The EMBO Journal vol.10 no.7 pp.1759-1766, 1991

Cytosolic and mitochondrial surface factor-independent import of a synthetic peptide into mitochondria

Shigeki Furuya, Katsuyoshi Mihara, Saburo Aimoto[1] and Tsuneo Omura

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Higashi-ku, Fukuoka, Fukuoka 812, and [1]Research Center for Protein Engineering, Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan

Communicated by W.Neupert

We chemically synthesized a peptide, 11β-45, which was composed of 45 amino acid residues including the whole extension peptide and some of the mature portion of bovine cytochrome P-450(11β) precursor. 11β-45 was imported into mitochondria *in vitro* depending on the mitochondrial membrane potential, but its import did not require extramitochondrial ATP. Although cytosolic protein factors in the high speed supernatant of reticulocyte lysate are known to stimulate the import of various precursor proteins into mitochondria, the import of 11β-45 was not stimulated by cytosolic factors in reticulocyte lysate. The import of the peptide did not require mitochondrial surface protein components because its import was not affected by trypsin treatment of mitochondria. On the other hand, trypsin treatment of mitoplasts resulted in a great reduction in the import of the peptide, indicating that 11β-45 interacts during the import process with some protein components located inside mitochondria. These observations indicated that the peptide 11β-45 was imported via the potential-dependent pathway as in the case of precursor proteins, but skipped the interactions with cytosolic factors and mitochondrial surface components normally required for the import of precursor proteins.

Key words: adrenal cortex mitochondria/cytosolic factors/mitochondrial protein import/P-450(11β) precursor/synthetic peptide

Introduction

Mitochondrial proteins except the products of the mitochondrial genome are encoded by nuclear genes and synthesized on cytosolic free polysomes. Most newly synthesized mitochondrial proteins carry an extension peptide (extra-peptide, presequence) composed of 20–60 amino acid residues at the amino terminus of the mature portion. They are post-translationally imported into mitochondria depending on the electrochemical potential and hydrolysis of ATP through the contact site between the outer and inner membranes, and subsequently processed to mature forms by specific processing protease(s) (for reviews, see Hartl *et al.*, 1989).

Several lines of evidence indicate that the information necessary for the import of precursor proteins into mitochondria is contained in the extension peptides. When some extension peptides were attached to the amino terminus of a cytosolic protein, the fusion proteins were imported into mitochondria (Hurt *et al.*, 1984; Horwich *et al.*, 1985a). Partial or whole chemically synthesized extension peptides inhibited the import of various precursors (Gillespie *et al.*, 1985; Furuya *et al.*, 1987). Because the precursors were not imported when the basic amino acids were substituted with neutral amino acids, the basic amino acids in the extension peptides are essential for the import (Horwich *et al.*, 1985b; Kumamoto *et al.*, 1987).

Recent studies have revealed several components which seem to participate in the import of precursor proteins into mitochondria. One group of these components is extra-mitochondrial factors. Requirements for HSP70 (Deshaies *et al.*, 1988) and other cytosolic proteins of reticulocyte lysate (Murakami and Mori, 1990; Ono and Tuboi, 1990b; Sheffield *et al.*, 1990) or yeast (Murakami *et al.*, 1988) in the import of various precursors have been shown genetically and biochemically. The other is mitochondrial factors.

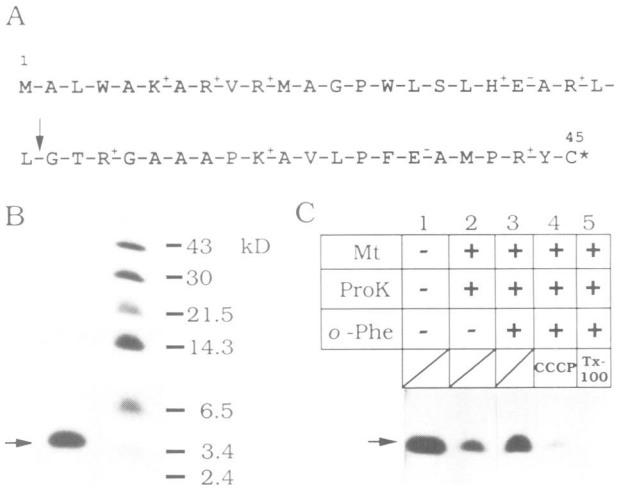

Fig. 1. Structure, electrophoretic pattern and import of 11β-45. **(A)** Amino acid sequence of 11β-45. The terminal cysteine residue marked with an asterisk was used for ^{3}H-labeling. The arrow indicates the cleavage point of the extension peptide. **(B)** Urea–SDS–PAGE of [^{3}H]11β-45. [^{3}H]11β-45 (24 000 d.p.m.) was electrophoresed followed by fluorography as described in Materials and methods. Positions of [^{3}H]11β-45 and [^{14}C]methylated protein molecular weight markers are indicated by an arrow and bars, respectively, in the figure. **(C)** Import of 11β-45 into isolated mitochondria. *In vitro* import of [^{3}H]11β-45 (54 pmol) into bovine adrenal cortex mitochondria (100 μg) was carried out as described in Materials and methods with (lanes 3, 4 and 5) or without (lane 2) preincubation with 0.5 mM *o*-phenanthroline. 40 μM CCCP was added to the import reaction mixture shown in lane 4. After the import reaction, mitochondria were treated with proteinase K in the absence (lanes 2, 3 and 4) or presence (lane 5) of 1% Triton X-100. The treated mitochondria were subjected to urea–SDS–PAGE and the band of [^{3}H]11β-45 was visualized by fluorography. 25% of the input peptide was also electrophoresed (lane 1). The arrow in the figure indicates the imported [^{3}H]11β-45.

Specific binding of several precursors to liposomes containing cardiolipin, mitochondria-specific acidic phospholipid, has been observed (Ou *et al.*, 1988). Putative mitochondrial surface receptor components have been identified by means of specific antibodies to outer membrane proteins with *Neurospora* (Söllner *et al.*, 1989, 1990) and yeast (Vestweber *et al.*, 1989; Hines *et al.*, 1990; Pain *et al.*, 1990), and also by means of affinity chromatography using a synthetic extension peptide with rat liver (Ono and Tuboi, 1990c). A processing protease has also been purified from *Neurospora* (Hawlistschek *et al.*, 1988), yeast (Yang *et al.*, 1988) and rat liver mitochondria (Ou *et al.*, 1989; Kleiber *et al.*, 1990). However, the molecular mechanism of the import of precursor proteins into mitochondria is still unclear.

Studies on the import of synthetic partial or whole extension peptides into mitochondria have recently been reported as a simple model system for the import of precursor proteins (Ono and Tuboi, 1988; Glaser and Cumsky, 1990b). In this study, we chemically synthesized a relatively long peptide, 11β-45, and used it as the artificial substrate for the import machinery. 11β-45 was composed of 45 amino acid residues corresponding to the amino-terminal portion of bovine cytochrome P-450(11β) precursor including the whole extension peptide (Kirita *et al.*, 1988) (Figure 1A). The use of 11β-45 enabled us to detect and analyze the import of the intact whole peptide with a suitable gel electrophoresis system.

11β-45 was imported into isolated bovine adrenal cortex mitochondria depending on the mitochondrial electrochemical potential. However, the import of 11β-45 was not stimulated by cytosolic factors in reticulocyte lysate and was not susceptible to trypsin treatment of mitochondria. Some characteristics of the import of 11β-45 were apparently different from those of precursor proteins, and seemed to skip one or a few initial steps involved in the whole import process of precursor proteins.

Results

Import of 11β-45 into isolated mitochondria

^{3}H-labeled 11β-45 could be detected and quantitated as a clear band by urea–SDS–PAGE followed by fluorography as shown in Figure 1B. The mobility of the labeled peptide on the gel was in agreement with its calculated molecular weight of 4967 daltons.

Mitochondria were incubated with ^{3}H-labeled 11β-45 and the labeled peptide was detected and quantitated after proteinase K treatment of the mitochondria as shown in Figure 1C, lanes 2 and 3. Addition of *o*-phenanthroline to the *in vitro* import system increased the amount of the proteinase K-resistant 11β-45 5-fold. The proteinase K-resistant peptide was greatly reduced by addition of 40 μM CCCP (Figure 1C, lane 4). 11β-45 was not detected when the mitochondria were treated with proteinase K in the presence of Triton X-100 (Figure 1C, lane 5). From these observations, we concluded that 11β-45 was imported into the inside of the mitochondria in an electrochemical potential-dependent manner.

Figure 2 shows the time course of the import of 11β-45.

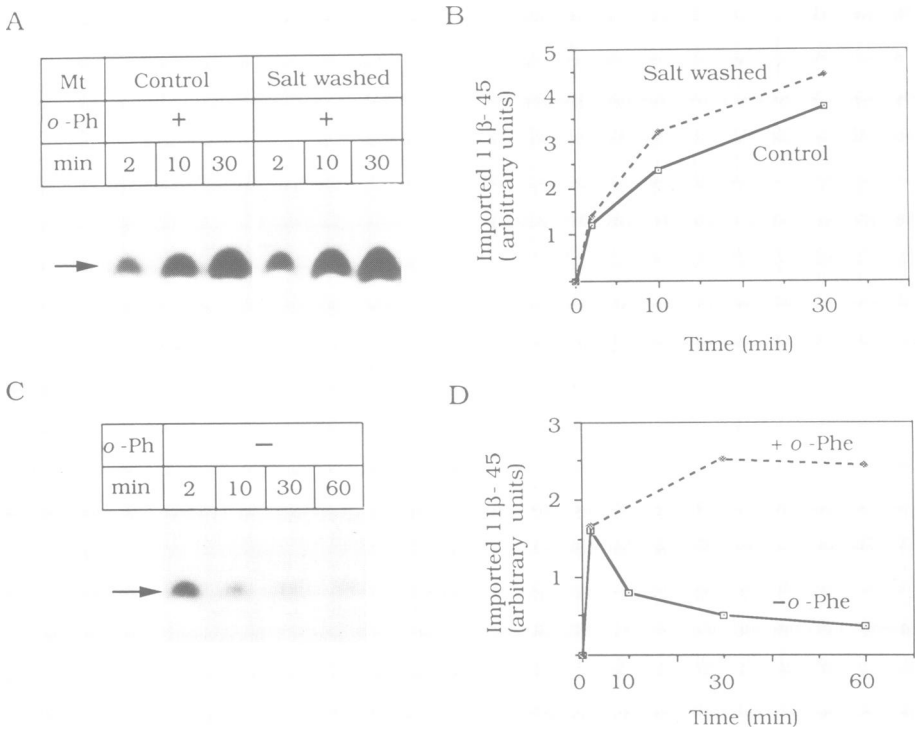

Fig. 2. Time course of the import of 11β-45 into isolated mitochondria in the presence (A and B) or absence (C and D) of 0.5 mM *o*-phenanthroline. (**A** and **B**) [^{3}H]11β-45 (135 pmol) was incubated with 100 μg of bovine adrenal cortex mitochondria at 30°C for 2, 10 and 30 min in the presence of 0.5 mM *o*-phenanthroline. Two preparations of mitochondria, one washed with SHE (control) and another washed with SHE containing 0.5 M potassium acetate (salt washed), were used. The import was analyzed (A) as described in the legend to Figure 1 and quantitated (B) by scanning densitometry of the bands with Shimadzu Double Wavelength Chromatoscanner CS-930. One unit on the ordinate of (B) corresponds to 5.5% of the total input peptide. (**C** and **D**) *In vitro* import of 116 pmol of [^{3}H]11β-45 into 100 μg of bovine adrenal cortex mitochondria was carried out for 2, 10, 30 and 60 min in the absence or presence of 0.5 mM *o*-phenanthroline. The import was analyzed (C) and quantitated (D) as described above. One unit on the ordinate of figure D corresponds to 10% of the total input peptide. The arrows in the figures indicate the imported [^{3}H]11β-45.

In the presence of *o*-phenanthroline, the amount of the imported 11β-45 increased in proportion to the incubation time (Figure 2A and B), reaching a plateau at 30 min (Figure 2D). Washing of the mitochondria with 0.5 M potassium acetate before the import reaction did not affect the import of 11β-45 (Figure 2A and B). When the metal chelator was not present, the amount of the imported 11β-45 was maximum at around 2 min and then decreased rapidly (Figure 2C and D).

Submitochondrial location of imported 11β-45

To examine intramitochondrial location of the imported 11β-45, proteinase K-treated mitochondria were subfractionated by hypotonic treatment followed by sonication after the import reaction. 36% of the imported 11β-45 was recovered in the matrix and intermembrane space fraction and the rest in the membrane fraction. Most of the membrane-associated 11β-45 was bound to the membranes by some mechanism other than ionic interactions because only 6% of the peptide was extracted from the membrane with 1 M NaCl. When proteinase K-treated mitochondria were treated with digitonin (0.25 mg/mg mitochondria) at which concentration most monoamine oxidase activities were solubilized (Hovius *et al.*, 1990), the imported 11β-45 was not solubilized (data not shown). Judging from these observations, the imported 11β-45 was mainly bound to the inner membrane.

Unlabeled 11β-45 inhibited the import of labeled 11β-45 and adrenodoxin precursor

The import of ^{3}H-labeled 11β-45 was inhibited by excess of unlabeled 11β-45 and the inhibition was ~80% at 30 μM (Figure 3A). The import of adrenodoxin precursor was also strongly inhibited by 10−30 μM unlabeled 11β-45 (Figure 3B). It was noted that the adrenodoxin precursor associated with mitochondria increased in the presence of unlabeled 11β-45 and that unidentified polypeptide bands appeared above the band of adrenodoxin precursor (Figure 3B, lanes 2 and 3). We could not explain these phenomena. The unidentified bands were, however, occasionally observed when the import of adrenodoxin precursor was inhibited by other synthetic peptides (S.Furuya and H.Aoyagi, unpublished observations).

AEP1-14, a synthetic partial extension peptide corresponding to the amino-terminal 14 amino acid residues of the extension peptide of adrenodoxin precursor, inhibited the import of adrenodoxin precursor and other precursors into mitochondria at 10−30 μM (Furuya *et al.*, 1987). Unexpectedly, however, the import of 11β-45 was inhibited by only 40% even at 30 μM AEP1-14 (Figure 3A), although the import of adrenodoxin precursor was almost completely inhibited, as reported previously (Figure 3B). The reason for incomplete inhibition of the import of 11β-45 by AEP1-14 is unclear. The inhibitory action of 11β-45 and AEP1-14 on the import was not due to the collapse of mitochondrial membrane potential, which was confirmed by the use of a membrane potential-sensitive cyano dye, 3,3′-dipropylthiocarbocyanine (Kinnally *et al.*, 1978 and data not shown).

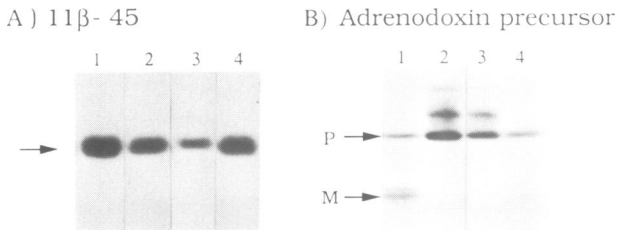

Fig. 3. Unlabeled 11β-45 inhibited the import of [^{3}H]11β-45 and adrenodoxin precursor. Various concentrations of unlabeled 11β-45 or AEP1-14 were preincubated with 100 μg of bovine adrenal cortex mitochondria at 0°C for 10 min, and then 60 pmol of [^{3}H]11β-45 (**A**) or 2 μl of *in vitro* translation products containing adrenodoxin precursor (**B**) were added to the mitochondrial suspensions. The import reaction was carried out and analyzed. The arrow in (A) indicates the imported [^{3}H]11β-45. P and M in (B) denote the precursor and the mature form of adrenodoxin, respectively. Lane 1, without unlabeled peptide; lane 2, 10 μM 11β-45; lane 3, 30 μM 11β-45; lane 4, 30 μM AEP1-14.

Energy requirement of the import of 11β-45

The import of precursor proteins into mitochondria requires the electrochemical potential of the inner membrane and NTPs (Pfanner and Neupert, 1986; Eilers *et al.*, 1987). We characterized the energy source of the import of 11β-45 (Figure 4). Isolated mitochondria without any supplements showed a low level of import. The import of 11β-45 was stimulated by the addition of ATP and a substrate of the mitochondrial electron transport chain, succinate, and the extent of stimulation was ~5-fold. When extramitochondrial ATP was depleted by treatment with potate apyrase and CAT while the membrane potential was maintained by succinate, the import was highly stimulated. This phenomenon could be related to an increase of intramitochondrial ATP by CAT in the presence of a membrane potential (Hwang and Schatz, 1989). On the other hand, when the membrane potential was dissipated by oligomycin and antimycin whereas ATP was supplied extramitochondrially, the extent of the import was lowered to the level of isolated mitochondria only. These observations indicated that the import of 11β-45 did not require extramitochondrial ATP but needed the existence of mitochondrial membrane potential.

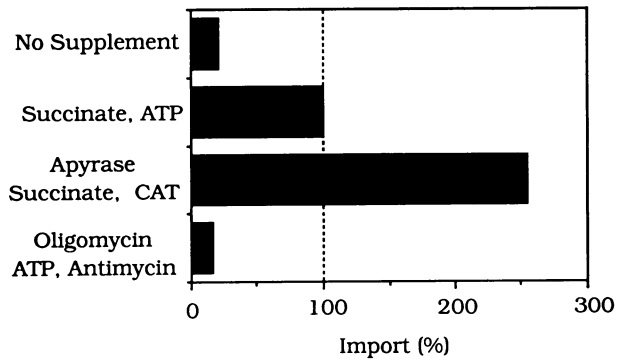

Fig. 4. Energy requirement for the import of 11β-45. Bovine adrenal cortex mitochondria were prepared and suspended in IMB without ATP and sodium succinate. 100 μg of mitochondria were incubated with 0.5 mM *o*-phenanthroline in a volume of 100 μl at 0°C for 15 min and the additives indicated in the figure were also included in the incubation medium. The final concentration of each additive was as follows; carboxyatractyloside (CAT) 40 μg/ml, oligomycin 25 μg/ml, antimycin 5 μg/ml, apyrase 8 U/100 μl, sodium succinate (succinate) 10 mM and ATP 2 mM. Apyrase was incubated with the mitochondria at 30°C for 10 min before the addition of other additives. [^{3}H]11β-45 (105 pmol) was added and the mixtures were incubated at 30°C for 30 min. After the incubation, the import of the peptide was analyzed and quantitated by counting the bands excised from the gel with a liquid scintillation counter in ACSII solution.

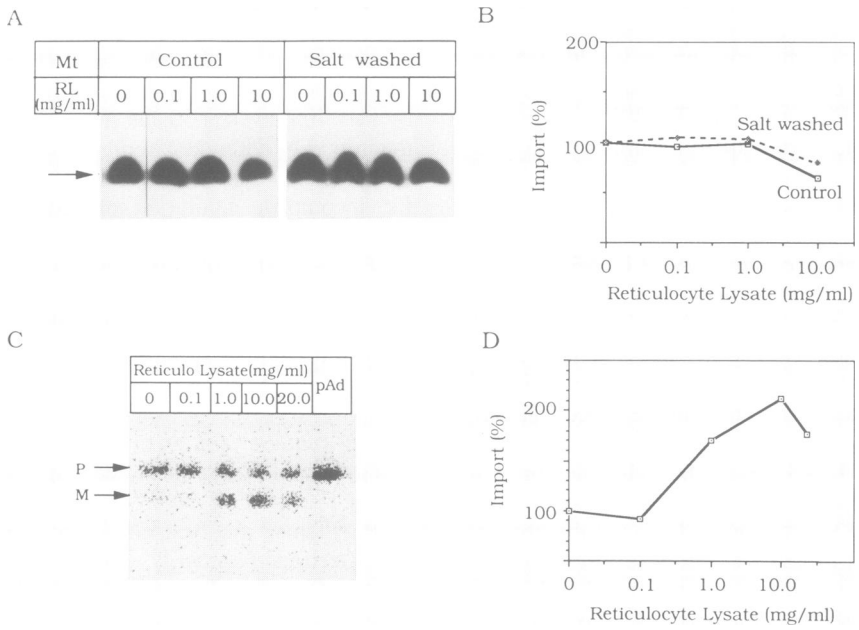

Fig. 5. Effect of a high speed supernatant of reticulocyte lysate on the import of 11β-45 and adrenodoxin precursor. (**A** and **B**) [^{3}H]11β-45 (135 pmol in 4 μl) and the high speed supernatant of reticulocyte lysate (0.1, 1.0 and 10.0 mg/ml) were incubated with 100 μg of mitochondria in a volume of 100 μl at 0°C for 10 min and then the import reaction was started by shifting the incubation temperature to 30°C. Two preparations of mitochondria, one washed with SHE (control) and another washed with SHE containing 0.5 M potassium acetate (salt washed), were used as in Figure 2A. After the incubation, the import was analyzed (A) and quantitated (B) as in Figure 2. The arrow in the figure indicates the imported [^{3}H]11β-45. (**C** and **D**) Purified adrenodoxin precursor (5 μl) and a high speed supernatant of the reticulocyte lysate (0.1, 1.0, 10 and 20 mg/ml) were incubated with 100 μg of bovine adrenal cortex mitochondria in 100 μl at 0°C for 10 min. The import reaction was carried out at 30°C for 30 min and then the mitochondria were washed and subjected to SDS−PAGE. The import of adrenodoxin precursor and analyzed by Fuji Bioimage Analyzer BAS2000. The precursor and the resultant mature form after the import reaction were visualized (C) by pictrography. The radioactivity of the mature form of adrenodoxin was quantitated (D) by BAS2000. 50% of the input precursor was electrophoresed and is shown in (C) (pAd). P and M in (C) denote the precursor and the mature form of adrenodoxin, respectively.

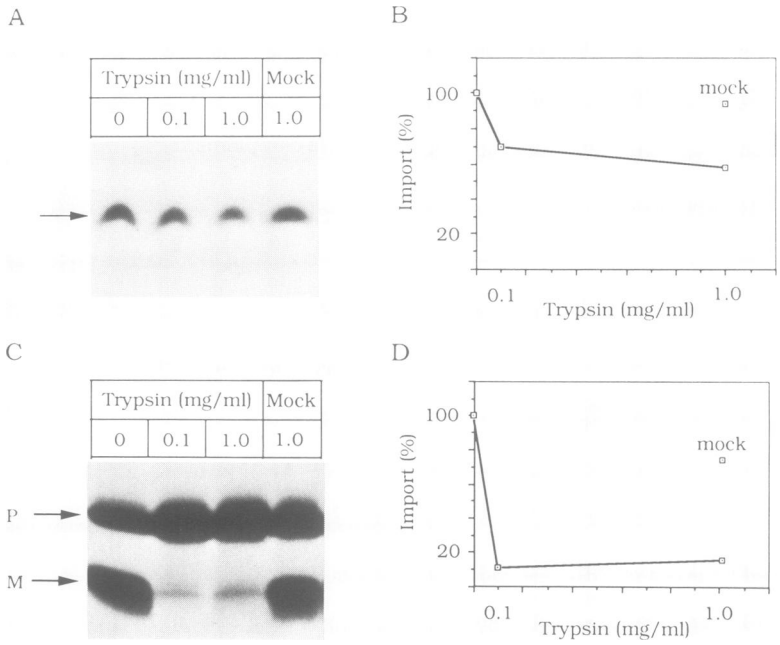

Fig. 6. Import of 11β-45 and adrenodoxin precursor into trypsin-treated mitochondria. Bovine adrenal cortex mitochondria were treated with 0.1 or 1.0 mg/ml trypsin. [^{3}H]11β-45 (116 pmol) or 2 μl of *in vitro* translation products containing adrenodoxin precursor were incubated with 100 μg of trypsin-treated mitochondria at 30°C for 30 min. The import of the peptide was analyzed (**A**) and quantitated (**B**) as in Figure 2. The import of the precursor was analyzed (**C**) and the radioactivity of the mature form was quantitated (**D**) with Fuji Bioimage Analyzer BAS2000. Mock in the figures indicates the experiments in which trypsin inhibitor and Trasylol were added before the addition of trypsin. The arrow in (A) indicates the imported [^{3}H]11β-45. P and M in (C) denote the precursor and the mature form of adrenodoxin, respectively.

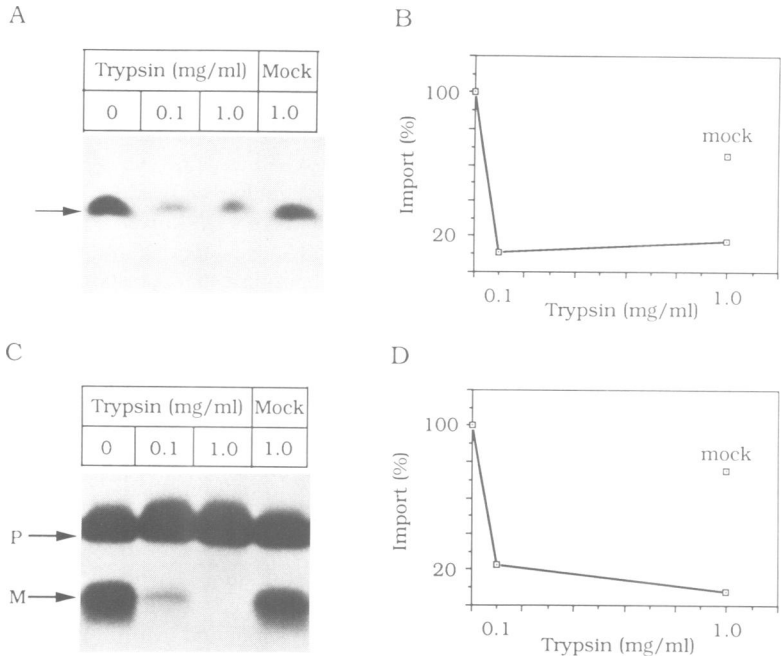

Fig. 7. Import of 11β-45 and adrenodoxin precursor into trypsin-treated mitoplasts. Bovine adrenal cortex mitoplasts were treated with 0.1 or 1.0 mg/ml trypsin. The import of [^{3}H]11β-45 and adrenodoxin precursor in trypsin-treated mitoplasts was carried out as described for Figure 6. The import of the peptide was analyzed (**A**) and quantitated (**B**). The import of the precursor was analyzed (**C**) and quantitated (**D**) as in Figure 6. The arrow in (A) indicates the imported [^{3}H]11β-45. P and M in (C) denote the precursor and the mature form of adrenodoxin, respectively.

Effects of cytosolic factors on the import of 11β-45 and adrenodoxin precursor

It has recently been reported from several laboratories that cytosolic factors stimulate the import of precursor proteins (Murakami *et al.*, 1988; Randall and Shore, 1989; Murakami and Mori, 1990) and a synthetic extension peptide (Ono and Tuboi, 1988) into isolated mitochondria. We examined the effect of cytosolic factors in reticulocyte lysate on the import of 11β-45. Assuming that some endogenous cytosolic factors might bind to the outer surface of the isolated mitochondria, we prepared mitochondria with and without washing with 0.5 M potassium acetate solution. Figure 5A and B shows that the high speed supernatant of reticulocyte lysate did not stimulate the import with either mitochondrial preparation. Different preparations of reticulocyte lysate and bovine adrenal cortex cytosol also showed no stimulation of the import of 11β-45 (data not shown). Neither 0.1 mg/ml rat liver HSP70 nor HSP70 with 1 mg/ml of high speed supernatant of reticulocyte lysate stimulated the import (data not shown). Binding of 11β-45 to the surface of isolated mitochondria at 4 °C was also not affected in the presence of reticulocyte lysate (data not shown). We concluded that the import of 11β-45 into isolated mitochondria was not dependent on cytosolic factors. On the other hand, the import of adrenodoxin precursor was stimulated by reticulocyte lysate as shown in Figure 5C and D.

The observation indicated that the reticulocyte lysate used in this study contained effective cytosolic factors which promoted the import of precursor proteins into mitochondria and that the import of 11β-45 was independent of the cytosolic factors.

Import of 11β-45 was not sensitive to the treatment of mitochondria with trypsin

It has been established that a protein component(s) on the outer surface of mitochondria is required for the import of various precursors (Zwizinski *et al.*, 1984; Ohba and Schatz, 1987a,b; Pfaller *et al.*, 1989). In agreement with this concept, the import of adrenodoxin precursor was greatly reduced by the treatment of mitochondria with only 0.1 mg/ml trypsin (Figure 6C and D). On the contrary, treatment of mitochondria with trypsin did not abolish the import of 11β-45; 70% of the control import level was still retained even after the same treatment of mitochondria (Figure 6A and B). These findings indicated that the protein receptor located on the surface of mitochondria was not essential for the import of 11β-45 but was necessary for the import of adrenodoxin precursor.

To investigate the requirement for protein component(s) other than the surface receptor in the import of 11β-45, trypsin treatment of mitoplasts was carried out and its effect on the import was examined. 11β-45 was imported into mitoplasts as efficiently as into intact mitochondria. However, the import of 11β-45 into mitoplasts was greatly reduced by the treatment with trypsin (Figure 7A and B). Trypsin treatment also abolished the import of adrenodoxin precursor into mitoplasts as is the case with intact mitochondria (Figure 7C and D). The membrane potential of mitoplasts was not affected by trypsin treatment (data not shown). The import of 11β-45 into mitochondria seems to be dependent on some protein component(s) which was accessible to trypsin after the formation of mitoplasts by hypotonic treatment.

Discussion

In this study, we demonstrated and characterized the mitochondrial import of a long synthetic peptide, 11β-45, which contained the extension peptide of cytochrome P-450(11β) precursor. The import of the peptide was dependent on the electrochemical potential of the inner membrane as in the case of various precursor proteins.

However, the import of 11β-45 was different from precursor proteins in that the former was not dependent on cytosolic factors and was not susceptible to trypsin treatment of mitochondria.

Degradation of the imported synthetic peptides

The imported 11β-45 accumulated in mitochondria until 30 min when *o*-phenanthroline was present, but it was degraded rapidly in the absence of the chelator. *o*-Phenanthroline is a membrane-permeable metal chelator and inhibits mitochondrial processing protease when added to mitochondrial suspension (Zwizinski and Neupert, 1983; Ogishima *et al.*, 1985). However, the degradation of 11β-45 in mitochondria was possibly not catalyzed by the precursor-specific processing protease but by some other metal protease since 11β-45 was not processed with the purified processing protease (data not shown). On the other hand, 11β-45 inhibited *in vitro* processing of the precursors by the purified processing protease (Ou,W.-J., Mihara,K., Ito,A., Kumamoto,T., Okasaki,H., Aimoto,S. and Omura,T., manuscript in preparation). We interpreted that 11β-45 was recognized by the processing protease but was not a cleavable substrate. Degradation of imported peptides was reported for the synthetic extension peptides of ornithine aminotransferase (OAT) (Ono and Tuboi, 1988) and cytochrome oxidase subunit IV (COX IV) precursors (Glaser and Cumsky, 1990b), albeit degradation of the imported COX IV peptide was weakly affected by metal chelators (Glaser and Cumsky, 1990b). It therefore appears that various synthetic peptides have different susceptibility to mitochondrial proteases.

Energy requirement for the import of synthetic peptides

The import of 11β-45 into mitochondria was dependent on the electrochemical potential of the inner membrane as in the case of various precursor proteins. The inhibition of the import of the COX IV peptide and the OAT peptide by valinomycin and CCCP, respectively, was reported (Ono and Tuboi, 1988; Glaser and Cumsky, 1990b). The step requiring the membrane potential in the import of the synthetic extension peptides seems to be common with precursor proteins.

The import of 11β-45 did not require extramitochondrial ATP. Non-dependence on extramitochondrial ATP was also reported for the OAT peptide (Ono and Tuboi, 1988). In the import of precursor proteins, however, ATP or other NTPs are required in addition to the membrane potential (Pfanner and Neupert, 1986; Eilers *et al.*, 1987). It was suggested that the hydrolysis of ATP or other NTPs keeps precursor proteins in an import competent, unfolded conformation in the cytosol or at the surface of mitochondria (Pfanner *et al.*, 1987). It is likely that 11β-45 was already in an import competent conformation in solution and did not need external ATP for unfolding in the import. In favor of this assumption, the shorter peptides corresponding to various portions of the extension peptide of P-450(SCC) precursor were reported to be in random conformations in aqueous solutions (Aoyagi *et al.*, 1987).

Requirement of cytosolic factors

Stimulation of the import of precursor proteins into isolated mitochondria by soluble factors in reticulocyte lysate or yeast cytosol was reported from several laboratories using bacterially expressed (Murakami and Mori, 1990; Sheffield *et al.*, 1990) or *in vitro* synthesized (Murakami *et al.*, 1988; Ono and Tuboi, 1990a; Randall and Shore, 1989) precursor proteins. Ono and Tuboi (1988) reported that the import of the OAT peptide was also stimulated by cytosolic factors in reticulocyte lysate. In striking contrast, however, the import of 11β-45 was not stimulated by the presence of the high speed supernatant of reticulocyte lysate. It is tempting to speculate that cytosolic factors recognize some particular conformation of the precursor molecules which include not only the extension peptide but also the mature protein, and keep them in an import competent state. Thus the import of 11β-45 was not dependent on cytosolic factors possibly because it was already in an import competent state. According to Randall and Shore (1989), the truncation of the carboxy-terminal 73 amino acid residues of ornithine transcarbamylase (OTC) precursor resulted in the loss of the dependence of its import on cytosolic factors, suggesting that recognition by cytosolic factors is not restricted to the extension peptide portion of the precursor. On the contrary, Murakami and Mori (1990) showed quite recently that presequence binding factor (PBF) which formed a soluble complex with OTC precursor was purified from reticulocyte lysate, and the formation of PBF−OTC precursor complex was inhibited by synthetic presequences (extension peptides) of OTC and other precursors.

Although the existence of the extension peptide is essential for the interaction of the precursor molecules with cytosolic factors, structural requirements for the interaction remain to be clarified.

Import of synthetic extension peptides skips some steps essential for the import of precursor proteins

Protein components located on the mitochondrial outer surface are indispensable for the import of various precursor proteins into mitochondria (Zwizinski *et al.*, 1984; Ohba and Schatz, 1987a,b; Pfaller *et al.*, 1989). However, the import of 11β-45 was resistant to trypsin treatment of the mitochondrial surface in contrast with that of adrenodoxin precursor. We suggest that collaboration of cytosolic factors and mitochondrial surface components is required for the import of precursor proteins. Since 11β-45 does not need cytosolic factors for import, it seems to skip the interaction with the mitochondrial surface components catalyzing the unfolding of precursor proteins. Glaser and Cumsky (1990a) also found that the import of a COX IV−DHFR fusion protein was inhibited by trypsin treatment of mitochondria, but was restored by denaturing the fusion protein.

Pfaller *et al.* (1989) found that trypsin-treated mitochondria showed residual import activities with various precursor proteins (~5−25% of the control level), and named it 'bypass import'. Judging from several lines of evidence, they concluded that 'bypass import' was less efficient than normal import because it omitted the initial surface receptor-mediated step. Since the import of 11β-45 was not much affected by trypsin treatment, we conclude that import of the peptide is originally independent of the surface receptor.

We found that 11β-45 could be imported into mitoplasts and the import was susceptible to trypsin treatment. This observation indicates that the import of 11β-45 is mediated by some protein components located inside of mitochondria,

which become accessible to trypsin on preparing mitoplasts. Judging from our observations and several reports, the import of various synthetic peptides into isolated mitochondria is carried out mainly via the potential-dependent pathway as is that of precursor proteins, but bypasses some steps which are necessary for the import of various precursor proteins. The import of precursor proteins into mitochondria can be dissected into several steps (reviewed by Pfanner and Neupert, 1990). We previously found that some synthetic peptides interfered with the translocation of adrenodoxin precursor into mitochondria, but had no effect on the binding of the precursor on the surface of mitochondria (Furuya *et al.*, 1987). Our observations indicated that 11β-45 enters the mitochondrial import pathway from somewhere after the surface components-mediated step. We consider that 11β-45 is a suitable probe for investigation of the import process after the initial surface components-mediated step. Function and location of mitochondrial protein components which interact with 11β-45 in the import remain to be identified.

After completion of this work, Pak and Weiner (1990) reported that the import of the synthetic extension peptides of ornithine transcarbamylase (OTC) and aldehyde dehydrogenase (ALDH) precursors into rat liver mitochondria did not require extramitochondrial ATP, cytosolic factors, mitochondrial surface components and the membrane potential. The import of 11β-45 was clearly dependent on the membrane potential. Dependence on the membrane potential was also reported for the import of the OAT peptide and the COX IV peptide. Since the import of various precursor proteins requires the membrane potential, the potential independent import of the OTC and ALDH peptides could be mediated by some mechanism other than the normal import pathway.

Materials and methods

Materials

[^{3}H]*N*-ethylmaleimide (1642.8 GBq/mmol), [^{3}H]iodoacetic acid (5.6 GBq/mmol) and [^{35}S]methionine (37 TBq/mmol) were purchased from Dupont/NEN Research Products. Rainbow ^{14}C-methylated protein molecular weight markers and ACSII were from Amersham International, UK. SP6 RNA polymerase and ribonuclease inhibitor were from Takara Syuzou Co., Kyoto, Japan. Apyrase (potate), trypsin (porcine pancreas) and trypsin inhibitor (soybean) were from Sigma Co., St Louis, USA. Other chemicals were all of reagent grade.

Synthesis and labeling of synthetic peptide 11β-45

Peptide 11β-45 corresponds to the whole extension peptide and amino-terminal 20 residues of the mature portion of bovine P-450(11β) precursor (Figure 1A). Try44 of the peptide is artificially inserted between the original Arg43 and the Cys44 of the precursor (Kirita *et al.*, 1988).

Peptide synthesis was carried out using a peptide synthesizer model 430A (Applied Biosystems Inc.). Starting from *p*-methylbenzhydrylamine resin, peptide chain elongation was performed according to the double coupling protocol of the standard t-Boc. A protected peptide resin was treated with anhydrous hydrogen fluoride in the presence of anisole and 1,4-butanedithiol. A crude product of [Cys(Acm)^{45}]11β-45 was purified on reverse-phase HPLC and the acetamidemethyl(Acm) group on the peptide was removed with mercuric acetate to give 11β-45. The amino acid composition of 11β-45 hydrolyzed with 4 M methanesulfonic acid at 110°C for 48 h was as follows: Thr 1.02, Ser 1.01, Glu 2.26, Pro 3.63, Gly 3.14, Ala 10.0, Cys 0.41, Val 1.90, Met 2.37, Leu 5.95, Tyr 0.96, Phe 1.17, Trp 1.89, His 1.07, Lys 2.07, Arg 4.07. The amino acid composition agreed with the values calculated from the amino acid sequence of 11β-45.

23 nmol of 11β-45 was labeled with 11.5 nmol of [^{3}H]*N*-ethylmaleimimide (NEM) according to the method of Riordan and Vallee (1967). The reaction was terminated by the addition of 200 nmol of unlabeled NEM. The peptide and free NEM was separated by gel filtration with Bio-Gel P-2 which was equilibrated with 20 mM Tris−HCl pH 7.4 containing 2 M urea and 50 mM NaCl. The specific radioactivities of the labeled peptide were 0.2−1.0 × 10^7 d.p.m./nmol. In some experiments, 50 nmol of 11β-45 were labeled with 600 nmol of [^{3}H]iodoacetic acid according to the method of Tamm (1986). The specific radioactivity of the labeled peptide was 0.9 × 10^6 d.p.m./nmol. Efficiencies of the import of the labeled peptide into isolated mitochondria were almost same with either type of the labeled peptides.

Preparation of mitochondria and mitoplast

Adrenal cortex mitochondria were prepared as described previously (Furuya *et al.*, 1987) except that an isolation buffer (SHE) consisting of 0.3 M sucrose, 10 mM HEPES−KOH pH 7.4, 1 mM EDTA, 1 μg/ml leupeptin and 10 U/ml Trasylol was used. After washing with SHE, mitochondria were suspended in the import buffer (IMB) consisting of 0.3 M sucrose, 10 mM HEPES−KOH pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM sodium succinate, 0.5 mM ATP, 1 μg/ml leupeptin and 10 U/ml Trasylol at a protein concentration of 20−30 mg/ml.

For preparing trypsin-treated mitochondria and mitoplasts, mitochondria were prepared as described above without leupeptin and Trasylol. Mitoplasts were prepared from the mitochondria according to the method of Ohba and Schatz (1987b), and suspended in SHE without leupeptin and Trasylol. 10 mg/ml of mitochondria and mitoplasts were treated with 0.1 or 1.0 mg/ml trypsin at 0°C for 30 min. The digestion was stopped by addition of soybean trypsin inhibitor (SBTI) and Trasylol to a final concentration of 2 mg/ml and 100 U/ml, respectively. They were washed once with SHE containing 0.2 mg/ml SBTI and 10 U/ml Trasylol, and suspended in IMB at a protein concentration of 10 mg/ml.

In vitro transcription−translation and affinity purification of adrenodoxin precursor

Adrenodoxin precursor was translated in the presence of [^{35}S]methionone with a nuclease-treated reticulocyte lysate as described previously (Furuya *et al.*, 1987). For affinity purification of the precursor, the *in vitro* translation product was incubated with anti-adrenodoxin IgG conjugated for Formyl Cellulofine (Seikagaku Kogyo Co.). Anti-bovine adrenodoxin IgG was kindly provided by Dr T.Hara (Nakamura Gakuen University). After the incubation, unadsorbed materials were washed out with 10 mM HEPES−KOH pH 7.4 containing 150 mM potassium acetate and 1 mM magnesium acetate. Further washing was carried out with the same buffer containing 1.5 M NaCl, subsequently with the same buffer containing 2 M urea. Adrenodoxin precursor was then eluted from the resin with 10 mM HEPES−KOH pH 7.4 containing 3 M NaSCN. The eluate was dialyzed against the buffer consisting of 10 mM HEPES−KOH pH 7.4, 2 M urea and 1 mM DTT. 0.2 mg/ml BSA was added to the solution of affinity purified adrenodoxin precursor containing BSA and stored at −80°C until use.

In vitro import of 11β-45 and adrenodoxin precursor

Freshly isolated bovine adrenal cortex mitochondria (1 mg/ml in IMB) were preincubated with 0.5 mM *o*-phenanthroline at 0°C for 15 min. The import reaction was started by addition of [^{3}H]11β-45 and the incubation was continued at 30°C for 30 min. After the incubation, the mitochondria were precipitated with a microfuge at 9000 r.p.m. for 10 min and suspended in SHE without leupeptin and Trasylol. The mitochondrial suspension was added with proteinase K (40 μg/ml) and incubated at 4°C for ~40 min. The treatment was terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mM. The treated mitochondria were precipitated and washed twice with SHE containing 0.2 mM PMSF and analyzed with urea−SDS−PAGE as described below. The import of the peptide into mitoplasts was carried out in the same way.

The import of adrenodoxin precursor was examined using either the purified precursor or the crude *in vitro* translation products. The reaction was carried out at 30°C for 30 min without the preincubation of mitochondria with *o*-phenanthroline. The mitochondria were recovered and washed as in the case of the peptide import. The import of the precursor was analyzed by SDS−PAGE followed by fluorography (Furuya *et al.*, 1987) without proteinase K treatment.

In some experiments, a high speed supernatant of reticulocyte lysate was prepared by centrifugation at 103 000 *g* for 30 min and its effect on the import was examined.

Gel electrophoresis

The import of 11β-45 was analyzed by urea−SDS−PAGE according to the method of Burr and Burr (1983) with minor modification. The stacking gel was composed of 3% acrylamide, 0.18% bisacrylamide, 8 M urea, 125 mM Tris−phosphate pH 6.8, and 0.1% SDS. The separating gel was composed of 17% acrylamide, 0.11% bisacrylamide, 8 M urea, 0.34 M Tris-phosphate pH 8.0 and 0.1% SDS. The sample buffer and running buffer

were those of Burr and Burr (1983). Laemmli's slab gel electrophoresis system (12.5% acrylamide) was used for the analysis of the import of adrenodoxin precursor (Laemmli, 1970).

Other methods

Proteins were measured by the BCA protein assay reagent (Pierce Chemical Co., Rockford, USA) and BSA as a standard. Fluorography was carried out according to the method of Chamberlain (1979).

Acknowledgements

This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science and Culture of Japan.

References

Aoyagi,H., Lee,S., Kanmera,T., Mihara,H. and Kato,T. (1987) *J. Biochem.*, **102**, 813–820.
Burr,F.A. and Burr,B. (1983) *Methods Enzymol.*, **96**, 239–244.
Chamberlain,J.P. (1979) *Anal. Biochem.*, **98**, 132–135.
Deshaies,R.J., Koch,B.D., Werner-Washburne,M., Craig,E.A. and Schekman,R. (1988) *Nature*, **332**, 800–805.
Eilers,M., Opplinger,W. and Schatz,G. (1987) *EMBO J.*, **6**, 1073–1077.
Furuya,S., Okada,M., Ito,A., Aoyagi,H., Kanmera,T., Kato,T., Sagara,Y., Horiuchi,T. and Omura,T. (1987) *J. Biochem.*, **102**, 821–832.
Gillespie,L.L., Aragan,C., Teneja,A.T., Hodges,R.S., Freeman,K.B. and Shore,G.C. (1985) *J. Biol. Chem.*, **260**, 16045–16048.
Glaser,S.M. and Cumsky,M.G. (1990a) *J. Biol. Chem.*, **265**, 8808–8816.
Glaser,S.M. and Cumsky,M.G. (1990b) *J. Biol. Chem.*, **265**, 8817–8822.
Hartl,F.-U., Pfanner,N., Nicholson,D.W. and Neupert,W. (1989) *Biochim. Biophys. Acta.*, **988**, 1–45.
Hawlitschek,G., Schneider,H., Schmidt,B., Tropschug,M., Hartl, F.-U. and Neupert,W. (1988) *Cell*, **53**, 795–806.
Hines,V., Brandt,A., Griffiths,G., Horstmann,H., Brütsch,H. and Schatz,G. (1990) *EMBO J.*, **9**, 3191–3200.
Horwich,A.L., Kalousek,F., Mellman,I. and Rosenberg,L.E. (1985a) *EMBO J.*, **4**, 1129–1135.
Horwich,A.L., Kalousek,F. and Rosenberg,L.E. (1985b) *Proc. Natl. Acad. Sci. USA*, **82**, 4930–4933.
Hovius,R., Lambrechts,H., Nicolay,K. and De Kruijff,B. (1990) *Biochim. Biophys. Acta.*, **1021**, 217–226.
Hurt,E.C., Pesold-Hurt,B. and Shatz,G. (1984) *FEBS Lett.*, **178**, 306–310.
Hwang,S.T. and Schatz,G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8432–8436.
Kinnally,K.W., Tedeschi,H. and Maloff,B.L. (1978) *Biochemistry*, **17**, 3419–3428.
Kirita,S., Morohashi,K., Hashimoto,T., Yoshioka,H., Fujii-Kuriyama,Y. and Omura,T. (1988) *J. Biochem.*, **104**, 683–686.
Kleiber,J., Kalousek,F., Swaroop,M. and Rosenberg,L.E. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7978–7982.
Kumamoto,T., Morohasi,K., Ito, A. and Omura,T. (1987) *J. Biochem.*, **102**, 833–838.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Murakami,K. and Mori,M. (1990) *EMBO J.*, **9**, 3201–3208.
Murakami,H., Pain,D. and Blobel,G. (1988) *J. Cell Biol.*, **107**, 2051–2057.
Ogishima,T., Okada,Y. and Omura,T. (1985) *J. Biochem.*, **98**, 781–791.
Ohba,M. and Shatz,G. (1987a) *EMBO J.*, **6**, 2109–2115.
Ohba,M. and Shatz,G. (1987b) *EMBO J.*, **6**, 2117–2122.
Ono,H. and Tuboi,S. (1988) *J. Biol. Chem.*, **263**, 3188–3193.
Ono,H. and Tuboi,S. (1990a) *Arch. Biochem. Biophys.*, **277**, 368–373.
Ono,H. and Tuboi,S. (1990b) *Arch. Biochem. Biophys.*, **280**, 299–304.
Ono,H. and Tuboi,S. (1990c) *J. Biochem.*, **107**, 840–845.
Ou,W.-J., Ito,A., Umeda,M., Inoue,K. and Omura,T. (1988) *J. Biochem.*, **103**, 589–595.
Ou,W.-J., Ito,A., Okazaki,H. and Omura,T. (1989) *EMBO J.*, **8**, 2605–2612.
Pain,D., Murakami,H. and Blobel,G. (1990) *Nature*, **347**, 444–449.
Pak,Y.K. and Weiner,H. (1990) *J. Biol. Chem.*, **265**, 14298–14307.
Pfaller,R., Pfanner,N. and Neupert,W. (1989) *J. Biol. Chem.*, **264**, 34–39.
Pfanner,N. and Neupert,W. (1986) *FEBS Lett.*, **209**, 152–156.
Pfanner,N., Tropschug,M. and Neupert,W. (1987) *Cell*, **49**, 815–823.
Pfanner,N. and Neupert,W. (1990) *Annu. Rev. Biochem.*, **59**, 331–353.
Randall,S.K. and Shore,G.C. (1989) *FEBS Lett.*, **250**, 561–564.
Riordan,J.F. and Vallee,B.L. (1967) *Methods Enzymol.*, **11**, 449–456.
Seffield,W.P., Shore,G.C. and Randall,S.K. (1990) *J. Biol. Chem.*, **265**, 11069–11076.
Söllner,T., Griffiths,G., Pfaller,R., Pfanner,N. and Neupert,W. (1989) *Cell*, **59**, 1061–1070.
Söllner,T., Pfaller,R., Griffiths,G., Pfanner,N. and Neupert,W. (1990) *Cell*, **62**, 107–115.
Tamm,L.K. (1986) *Biochemistry*, **25**, 7470–7476.
Vestweber,D., Brunner,J., Baker,A. and Schatz,G. (1989) *Nature*, **341**, 205–209.
Yang,M., Jensen,R.E., Yaffe,M.P., Oppliger,W. and Schatz,G. (1988) *EMBO J.*, **7**, 3857–3862.
Zwizinski,C. and Neupert,W. (1983) *J. Biol. Chem.*, **258**, 13340–13346.
Zwizinski,C., Schleyer,M. and Neupert,W. (1984) *J. Biol. Chem.*, **259**, 7850–7856.

Received on January 11, 1991; revised on March 4, 1991