Mitochondria Can Import Artificial Precursor Proteins Containing a Branched Polypeptide Chain or a Carboxy-terminal Stilbene Disulfonate

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Abstract. A purified, artificial precursor protein was used as a transport vehicle to test the tolerance of the mitochondrial protein import system. The precursor was a fusion protein consisting of mouse dihydrofolate reductase linked to a yeast mitochondrial presequence; it contained a unique cysteine as its COOH-terminal residue. This COOH-terminal cysteine was covalently coupled to either a stilbene disulfonate derivative or, with the aid of a bifunctional cross-linker, to one of the free amino groups of horse heart cytochrome c. Coupling to horse heart cytochrome c generated a mixture of branched polypeptide chains since this cytochrome lacks a free alpha-amino group. Both adducts were imported and cleaved by isolated yeast mitochondria. The mitochondrial protein import machinery can thus transport more complex structures and even highly charged "membrane-impermeant" organic molecules. This suggests that transport occurs through a hydrophilic environment.

A large body of evidence suggests that tightly folded proteins cannot be transported across a biological membrane (Zimmermann and Meyer, 1986; Eilers and Schatz, 1988). It is not clear, however, how completely a protein must unfold in order to be accommodated by the translocation machinery. To test the tolerance of this machinery, we have constructed an artificial mitochondrial precursor protein which contains a unique cysteine residue at its COOH terminus. This precursor can be easily and specifically coupled to a variety of molecules and the resulting adducts can be tested for their ability to import into mitochondria.

We show that this artificial precursor can function as the transport vehicle for a "membrane-impermeant" stilbene disulfonate and for cytochrome c that is attached via a branched-chain configuration. This result suggests that import of proteins into mitochondria occurs through a hydrophilic pore which can accommodate structures different from, and more complex than, fully extended linear polypeptide chains.

Materials and Methods

Construction of the Fusion Protein

The starting material was the gene-encoding fusion protein 7/188/189 (Vestweber and Schatz, 1988*a*) inserted into plasmid pDS5/2-1 (Stueber et al., 1984; Hurt et al., 1985). The fusion protein consisted of the first 16 residues of the yeast cytochrome oxidase subunit IV precursor linked to the NH_2 terminus of a modified mouse dihydrofolate reductase (DHFR)[1] whose COOH terminus had been extended by the addition of glutamine and cystein. The 5′ half of the fusion gene was excised as an Eco RI–Sac I fragment and replaced by the corresponding fragment from plasmid pDS5/2-1-pcox IV-DHFR (Hurt et al., 1984); the fusion gene carried by that plasmid encoded the first 22 residues of the cytochrome oxidase subunit IV precursor linked to DHFR. The new fusion gene generated by the exchange of Eco RI–Sac I fragments was excised as an Eco RI–Hind III fragment and inserted into M13 mp 10. The two cysteine codons (corresponding to position 19 of the presequence and position 7 of DHFR) were replaced by serine codons (Zoller and Smith, 1983). No selection step was used in this method except that DNA obtained by primer extension was transformed into the repair-deficient *Escherichia coli* strain BMH 71-18 *mutS* (Kramer et al., 1984). The resulting fusion gene was excised as an Eco RI–Hind III fragment and inserted into the *E. coli* expression vector pKK 223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Labeling and Purification of the Fusion Protein

The fusion gene carried on plasmid pKK 223-3 was expressed in *E. coli* strain W3110 in the presence of $[^{35}S]O_4^{--}$ as follows. 20 ml of a stationary *E. coli* culture in LB medium (Maniatis et al., 1982) were harvested and the cells were resuspended in 200 ml of minimal medium (van Loon et al., 1983) supplemented with 1 mM of each amino acid except cysteine and methionine. The cells were allowed to grow for 3 h at 37°C, harvested, washed once with water, and transferred to 200 ml of $MgSO_4$-free minimal medium containing 1 mM isopropyl thiogalactopyranoside. After 15 min at 37°C, 6 mCi of $[^{35}S]O_4^{--}$ (>1,000 Ci/mmol) was added and incubation was continued for another 15 min at 37°C. From then on, all steps were performed at 0°C. The cells were sedimented and washed first in 2 ml of 30% sucrose, 20 mM KPi, pH 8.0, 1 mM EDTA, and then in 2 ml of water. Lysis of the cells, precipitation of DNA by protamine sulfate, and chromatography of the extracted proteins on a CM-Sephadex C50 column was done as described by Eilers and Schatz (1986). Fractions were analyzed by SDS-12% PAGE and fluorography, pooled, and dialyzed against 1 liter of 0.25 M KCl, 20 mM Hepes-KOH, pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10% (vol/vol) glycerol for 10 h, and then twice for 12 h each against 1 liter of the same buffer without DTT. Aliquots were frozen in liquid nitrogen and stored at −70°C. They

1. *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; MBS, maleimido-benzoyl-*N*-hydroxysuccinimide ester.

could be stored for ≥1 mo without detectable loss of import efficiency. The yield was 60–75 μg and the specific activity 6–7 × 10^5 dpm/μg protein.

Derivatization of the Purified Fusion Protein

Horse heart cytochrome c (type VI; Sigma Chemical Co., St. Louis, MO) was dissolved to 10 mg/ml in 100 μl of 20 mM NaPi, pH 7.0, and reacted for 10 min at room temperature with a fivefold molar excess of maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). MBS was added as an 80-mM solution in DMSO. Excess cross-linker was removed by gel filtration through a Sephadex G-10 spin-column (Pharmacia Fine Chemicals) that was centrifuged for 5 min at 1,000 *g* in a 1-ml plastic syringe. Residual cross-linker in the eluate (100 μl) was blocked by adding 10 μl of 1 M Tris/HCl, pH 7.0, incubating for 10 min at room temperature, and repeating the gel filtration step. The MBS-derivatized cytochrome c was added in 200-fold molar excess to the purified fusion protein. After 30 min at room temperature, remaining free maleimide groups were blocked by adding DTT to 2 mM and incubating for 5 min at room temperature.

The membrane-impermeant fluorescent dye, 4-acetamido-4′-maleimidyl-stilbene-2,2′-disulfonic acid (Molecular Probes Inc., Junction City, OR) was dissolved to 4.9 mg/ml (10 mM) in 50 mM NaPi, pH 7.0, and added in 1,000-fold molar excess to the purified fusion protein for 1 h at room temperature. Excess maleimide groups were blocked by 2 mM DTT and incubation for 5 min at room temperature.

Miscellaneous

Mitochondria were isolated from the wild-type *Saccharomyces cerevisiae* strain D273-10B (ATCC 25657; MAT α) as described (Daum et al., 1982). Import experiments were done as outlined in the accompanying paper (Vestweber and Schatz, 1988*b*) with 200 μg of mitochondria and 20 μl of free or derivatized fusion protein (corresponding to 250 μg of purified fusion protein). All other methods are outlined in the accompanying paper (Vestweber and Schatz, 1988*b*).

Results

Construction of the Artificial Precursor Protein

The artificial precursor protein used in this study was a fusion protein whose single cysteine residue was the COOH-terminal amino acid. The protein could thus easily and specifically be derivatized at its COOH terminus. The protein is a variant of the previously described fusion protein which consists of a mitochondrial presequence (the first 22 residues of the yeast cytochrome oxidase subunit IV precursor) linked to mouse DHFR. This protein is readily imported and cleaved by mitochondria in vitro and in vivo (Hurt et al., 1984, 1985). The protein's two cysteine residues (in position 19 of the presequence and in position 7 of DHFR) were replaced by serine residues, and its COOH terminus was extended by glutamine and cysteine. The protein was expressed and labeled with [^{35}S]O_4^{--} in *E. coli*, and purified. A typical purification yielded between 60 and 75 μg protein (from 200 ml culture). Its specific radioactivity was ~6–7 × 10^8 dpm/mg and its purity >95% as judged by silver staining of an SDS–polyacrylamide gel (Fig. 1).

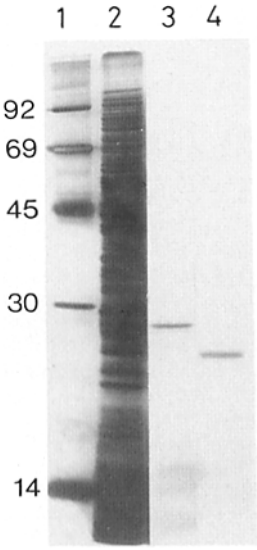

Figure 1. Purification of the radiolabeled fusion protein. The photograph of a SDS–12% polyacrylamide gel stained with silver is shown. Lane *1*, molecular mass markers (molecular masses in kD are marked on the left); lane *2*, 0.05% of the total *E. coli* extract; lane *3*, 125 ng of the purified fusion protein; and lane *4*, purified authentic mouse DHFR.

COOH-Terminal Modification of the Fusion Protein

The first coupling reaction involved covalent attachment of 4-acetamido-4′-maleimidyl-stilbene-2,2′-disulfonate (Fig. 2) to the protein's COOH-terminal sulfhydryl group. The reaction was essentially quantitative with respect to the fusion protein. The product migrated more slowly on SDS–polyacrylamide gels (Fig. 3, compare lanes *1* and *2*). Although the structure of the adduct was not checked further, the properties of the two reactants predict a single, well-defined product.

The second coupling reaction involved covalent attachment of cytochrome c. Horse heart cytochrome c was first derivatized with the bifunctional cross-linker MBS at one of its many internal lysine residues and then coupled via the free maleimide group of the cross-linker to the fusion protein's COOH-terminal cysteine (horse heart cytochrome lacks a free sulfhydryl group). Since the alpha-amino group of horse heart cytochrome c is acetylated (Margoliash et al., 1961), this coupling reaction should generate branched-chain isomers. We did not characterize these isomers in more detail. However, of the 19 lysine residues in horse heart cytochrome c, one is at position 5 and another five residues upstream of the COOH terminus; the minimal branch size of the adducts is thus four amino acid residues. Analysis of the reaction product by SDS–12% PAGE confirmed that >90% of the fusion protein was converted to a discrete band of lower mobility. Adducts containing more than one cytochrome c per molecule were not detected in significant amounts (Fig. 4, compare lanes *1* and *2*).

Both Precursor Derivatives Are Imported and Cleaved by Isolated Mitochondria

When the precursor derivatives were incubated with energized yeast mitochondria, ~1/3 of the molecules were cleaved to a smaller size (Fig. 3, lane *6*; Fig. 4, lane *3*). The cleaved molecules were inaccessible to externally added protease (Fig. 3, lane *7*; Fig. 4, lane *4*) unless the mitochondrial membranes were lysed with Triton X-100 (Fig. 3, lane *8*; Fig. 4, lane *5*). Deenergized mitochondria failed to cleave or internalize the precursor derivatives (Fig. 3, lanes *9* and *10*; Fig. 4, lanes *6* and *7*). By these criteria, both precursor derivatives are imported into isolated mitochondria.

In the derivative containing cytochrome c, the cytochrome c moiety is unlabeled. Resistance of the imported precursor

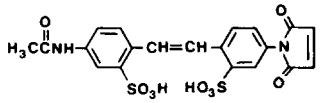

Figure 2. Structure of the charged fluorescent dye 4-acetamido-4′-maleimidyl-stilbene-2,2′-disulfonic acid.

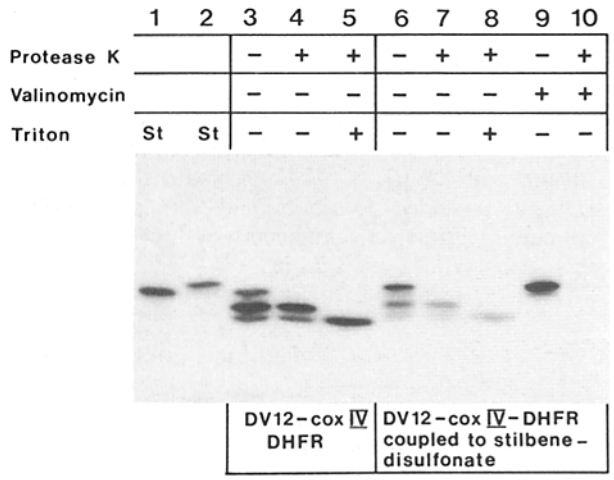

Figure 3. The fusion protein containing a COOH-terminal stilbene disulfonate is imported by isolated mitochondria. The ^{35}S-labeled adduct was presented to energized yeast mitochondria and the mitochondria were then analyzed by SDS-12% PAGE and fluorography. Lane *1*, underivatized fusion protein; lane *2*, derivatized fusion protein (>95% appears to be derivatized as indicated by the mobility shift). Lanes *1* and *2*, 20% of the amount of fusion protein that was added to each import assay. Lanes *3* and *6*, mitochondria that had imported the fusion protein or its adduct; lanes *4* and *7*, same as lanes *3* and *6* but mitochondria treated with 500 μg proteinase K/ml for 15 min followed by addition of 2 mM PMSF before analysis; lanes *5* and *8*, same as lane *4* but protease treatment performed on mitochondria that had been lysed by 1% Triton X-100; lane *9*, mitochondria incubated with the derivatized fusion protein in the presence of valinomycin; lane *10*, same as lane *9* but mitochondria treated with proteinase K immediately before analysis. A fluorogram of the dried SDS-polyacrylamide gel is shown. The fusion protein used here (DV12-cox IV-DHFR) is cleaved twice, probably because of the mutation that had been introduced two residues upstream of the normal cleavage site. As noted before (Eilers and Schatz, 1986), the imported DHFR moiety is cleaved to a protease-resistant fragment in the presence of Triton X-100 and proteinase K. This fragment migrates only slightly faster than the smaller of the two "mature" forms of the fusion protein generated by the matrix-located processing protease.

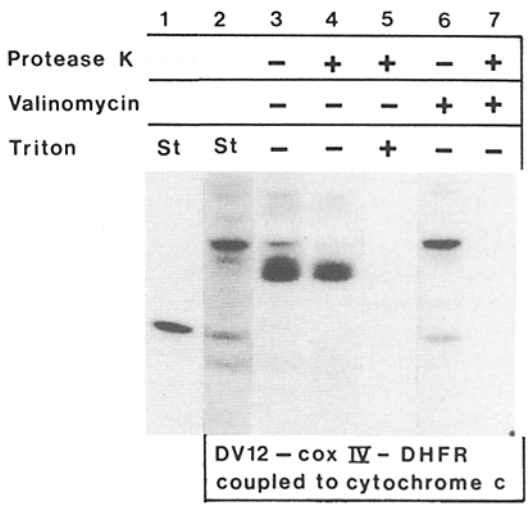

Figure 4. The fusion protein coupled to cytochrome c is imported by isolated mitochondria. Experimental details were as described in Fig. 3. Lane *1*, underivatized fusion protein; lane *2*, 20% of the cytochrome c adduct that was added to each of the import assays; lane *3*, mitochondria that had imported the adduct; lane *4*, same as lane *3* but after treating the mitochondria with 500 μg proteinase K/ml for 15 min at 0°C, followed by addition of 2 mM PMSF; lane *5*, same as lane *3* but after treating the mitochondria with protease in the presence of 1% Triton X-100; lane *6*, mitochondria that had been incubated with the adduct in the presence of the uncoupler valinomycin; lane *7*, same as lane *6* but after treating the mitochondria with protease. In this, the protease-resistant DHFR fragment (see legend to Fig. 3) is not observed (lane *5*), perhaps because the attached cytochrome c had impaired tight folding of the DHFR moiety.

derivative to externally added proteinase K in the absence of Triton, thus, does not exclude the possibility that the cytochrome c moiety had remained outside the mitochondria, but had escaped digestion merely because it is inherently resistant to proteinase K. This possibility was rendered unlikely by the observation that iodinated horse heart cytochrome c was readily degraded by proteinase K (not shown). Also, any conformational alteration induced by the coupling procedure should increase, rather than decrease, the susceptibility to protease.

Upon subfractioning the mitochondria by controlled lysis with detergent (Fig. 5), the cleaved underivatized fusion protein and the cleaved fusion protein derivatized with the stilbene dilsulfonate were completely recovered with the soluble matrix; similarly, 80–90% of the cleaved fusion protein coupled to cytochrome c was recovered with the soluble matrix contents. Under these conditions, >90% of two mitochondrial membrane proteins remain in the pellet (Vestweber and Schatz, 1988*b*). The COOH-terminally modified fusion proteins, thus, do not become stuck in the membrane, but appear to be completely transported across both mitochondrial membranes.

Import Kinetics of the Derivatized Fusion Proteins

Isolated mitochondria imported the two derivatives with approximately the same rate and similar efficiency as the underivatized fusion protein (Fig. 6). The underivatized fusion protein, in turn, is imported into mitochondria at a similar rate and efficiency as the F_1-ATPase β-subunit (Eilers, M., and T. Endo, unpublished observations). We conclude that all these proteins use identical or very similar import mechanisms. The data reported in the accompanying paper (Vestweber and Schatz, 1988*b*) strongly support this view.

Discussion

Earlier work in several laboratories has shown that the information for translocating a precursor protein across a membrane resides predominantly, if not exclusively, in the precursor's signal sequence (Lingappa et al., 1984; Hurt et al., 1984; Horwich et al., 1985; van den Broeck et al., 1985). The attached polypeptide chain may interfere with the function of the signal sequence or determine the ultimate transmembrane orientation of the protein, but it does not appear to contribute essential information to the translocation process itself. Once the translocation machinery has engaged a precursor protein via the signal sequence, it seems to be "blind" to the nature of the passenger protein.

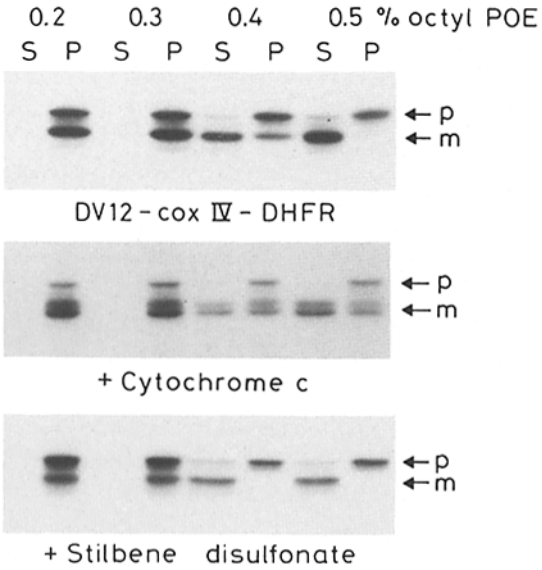

Figure 5. Both derivatives of the fusion protein are imported into the soluble matrix space. Mitochondria were allowed to import the underivatized fusion protein (DV12–cox IV–DHFR), its adduct with cytochrome c (+ Cytochrome c), and its adduct with the stilbene disulfonate (+ Stilbene disulfonate). They were then reisolated by centrifugation and lysed with the indicated concentrations of the nonionic detergent octyl-polyoxyethylene (*octyl-POE*) as described in the accompanying paper (Vestweber and Schatz, 1988*b*). After ultracentrifugation, pellets (*P*) and supernatants (*S*) were analyzed by SDS–12% PAGE and fluorography for the distribution of uncleaved (*p*) and cleaved (*m*) fusion protein.

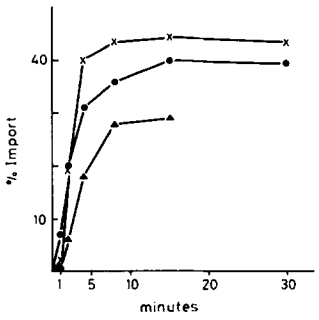

Figure 6. Time course of import into mitochondria for the underivatized fusion protein and its adducts with cytochrome c and the stilbene disulfonate. Precursors were incubated with isolated mitochondria for the indicated times at 30°C and import was stopped by addition of 10 μg/ml valinomycin. Mitochondria were subsequently treated with 0.5 mg/ml proteinase K for 15 min at 0°C. The protease was stopped by addition of 2 mM PMSF. Mitochondria were reisolated, lysed in gel electrophoresis sample buffer, and analyzed by SDS–12% PAGE and fluorography. The mature size precursor band was scanned, the amount of added precursor to the import reaction was taken as 100%. (×) Underivatized fusion protein; (●) stilbene dilsulfonate adduct; (▲) cytochrome c adduct.

The present study shows that the mitochondrial protein import system even tolerates passenger proteins that are branched or that carry highly charged organic molecules which are normally considered to be membrane impermeant. This makes it unlikely that a polypeptide chain is moved across the membrane by specific recognitions of peptide bonds and ratchet-like transposition of these bonds along a fully extended polypeptide chain. If this were true, the branched cytochrome c adducts would be translocated only with difficulty, if at all.

Transport of branched polypeptide chains may even be a physiological process. It has been shown that cell surface proteins such as the lymphocyte homing receptor gp90^{MEL14} is conjugated to ubiquitin, which is probably attached via an isopeptide bond (Siegelman et al., 1986). Since ubiquitin is a cytosolic protein, ubiquitination of cell surface proteins requires the transport of a branched polypeptide into the lumen of the endoplasmic reticulum. While there is no direct evidence for such a process in the endoplasmic reticulum, our findings with the cytochrome c adduct show that branched polypeptide chains can be transported across a biological membrane.

During their translocation across the endoplasmic reticulum or the two mitrochondrial membranes, precursor proteins remain accessible to urea and alkaline pH (Gilmore and Blobel, 1985; Pfanner et al., 1987). This has been interpreted as evidence that the polypeptide chains are translocated through a hydrophilic channel which remains accessible to water-soluble protein denaturants. Our present data support and extend this conclusion; the fact that a stilbene disulfonate readily passes a membrane if attached to an appropriate precursor protein places a lower limit on the size of the proposed hydrophilic channel. It also raises the intriguing possibility that precursor proteins may pass a membrane after they have been posttranslationally modified by phosphorylation or ADP-ribosylation. It is even conceivable that nucleotides or nucleic acids may be transported across a membrane provided they are attached to an appropriate precursor protein. This possibility deserves closer study since there is strong, if still indirect, evidence that mitochondria import some of their RNA species from the cytoplasm (Attardi and Schatz, 1988). The tolerance of the protein translocation machinery is not limitless, however; the accompanying paper shows that a tightly folded 6-kD protein domain cannot pass mitochondrial membranes (Vestweber and Schatz, 1988*b*). Similarly, artificial precursors containing a small domain with an intramolecular disulfide bond attached to prepromelittin, or a tightly folded 6-kD metallothionein domain fused to a truncated F_1-ATPase β-subunit precursor, fail to pass the endoplasmic reticulum or the mitochondrial membranes, respectively (Müller and Zimmermann, 1988; Chen and Douglas, 1987).

We did not expect that the cytochrome c adduct described here would be translocated. Cytochrome c is not only a stably folded globular protein, but is internally cross-linked: two thioether bonds originating from internal cysteine residues attach the protoheme group to the holocytochrome, thereby generating a covalent internal loop. This stable structure has frequently been invoked to explain why only the heme-free apocytochrome c interacts with lipid model systems and passes the mitochondrial outer membrane (Korb and Neupert, 1978; Rietveld and de Kruijff, 1984; Pilon et al., 1987; Dumont and Richards, 1984). While it is highly likely that the cytochrome c moiety in our cytochrome c adduct had retained the covalently bound heme group, we have not proved this directly since the small amounts of adduct available to us made such a proof difficult. If the heme group is still present, then holocytochrome c is not inherently unable to pass a membrane; its inability to do so in vitro may rather be caused by "hiding" of its noncleaved signal sequence. The normal import pathway of apocytochrome c is probably different from that of the vast majority of mitochondrial precur-

sor proteins since apocytochrome c import does not need a membrane potential. The complete import inhibition for the cytochrome c adduct in the absence of a membrane potential therefore indicates that this construct is not imported by the normal apocytochrome c pathway, but via the pathway used by the great majority of mitochondrial proteins.

By attaching various ligands to its COOH terminus and testing the translocation competence of the resulting adducts, the artificial precursor described here could be used as a convenient yardstick to study the steric properties of the mitochondrial protein import system.

We wish to thank the colleagues in our laboratory for critically reading the manuscript, M. Probst for secretarial assistance, and L. Müller and M. Jäggi for the photography and artwork.

This study was supported by grants 3.335.0.86 from the Swiss National Science Foundation and CBY-1 1 R01 GM37803-01 from the US Public Health Service; and an European Molecular Biology Organization long-term fellowship to D. Vestweber.

Received for publication 16 June 1988, and in revised form 26 July 1988.

References

Attardi, G., and G. Schatz. 1988. The biogenesis of mitochondria. *Annu. Rev. Cell Biol.* In press.

Chen, W.-J., and M. G. Douglas. 1987. The role of protein structure in the mitochondrial import pathway. Unfolding of mitochondrially bound precursors is required for membrane translocation. *J. Biol. Chem.* 262:15605–15609.

Daum, G., P. C. Boehni, and G. Schatz. 1982. Import of proteins into mitochondria: cytochrome b_2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* 257:13028–13033.

Dumont, M. E., and F. M. Richards. 1984. Insertion of apocytochrome c into lipid vesicles. *J. Biol. Chem.* 259:4147–4156.

Eilers, M., and G. Schatz. 1986. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature (Lond.).* 322:228–232.

Eilers, M., and G. Schatz. 1988. Protein unfolding and the energetics of protein translocation across biological membranes. *Cell.* 52:481–483.

Gilmore, R., and G. Blobel. 1985. Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. *Cell.* 42:497–505.

Horwich, A. L., F. Kalousek, I. Mellmann, and L. E. Rosenberg. 1985. A leader peptide is sufficient to direct mitochondrial import of a chimeric protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1129–1135.

Hurt, E. C., B. Pesold-Hurt, and G. Schatz. 1984. The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3149–3156.

Hurt, E. C., B. Pesold-Hurt, K. Suda, W. Oppliger, and G. Schatz. 1985. The first twelve amino acids (less than half of the pre-sequence) of an imported mitochondrial protein can direct mouse cytosolic dihydrofolate reductase into the yeast mitochondrial matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2061–2068.

Korb, H., and W. Neupert. 1978. Biogenesis of cytochrome c in Neurospora crassa. Synthesis of apocytochrome c, transfer to mitochondria and conversion to holocytochrome c. *Eur. J. Biochem.* 91:609–620.

Kramer, B., W. Kramer, and H.-J. Fritz. 1984. Different base/base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E. coli*. *Cell.* 38:879–887.

Lingappa, V. R., J. Chaidez, C. S. Yost, and J. Hedgpeth. 1984. Determinants for protein localization: betalactamase signal sequence directs globin across microsomal membranes. *Proc. Natl. Acad. Sci. USA.* 81:456–460.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Margoliash, E., E. L. Smith, G. Kreil, and H. Tuppy. 1961. Amino-acid sequence of horse heart cytochrome c. *Nature (Lond.).* 192:1125–1127.

Müller, G., and R. Zimmermann. 1988. Import of honeybee pre-promellitin into the endoplasmic reticulum: energy requirements for membrane insertion. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:639–648.

Pfanner, N., F.-U. Hartl, B. Guiard, and W. Neupert. 1987. Mitochondrial precursor proteins are imported through a hydrophilic membrane environment. *Eur. J. Biochem.* 169:289–293.

Pilon, M., W. Jordi, B. de Kruijff, and R. A. Demel. 1987. Interactions of mitochondrial precursor protein apocytochrome c with phosphatidylserine in model membranes. A monolayer study. *Biochim. Biophys. Acta.* 902:207–216.

Rietveld, A., and B. de Kruijff. 1984. Is the mitochondrial precursor protein apocytochrome c able to pass a lipid barrier? *J. Biol. Chem.* 259:6704–6707.

Siegelman, M., M. W. Bond, W. M. Gallatin, T. St. John, H. T. Smith, V. A. Fried, and I. L. Weissman. 1986. Cell surface molecule associated with lymphocyte homing is a ubiquitinated branched-chain glycoprotein. *Science (Wash. DC).* 231:823–829.

Stueber, D., I. Ibrahimi, D. Cutler, B. Dobberstein, and H. Bujard. 1984. A novel in vitro transcription–translation system: accurate and efficient synthesis of single proteins from cloned DNA sequences. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3143–3148.

van den Broeck, G., M. P. Timko, A. P. Kausch, A. R. Cashmore, M. van Montagu, and L. Herrera-Estrella. 1985. Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase. *Nature (Lond.).* 313:358–363.

van Loon, A. P. G. M., A. C. Maarse, H. Riezman, and L. A. Grivell. 1983. Isolation, characterization and regulation of expression of the nuclear genes for the core II and Rieske iron–sulphur proteins of the yeast ubiquinol–cytochrome c reductase. *Gene (Amst.).* 26:261–272.

Vestweber, D., and G. Schatz. 1988*a*. Point mutations destabilizing a precursor protein enhance its post-translational import into mitochondria. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1147–1151.

Vestweber, D., and G. Schatz. 1988*b*. A chimeric mitochondrial precursor protein with internal disulfide bridges blocks import of authentic precursors into mitochondria and allows quantitation of import sites. *J. Cell Biol.* 107: 2037–2043.

Zimmermann, R., and D. I. Meyer. 1986. 1986: a year of new insights into how proteins cross membranes. *Trends Biochem. Sci.* 11:512–514.

Zoller, M. J., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* 100:468–500.