-Gln-Arg-Gln-Ser-Arg-Gln-Gln-Ser-Gln-Arg-Gln-Ser-Arg-Tyr-Leu-Leu-CONH2

SynC
H_3N-Met-Leu-Ser-Ser-Leu-Leu-Arg-Leu-Arg-Ser-Leu-Ser-Leu-Leu-Arg-Leu-Arg-Leu-Ser-Arg-Trp-Gly-Ser-Gly-Ile-DHFR
H_3N-Met-Leu-Ser-Ser-Leu-Leu-Arg-Leu-Arg-Ser-Leu-Ser-Leu-Leu-Arg-Leu-Arg-Leu-Ser-Arg-Tyr-Leu-Leu-CONH2

Fig. 1. Sequences of the fusion proteins and synthetic peptides analyzed in this study. DHFR = mouse dihydrofolate reductase (Stone *et al.*, 1979). WT, presequence of yeast cytochrome oxidase subunit IV; Δ11,12, deletion mutant lacking residues 11 and 12 of cytochrome oxidase subunit IV; SynA2, SynB2, SynC, artificial presequences described in Allison and Schatz (1986).

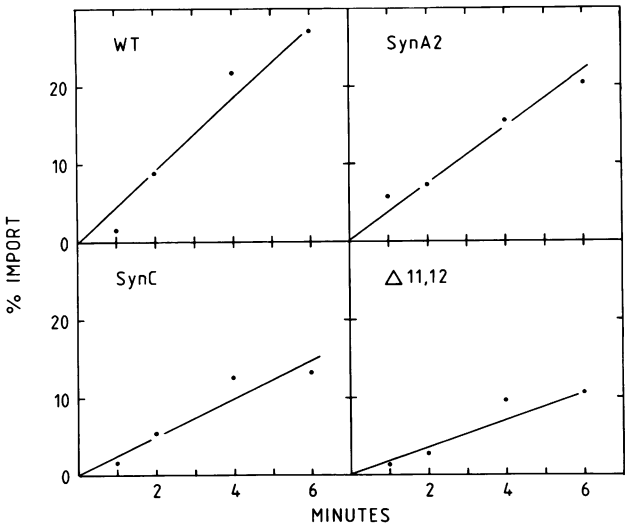

Fig. 2. Time course of import of DHFR fusion proteins. DHFR attached to SynB2 was not imported and is not shown here. Results shown are the average of two experiments.

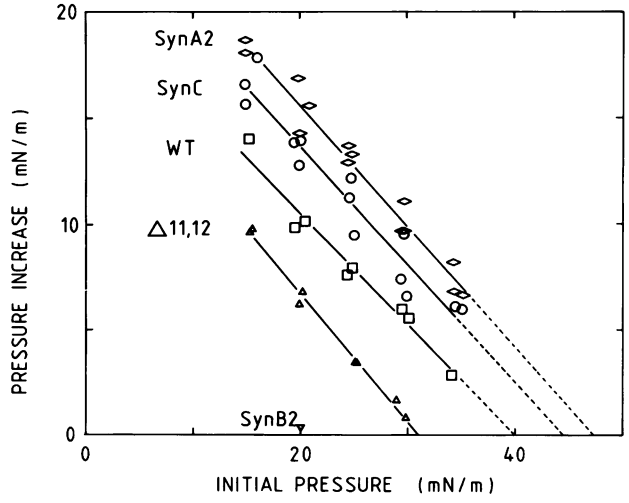

Fig. 3. Functional presequence peptides are surface active. Synthetic peptides were compared for their ability to insert into phospholipid monolayers on a Langmuir trough. Peptide concentrations were: WT = 150 nM; Δ11,12 = 158 nM; SynA2 = 75 nM; SynB2 = 300 nM; SynC = 88 nM.

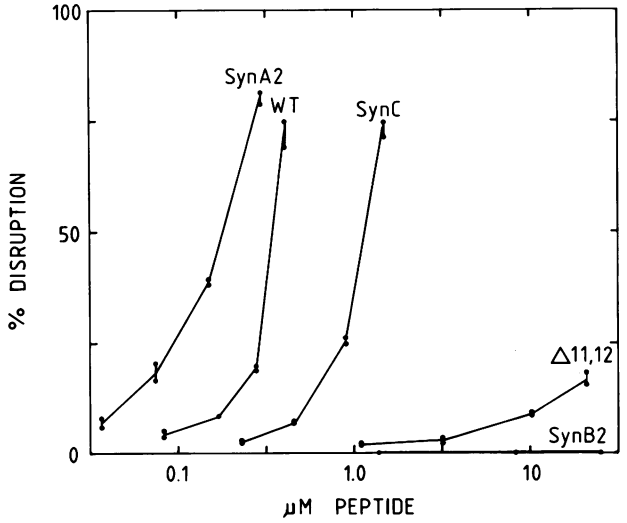

Fig. 4. Functional presequence peptides disrupt phospholipid vesicles. Disruption of vesicles was measured as fluorescence increase (excitation 490 nm, emission 525 nm) after 1 min. Triton X-100 (0.1% final) was added to determine the 100% disruption value. Final lipid concentration was 73 μM.

in studying the physical properties of mitochondrial presequences.

Since the SynA2 and SynC presequences also direct attached CoxIV into mitochondria (Allison and Schatz, 1986) their effectiveness with DHFR as a 'passenger' protein does not simply reflect exposure of a cryptic targeting sequence within DHFR (Hurt and Schatz, 1987). The fact that the targeting efficiencies are different with the two different 'passenger' proteins is in line with the observation that attached proteins may modulate the effectiveness of a mitochondrial presequence (van Steeg *et al.*, 1986).

To test whether amphiphilic helicity is a requirement for efficient import signals, we have synthesized peptides corresponding to the four novel presequences described above. Since the artificially created sequences contain roughly the same charge density as the CoxIV presequence, but differ widely in their efficiencies as mitochondrial import signals, we hoped to identify biophysical parameters which would correlate with biological function. Ability to insert into phospholipid monolayers is one criterion for peptide amphi-

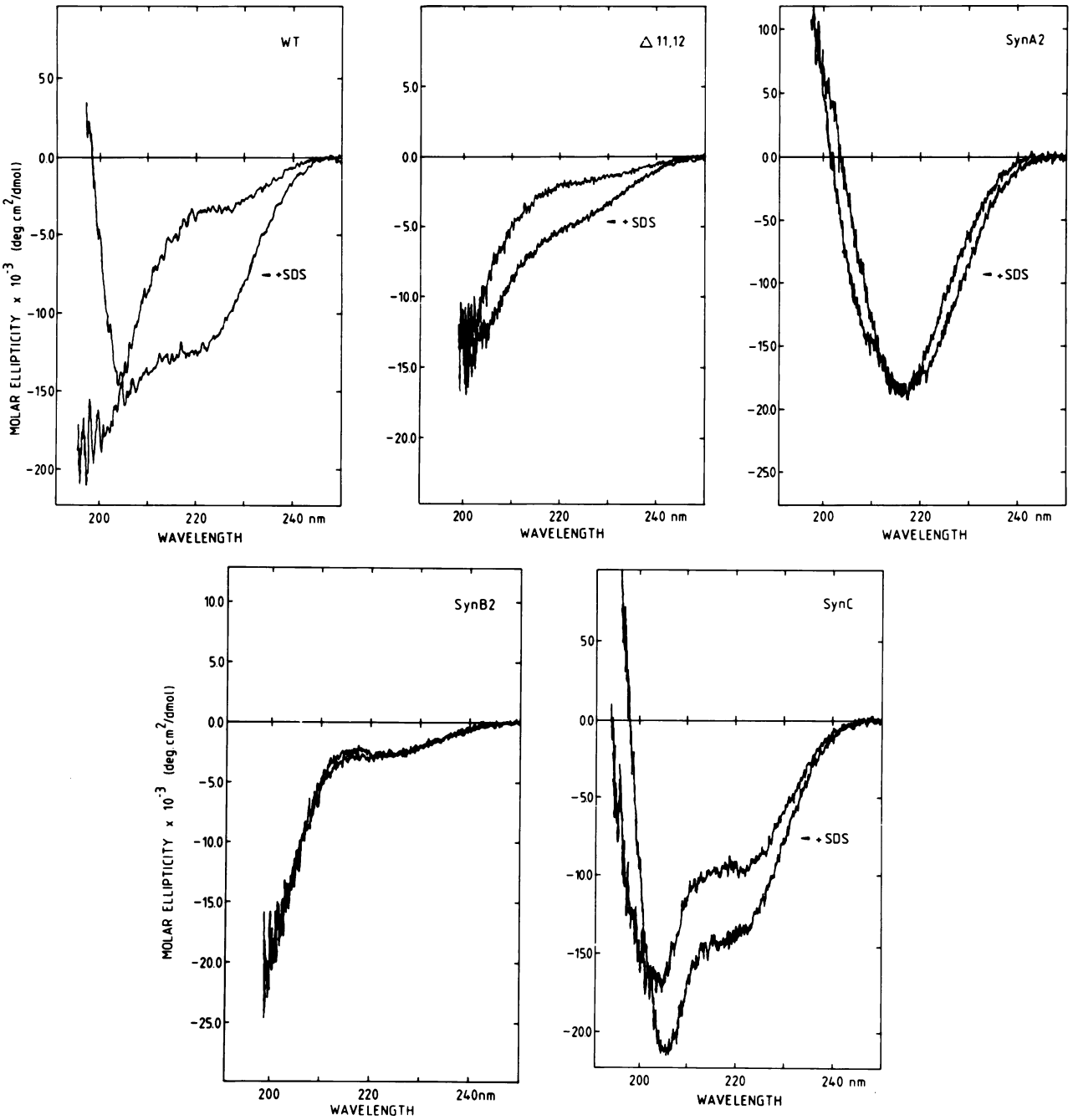

Fig. 5. Circular dichroism reveals the peptide's secondary structures. SynB2 spectra were essentially identical in the presence or absence of SDS. Peptide concentrations were: WT = 0.085 mg/ml; Δ11,12 = 0.093 mg/ml; SynA2 = 0.079 mg/ml; SynB2 = 0.085 mg/ml; SynC = 0.098 mg/ml. Calculated helical contents were: WT = 41% helix; Δ11,12 = 17% helix; SynB2 = 10% helix; SynC = 45% helix. Helical content of SynA2 could not be estimated because of the large amount of β-sheet present.

philicity. Figure 3 shows the peptide-induced increase in the surface pressure of the monolayer as a function of the initial surface pressure. Such plots allow estimation of limiting insertion pressures for each peptide, i.e. the initial pressure against which a peptide would no longer be able to insert into the monolayer. All four functional presequence peptides showed high limiting pressures of insertion into the monolayer, suggesting that they would also have high affinities for binding to phospholipid bilayers. The hydrophilic SynB2 presequence showed no affinity for the monolayer even at low surface pressure. This result correlates well with this peptide's inability to import attached proteins or to bind attached CoxIV to de-energized mitochondria (Allison and Schatz, 1986). Since the peptides SynA2 and SynC had even higher limiting insertion pressures than the wild-type peptide, yet were less efficient as presequences, peptide insertion into monolayers cannot by itself be used as a parameter to predict import function.

As a second criterion for peptide amphiphilicity, disruption of sonicated unilamellar vesicles (SUV) by the peptides was measured. Figure 4 shows rates of release of trapped fluorescent dye from vesicles as a function of peptide concentration. All the functional presequence peptides induced rapid vesicle disruption. The mechanism of this effect is not

known, but it is commonly observed for peptides which insert spontaneously into membrane bilayers (Bernheimer and Rudy, 1986). With the exception of SynA2, the efficiency of vesicle disruption by a peptide corresponded to its efficiency as an import signal. Vesicle disruption is the second criterion by which the functional presequences were found to be amphiphilic.

The presence of secondary structures in these peptides was investigated by circular dichroism measurements (Figure 5). The spectra were determined in the presence and absence of detergent micelles which provide an amphiphilic surface with which the peptides can interact. The wild-type, Δ11,12, and SynB2 peptides showed little secondary structure in the absence of detergent. Under these conditions the SynA2 peptide was present almost completely as a β-sheet, and the SynC peptide was a mixture of α and β structure. Upon addition of SDS, varying degrees of α-helicity were induced. The order of helix induction (based on the change in molar ellipticity at 222 nm) is roughly wild-type > SynC > Δ11,12 > SynA2 > SynB2. Use of lysolecithin instead of SDS to provide the surface for binding resulted in essentially the same spectra (not shown). Although a peptide's structure in the absence of detergent may depend strongly on its concentration and may not reflect the actual unbound presequence structure at extremely low concentrations, the structures induced by the large detergent:peptide ratios used here are likely to represent the actual surface-bound, monomeric conformation of these peptides.

Discussion

The five presequences compared in this study displayed a broad range of efficiencies as import signals, and a comparison of their physical properties could thus suggest common features required for presequence function. The observation that all functional presequence peptides were able to insert into phospholipid monolayers and disrupt phospholipid vesicles supports the view that amphiphilicity is required for presequence function. The CD data revealed that the SynA2 peptide exists in detergent micelles primarily as a β-sheet, suggesting that an α-helical structure is not absolutely required for presequence function. Amphiphilic β-sheets have previously been described (Osterman *et al.*, 1984), and the SynA2 primary sequence suggests that such a structure would be likely with this peptide. The SynC presequence was designed to be a poor amphiphilic helix (Allison and Schatz, 1986), and although the CD spectra of the corresponding peptide showed significant helical content, it seems unlikely that a non-distorted helix with this sequence would show such high surface activity. Clearly a more detailed structural analysis of the peptides is necessary.

Perhaps the most revealing comparison is between the wild-type sequence and its two-residue deletion (Δ11,12). This minor change in primary sequence caused a 2.5-fold decrease in rate of import of attached DHFR (Figure 2). In addition, the corresponding peptide showed a significant decrease in its surface activity (Figure 3) and its ability to disrupt vesicles (Figure 4), as well as a 2-fold decrease in detergent-induced helical content (Figure 5) (all comparisons are relative to the wild-type peptide). Since the penetration of presequences across the mitochondrial inner membrane requires a membrane potential negative inside (Pfanner and Neupert, 1985), part of the lowered efficiency of the Δ11,12 presequence may be due to its slightly decreased positive charge. However, the presequence's lower amphiphilicity probably also contributes to its decreased import efficiency.

What are the structural requirements for functional mitochondrial presequences? Until now, most information has come from predictive methods using known presequence primary structures. An examination of all known natural mitochondrial presequences showed that most of them can potentially form amphiphilic α-helices (von Heijne, 1986). Several reports have described the effects of changes in mitochondrial presequence primary structure on import (Hurt *et al.*, 1985, 1987; Horwich *et al.*, 1985, 1986; Pilgrim and Young, 1987), and the observed decreases in import efficiency can generally be explained as being caused by disruption of portions of a presequence's positively charged, amphiphilic helical structure. Conversely, an analysis of mutations within a non-functional, truncated presequence which restore the ability of the presequence to effect import suggests that these changes increase the ability of the sequence to form a positively charged, amphiphilic helix (Vassarotti *et al.*, 1987). Is such a presequence structure necessary for its function? Although not directly tested in this study, positive charge seems to be a common feature of all presequences (von Heijne, 1986; Baker and Schatz, 1987). This is probably important to provide a thermodynamic driving force for presequence translocation across the inner membrane (Pfanner and Neupert, 1985): transfer of a single charge across a membrane maintaining a 180 mV potential (typical for the mitochondrial inner membrane) provides ~3.6 kcal/mol (Kempf *et al.*, 1982). However, this energy can only be utilized if a kinetically accessible pathway exists for transfer of the charges through the membrane; the ability of a presequence to insert into and perhaps partially disrupt a bilayer may be necessary to accelerate trans-membrane charge transfer. The present results suggest that such properties (i.e. amphiphilicity) are common to all functional mitochondrial presequences. Amphiphilic properties are not limited to peptides having α-helical structure however, and there may be considerable flexibility in the structural requirements for presequences. We conclude that positively-charged, amphiphilic (but not necessarily helical) structure is essential for mitochondrial presequence function.

Materials and methods

All peptides used in this study were synthesized as previously described (Roise *et al.*, 1986). Lipids were obtained from Avanti Polar Lipids, Birmingham, AL, USA. All other chemicals and reagents were of the highest quality available.

The plasmid containing DHFR attached to the wild-type (WT) CoxIV presequence has been described previously (Hurt *et al.*, 1985). The Δ11,12 deletion protein was created by site-directed mutagenesis of the wild-type CoxIV gene in M13 phage using plasmid M13mp11-R/HCOXIV (Allison and Schatz, 1986). This presequence, as well as the three artificial ones, was then fused to DHFR by the following procedure. The gene coding for DHFR was excised from peptide pDS5/2 (Stueber *et al.*, 1984) as a *Bam*HI/*Hin*dIII fragment. This was inserted into plasmid pJF118EH (Fuerste *et al.*, 1986) which had been cut with *Hin*dIII and *Bam*HI. Presequences contained in replicative form phage M13mp11−CX11 derivatives (Allison and Schatz, 1986) were excised as small *Eco*RI−*Eco*RV fragments and inserted in the DHFR-containing pJF118-EH peptide which had been opened with *Eco*RI and *Sma*I. This created an in-frame fusion to the DHFR sequence. All DNA manipulations were carried out essentially as described (Maniatis *et al.*, 1982). For imports, the fusion proteins were expressed in an *in vitro* transcription/translation system as previously described (Hurt *et al.*, 1984)

and then incubated at 30°C with energized yeast mitochondria (Gasser *et al.*, 1982) for the indicated times. Import was stopped by addition of valinomycin (1 μg/ml final) and transfer to ice, and non-imported proteins were digested by treatment with proteinase K (100 μg/ml; 30 min at 0°C). The protease was inhibited by 1 mM phenylmethyl sulfonyl fluoride and the mitochondria were re-isolated through a 20% sucrose cushion (12 000 *g*, 10 min at 4°C). Pellets were dissolved in sodium dodecylsulfate (SDS) sample buffer and analyzed by 12.5% SDS–PAGE. Gels were soaked in 1 M sodium salicylate, dried, and radioautographed with Kodak X-Omat S film. Fluorograms were quantified by scanning, and internally calibrated against a lane containing 10% of the amount of precursor offered to the mitochondria.

The surface activity of the synthetic peptides was measured on a Langmuir trough as previously described (Roise *et al.*, 1986) except that the lipid was 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC):1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) 4:1. Sub-phase was 150 mM NaCl, 10 mM Na-Hepes pH 7.2.

Vesicle disruption experiments were performed essentially as described (Roise *et al.*, 1986). Vesicles were made by sonication of POPC:POPG 4:1 (10 mg/ml) in 50 mM K-carboxyfluorescein, 75 mM K-tartrate, 10 mM K-Hepes, pH 7.2 as described. External dye was removed by Sephadex G-50 gel filtration into 150 mM K-tartrate, 10 mM K-Hepes, pH 7.2. Assays were performed in 2.5 ml of the same buffer in stirred cuvettes at 20°C.

CD spectra were measured using a 1 mm cell on a modified Cary 61 Circular Dicrograph at 20°C. Buffer was 150 mM NaCl, 10 mM Na-Hepes, pH 7.2, with 20 mM SDS as indicated. α-Helix content of each peptide in SDS was calculated from θ_{222} (mean residual ellipticity) according to Chen *et al.* (1974)

Acknowledgements

We would like to acknowledge gratefully the use of the Microchemical Facility in the laboratory of Professor Leroy E.Hood at Caltech. We would also like to thank Dr E.Lanka for plasmid pJF118-EH. This study was supported by grant 3.335-0.86 from the Swiss National Science Foundation, grant CBY-11R01 GM37803-01 from the US Public Health Service and a postdoctoral fellowship (to D.R.) from the Jane Coffin Childs Memorial Fund for Medical Research.

References

Allison,D.S. and Schatz,G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9011–9015.

Baker,A. and Schatz,G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3117–3121.

Bernheimer,A.W. and Rudy,B. (1986) *Biochim. Biophys. Acta*, **864**, 123–141.

Chen,Y.-H., Yang,J.T. and Chau,K.H. (1974) *Biochemistry*, **13**, 3350–3359.

Epand,R.M., Hui,S.-W., Argan,C., Gillespie,L.L. and Shore,G.C. (1986) *J. Biol. Chem.*, **261**, 10017–10020.

Fuerste,J.P., Pansegrau,W., Frank,R., Bloecker,H., Scholz,P., Bagdasarian,M. and Lanka,E. (1986) *Gene*, **48**, 119–131.

Gasser,S.M., Daum,G. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13034–13041.

Horwich,A.L., Kalousek,F. and Rosenberg,L.E. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4930–4933.

Horwich,A.L., Kalousek,F., Fenton,W.A., Pollock,R.A. and Rosenberg,L.E. (1986) *Cell*, **44**, 451–459.

Hurt,E.C. and Van Loon,A.P.G.M. (1986) *Trends Biochem. Sci.*, **11**, 204–207.

Hurt,E.C. and Schatz,G. (1987) *Nature*, **325**, 499–503.

Hurt,E.C., Pesold-Hurt,B. and Schatz,G. (1984) *EMBO J.*, **3**, 3149–3156.

Hurt,E.C., Pesold-Hurt,B., Suda,K., Oppliger,W. and Schatz,G. (1985) *EMBO J.*, **4**, 2061–2068.

Hurt,E.C., Allison,D.S., Mueller,U. and Schatz,G. (1987) *J. Biol. Chem.*, **262**, 1420–1424.

Kempf,C., Klausner,R.D., Weinstein,J.N., van Renswoude,J., Pincus,M. and Blumenthal,R. (1982) *J. Biol. Chem.*, **257**, 2469–2476.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Myers,M., Mayorga,O.L., Emtage,J. and Freire,E. (1987) *Biochemistry*, **26**, 4309–4315.

Osterman,D., Mora,R., Kézdy,F.J., Kaiser,E.T. and Meredith,S.C. (1984) *J. Am. Chem. Soc.*, **106**, 6845–6847.

Pfanner,N. and Neupert,W. (1985) *EMBO J.*, **4**, 2819–2825.

Pilgrim,D. and Young,E.T. (1987) *Mol. Cell. Biol.*, **7**, 294–304.

Roise,D., Horvath,S.J., Tomich,J.M., Richards,J.H. and Schatz,G. (1986) *EMBO J.*, **5**, 1327–1334.

Stone,D., Paterson,S.J., Raper,J.H. and Philips,A.W. (1979) *J. Biol. Chem.*, **254**, 480–488.

Stueber,D., Ibrahimi,I., Cutler,D., Dobberstein,B. and Bujard,H. (1984) *EMBO J.*, **3**, 3143–3148.

van Steeg,H., Oudshoorn,P., van Hell,B., Polman,J.E.M. and Grivell,L.A. (1986) *EMBO J.*, **5**, 3643–3650.

Vassarotti,A., Stroud,R. and Douglas,M. (1987) *EMBO J.*, **6**, 705–711.

von Heijne,G. (1986) *EMBO J.*, **5**, 1335–1342.

Watson,M.E.E. (1984) *Nucleic Acids Res.*, **12**, 5145–5164.

Received on November 4, 1987; revised on December 23, 1987