The EMBO Journal vol.9 no.10 pp.3201–3208, 1990

Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein

Kaoru Murakami and Masataka Mori

Institute for Medical Genetics, Kumamoto University Medical School, Kuhonji, Kumamoto 862, Japan

Communicated by G.Schatz

In vitro **mitochondrial import of the purified precursor form (pOTC) of rat ornithine carbamoyltransferase (OTC) is stimulated by a cytosolic factor(s) contained in rabbit reticulocyte lysate. A protein factor that binds to pOTC but not to mature OTC and was named presequence binding factor or PBF, was purified 91 000-fold from the lysate by affinity chromatography using pOTC-bound Sepharose, DEAE–5PW HPLC and sucrose gradient centrifugation. The purified PBF migrated as a single polypeptide of 50 000 daltons on SDS–PAGE. On sucrose gradients, urea-denatured pOTC sedimented to the bottom, whereas PBF sedimented with an $s_{20,w}$ value of 5.5S. When pOTC and PBF were centrifuged together, both polypeptides sedimented as a complex of 7.1S. Formation of the pOTC–PBF complex was inhibited by micromolar concentrations of the synthetic presequence of pOTC and those of other mitochondrial precursor proteins. The purified PBF markedly stimulated the import of purified or** ***in vitro*** **synthesized pOTC into the mitochondria. PBF-stimulated pOTC import was further enhanced by a 70 kd heat shock protein (hsp70) purified from yeast; the hsp70 alone had little effect. Thus, PBF binds to the presequence portion of the precursors and may hold them in a transport-competent form in cooperation with hsp70.**

Key words: cytosolic factor/hsp70/mitochondrial protein import/ornithine carbamoyltransferase precursor/presequence binding factor

Introduction

The great majority of mitochondrial proteins are encoded by nuclear genes. In recent studies much has been learned about the mechanism of protein import into the mitochondria (for reviews, see Douglas *et al.*, 1986; Pfanner *et al.*, 1988; Vener and Schatz, 1988). Most mitochondrial proteins are synthesized on cytosolic free ribosomes as larger precursors with NH_2-terminal presequences. The precursor proteins are released into a cytosolic pool and then imported into the mitochondria with half-lives of a few minutes. During or immediately after the import, the NH_2-terminal presequences are removed by a chelator-sensitive processing protease present in the mitochondrial matrix. The binding of precursor proteins to the mitochondrial outer surface appears to be mediated by a 'receptor'. The binding and import of precursor proteins require an energized inner membrane and ATP. Recent studies showed that precursor proteins cannot be transported into mitochondria in a tightly folded state (Eilers and Schatz, 1986; Chen and Douglas, 1987; Schleyer and Neupert, 1987; Eilers *et al.*, 1988; Vestweber and Schatz, 1988a,b). Therefore, the precursor proteins released into the cytosol must be unfolded or be maintained in a transport-competent loose conformation before being imported into the mitochondria. ATP appears to participate, directly or indirectly, in the unfolding of the precursor proteins for their transport into the mitochondria (Pfanner *et al.*, 1987a,b; Vener and Schatz, 1987). Other studies showed that a heat shock protein, hsp70, is involved in the transport of mitochondrial precursor proteins across membranes in yeast (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Murakami *et al.*, 1988b). Thus, unfolding may be mediated by heat shock-like proteins.

Studies in our laboratory (Miura *et al.*, 1983; Murakami *et al.*, 1988a) and elsewhere (Ohta and Schatz, 1984; Argan and Shore, 1985; Ono and Tuboi, 1988; Murakami *et al.*, 1988b) suggested that import of precursor proteins into mitochondria involves a cytosolic factor(s) present in a rabbit reticulocyte lysate or yeast cytosolic fraction. To comprehend better the mechanism of the import of the precursor proteins, purification and characterization of the cytosolic factor(s) and elucidation of its function are important. We reported a high level expression of the precursor form (pOTC) of rat ornithine carbamoyltransferase (OTC) in *Escherichia coli* and established an *in vitro* import system in which the purified recombinant pOTC was imported into isolated mitochondria (Murakami *et al.*, 1988a). Using this system, we showed that a protein factor(s) in the reticulocyte lysate is required for the import of the purified pOTC, and that this factor(s) prevents pOTC from forming a large aggregate and holds it in a soluble complex. Acquisition of large amounts of purified pOTC paved the way for isolation of this cytosolic factor which was found to bind the presequence portion of pOTC. We refer to this factor as presequence binding factor (PBF). We now report the purification of PBF from rabbit reticulocyte lysate and rat liver and heart cytosol and the association with purified pOTC. The purified PBF stimulated *in vitro* import of purified or *in vitro* synthesized pOTC into mitochondria. The PBF-stimulated pOTC import was further enhanced by purified yeast hsp70.

Results

Purification of PBF from reticulocyte lysate

Like most mitochondrial proteins, OTC, a mitochondrial matrix enzyme of the urea cycle, is initially synthesized as a larger precursor, pOTC (Conboy *et al.*, 1979; Mori *et al.*, 1980) with an NH_2-terminal presequence of 32 amino acid residues (Horwich *et al.*, 1984; Takiguchi *et al.*, 1984). pOTC is then imported into the mitochondrial matrix (Mori *et al.*, 1981a; Morita *et al.*, 1982) with a half-life of 1–2 min (Mori *et al.*, 1981b) and processed to the mature form by a matrix localized processing protease (Miura *et al.*, 1982). We reported a high level expression in *E.coli*

© Oxford University Press

Table I. Purification of PBF from reticulocyte lysate

Purification step	Total proteins (mg)	Total activity (units)	Sp. act. (units/mg protein)	Purification (fold)	Yield (%)
Lysate	30 000	3 300	0.1	1	100
Affinity chromatography	21.6	2 400	110	1 000	73
DEAE−5PW HPLC	0.81	2 250	2 800	25 000	68
Sucrose gradient centrifugation	0.21	2 100	10 000	91 000	64

and purification of rat pOTC, and showed that a cytosolic protein factor(s) in the reticulocyte lysate stimulates import of the purified pOTC into isolated mitochondria (Murakami *et al.*, 1988a). This factor (or one of the factors) binds to the presequence portion of pOTC and forms a complex with pOTC (see below). We refer to this factor as PBF.

PBF was purified from the lysate by affinity chromatography using pOTC as a ligand. PBF activity was assayed for its ability to form a complex with ^{35}S-labeled and purified pOTC (see Materials and methods). PBF bound to pOTC-conjugated Sepharose, but not to mature OTC-conjugated Sepharose which was prepared in a similar way to pOTC−Sepharose. PBF was eluted from the pOTC−Sepharose with 0.5 M KCl. PBF activity was recovered quantitatively in the eluate and the non-absorbed fraction had little activity. This affinity purification step resulted in a 1000-fold purification. Further purification was achieved by HPLC with DEAE−5PW and sucrose gradient centrifugation.

Typical results of the purification are shown in Table I. The overall purification is 91 000-fold from the reticulocyte lysate with a yield of 64%; 210 μg of nearly homogeneous PBF was obtained from 200 ml of the lysate (see below).

Figure 1 shows the results of SDS−PAGE of PBF fractions at each step of the purification. The purified factor was nearly homogeneous and migrated as a single polypeptide with an apparent M_r of ~50 000. We identified this polypeptide to be PBF as follows. First, this polypeptide was enriched throughout the purification together with PBF activity on DEAE−5PW HPLC and sucrose gradient centrifugation (data not shown). Second, PBF purified from rat liver and heart was composed of a polypeptide of apparently the same size (see below). Third, this polypeptide formed a complex with purified pOTC (see below). M_r of PBF was estimated to be 390 000 by HPLC gel filtration. These results suggest that PBF is an oligomeric protein composed of several identical polypeptides.

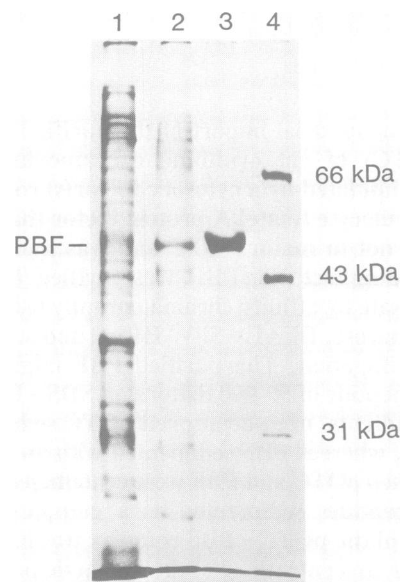


Fig. 1. Purification of PBF. PBF was purified as described in Materials and methods. Samples of PBF at each purification step were analyzed by 12% SDS−PAGE and silver staining. Lane 1, affinity purified fraction; lane 2, DEAE−5PW active fraction; lane 3, sucrose gradient fraction. Lane 4, protein molecular mass markers; rabbit muscle phosphorylase *b* (97 kd), bovine serum albumin (66 kd), hen egg white ovalbumin (43 kd) and bovine carbonic anhydrase (31 kd).

PBF forms a soluble complex with purified pOTC

Figure 2 shows the effects of purified PBF on sedimentation of purified [^{35}S]pOTC. When urea-denatured pOTC alone was subjected to sucrose gradient centrifugation, it formed a large aggregate and sedimented to the bottom (see also Murakami *et al.*, 1988a). When increasing amounts of purified PBF were added, precipitation of pOTC was prevented and pOTC sedimented with an $s_{20,w}$ value of 7.1S. About 200 ng of the purified PBF was required to maintain most of the added pOTC (70 ng, 3.5 × 10^4 d.p.m.) in the 7.1S form. These results indicate that PBF binds to pOTC and forms a soluble complex of 7.1S. ATP had no effect on formation of this complex or on its dissociation. On the other hand, PBF did not form a complex with purified mature OTC (data not shown).

Figure 3 shows association of pOTC and PBF. When urea-denatured pOTC alone was subjected to sucrose gradient centrifugation, it sedimented to the bottom of the tube. The purified PBF sedimented with an $s_{20,w}$ value of 5.5S. When pOTC and PBF were mixed and centrifuged, both polypeptides cosedimented with an $s_{20,w}$ value of 7.1S. These results indicate that pOTC and PBF associate and form a complex of 7.1S. Densitometric scanning of the silver stain showed that the ratio of the pOTC polypeptide to the PBF polypeptide was about 1:1, assuming that both polypeptides were stained similarly.

Synthetic presequence of pOTC inhibits formation of the PBF−pOTC complex

PBF bound to pOTC−Sepharose and formed a complex with pOTC, but did not bind to OCT−Sepharose and did not form a complex with OTC. This suggests that PBF binds to the presequence portion of the precursor protein. To confirm this, we performed competition experiments using the synthetic presequence of pOTC (Figure 4). This presequence is composed of NH_2-terminal 32 amino acid residues of pOTC (Takiguchi *et al.*, 1984). The presequence inhibited the formation of the PBF−pOTC complex of 7.1S, partially at 2−20 μM and completely at 50 μM. Purified recombinant pOTC strongly inhibited the complex formation at 50 μM,

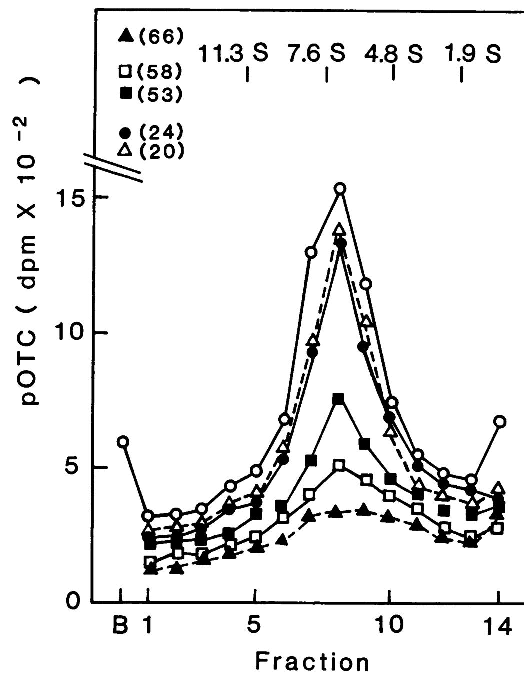


Fig. 2. Effect of purified PBF on sedimentation of pOTC. ^{35}S-labeled and purified pOTC (70 ng, 3.5×10^4 d.p.m.), dissolved in 1 μl of 8 M urea, was diluted in 50 μl of 20 mM potassium HEPES (pH 7.6) containing none (▲), 50 ng (□), 100 ng (■), 200 ng (●), 400 ng (△) or 2 μg (○) of purified PBF. The mixtures were subjected to sucrose gradient centrifugation as described in Materials and methods (see *Assay of PBF activity*). Fractions (0.35 ml) were collected and 0.1 ml portions were counted for radioactivity. Vertical lines show the positions of bovine catalase (11.3S), yeast alcohol dehydrogenase (7.6S), bovine serum albumin (4.8S) and hen egg white lysozyme (1.9S). Numbers in parentheses show radioactivities of pOTC that were out of scale in the figure. B, bottom fraction.

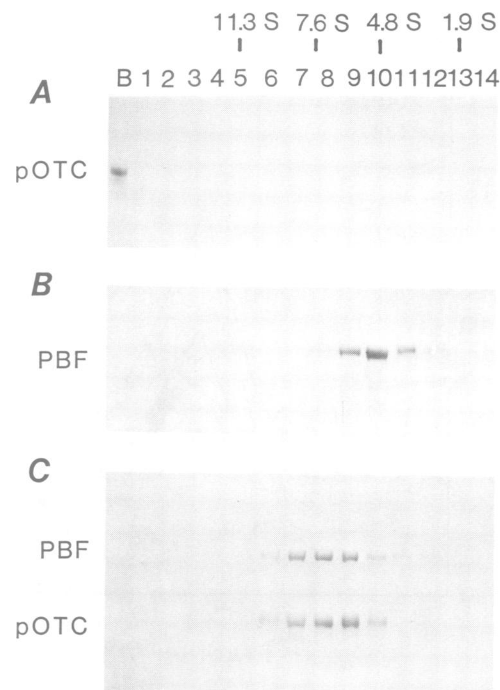


Fig. 3. Cosedimentation of pOTC and PBF. Mixtures (50 μl of 20 mM potassium HEPES, pH 7.6) containing 1 μg of purified pOTC dissolved in 1 μl of 8 M urea (**A**), 2 μg of purified PBF (**B**) or both (**C**), were subjected to sucrose gradient centrifugation as described in Materials and methods. Fractions (0.35 ml) were collected and concentrated to ~30 μl by using Ultra free C3GC (Millipore). Concentrated fractions were subjected to SDS−PAGE and silver staining. Relevant portions of the stained gels are shown. Vertical lines show the positions of standard proteins which are the same as for Figure 2. B, bottom fraction.

whereas purified recombinant mature OTC did not (Murakami *et al.*, 1990). All these results indicate that PBF binds to the presequence portion of pOTC.

Presequences of other mitochondrial precursor proteins inhibit complex formation

To determine whether or not PBF is also involved in the import of other mitochondrial precursor proteins, the effect of synthetic peptides corresponding to the presequences of mouse mitochondrial malate dehydrogenase (Joh *et al.*, 1987) and pig mitochondrial aspartate aminotransferase (Joh *et al.*, 1985) on formation of the PBF−pOTC complex was examined. The two presequences are composed of 24 and 29 amino acid residues, respectively. The malate dehydrogenase presequence inhibited complex formation, partially at 2 and 20 μM and completely at 50 μM (Figure 5A). The aspartate aminotransferase presequence also inhibited complex formation (Figure 5B). Its own presequence of pOTC and the presequences of other mitochondrial proteins exerted similar inhibitions. A control peptide corresponding to residues 269−290 of rat OTC did not affect the complex formation at 50 μM. These results indicate that PBF interacts specifically with presequences of several distinct precursor proteins.

Purified PBF stimulates import of purified pOTC into the mitochondria

We reported that a cytosolic factor(s) in the reticulocyte lysate stimulates import of purified pOTC into the isolated mitochondria (Murakami *et al.*, 1988a). To observe whether or not PBF is responsible for this stimulation, the effect of purified PBF as well as of dialyzed reticulocyte lysate on pOTC import was examined (Figure 6). Import was assessed by pOTC processing to the mature form, because processing occurs in the mitochondrial matrix following the import. When urea-denatured purified pOTC alone was incubated with isolated rat liver mitochondria for 90 min at 25°C, little processing of pOTC to the mature form was observed (Figure 6A, lanes 2 and 6). pOTC import increased with increasing amounts of the reticulocyte lysate up to 30 μl (lanes 2−5 and lower panel). pOTC import could be supported by the purified PBF. The import increased with increasing amounts of PBF up to 600 ng, where 32% of the added pOTC was imported and processed (lanes 6−10 and lower panel). The PBF-stimulated import was completely inhibited by an uncoupler dinitrophenol (lanes 11 and 12). Therefore, we conclude that PBF is essential for the transport of pOTC into the mitochondria.

In the presence of 30 μl of the reticulocyte lysate, pOTC import increased with time up to 30 min and reached a plateau in 30−60 min (Figure 6B, lanes 2−7 and lower panel). On the other hand, in the presence of 400 ng of purified PBF, pOTC import increased slowly with time up to 120 min (lanes 8−13). The results suggest that a discrete factor(s) present in the reticulocyte lysate is required for a more efficient import of pOTC.

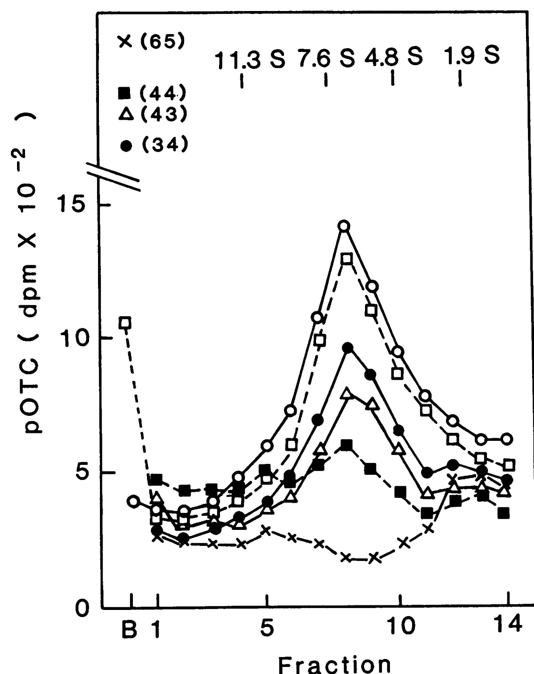


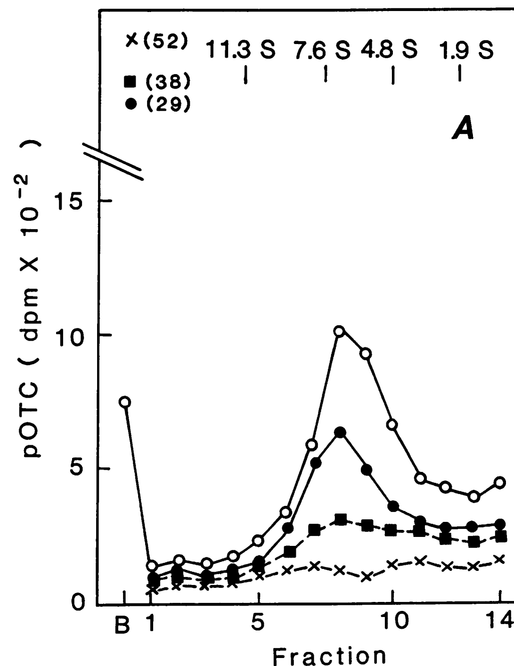
Fig. 4. Effect of synthetic presequence of pOTC on formation of the pOTC–PBF complex. ^{35}S-labeled and purified pOTC (80 ng in 1 μl of 8 M urea, 4 × 10^4 d.p.m.) was diluted in 50 μl of 25 mM potassium HEPES (pH 7.6) containing 200 ng (2 units) of PBF plus 0 μM (○), 0.2 μM (□), 2 μM (●), 5 μM (△), 20 μM (■) or 50 μM (×) synthetic presequence of pOTC. Sucrose gradient centrifugation and radioactivity measurement were performed as described for Figure 2.

Hsp70 stimulates pOTC import in the presence of PBF

Recently hsp70 was shown to be involved in the import of precursor proteins into mitochondria in yeast (Deshaies *et al.*, 1988; Murakami *et al.*, 1988b). We examined the effect of hsp70 on pOTC import in combination with PBF (Figure 7). In the presence of PBF alone, pOTC import increased linearly up to 60 min and slowly thereafter (lanes 2–6 and lower panel). Addition of purified yeast hsp70 further stimulated the pOTC import (lanes 7–11 and lower panel). The stimulation by hsp70 was ~3-fold at 10 and 30 min of incubation. No import of pOTC was observed in the presence of the hsp70 alone (lanes 12–16). These results indicate that hsp70 is required for a more efficient import of pOTC into the mitochondria.

PBF-depleted reticulocyte lysate does not support pOTC import into the mitochondria

To examine whether PBF is required for import of *in vitro* synthesized pOTC as well as of purified and urea-denatured pOTC into the mitochondria, pOTC was synthesized in a PBF-depleted reticulocyte lysate which was prepared by treating the lysate with pOTC–Sepharose. Untreated lysate and OTC–Sepharose treated lysate were used as controls. Treatment of the lysate with pOTC–Sepharose or OTC–Sepharose had little effect on pOTC synthesis. pOTC synthesized in the untreated lysate or OTC–Sepharose treated lysate was efficiently imported into the mitochondria, whereas pOTC synthesized in the pOTC–Sepharose treated lysate was not (Figure 8). Addition of the purified PBF to the pOTC–Sepharose treated lysate fully restored the import. These results indicate that PBF is involved in import of *in vitro* synthesized pOTC into the mitochondria.



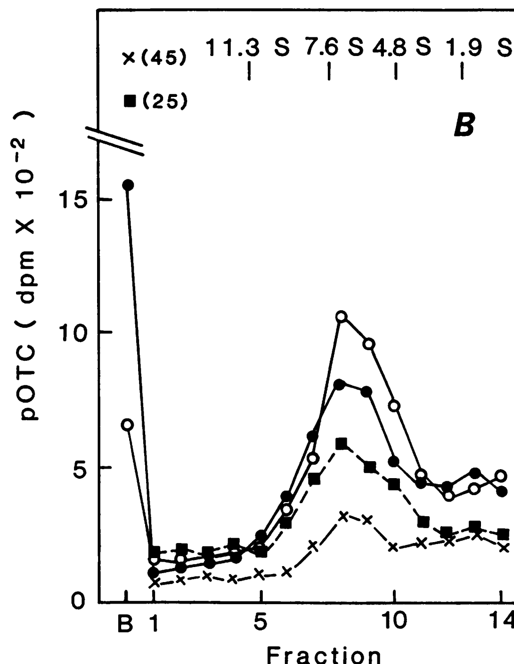


Fig. 5. Effect of heterologous mitochondrial presequences on formation of the pOTC–PBF complex. ^{35}S-labeled and purified pOTC (40 ng in 1 μl of 8 M urea, 2 × 10^4 d.p.m.) was diluted in 50 μl of 25 mM potassium HEPES (pH 7.6) containing 200 ng (2 units) of PBF plus 0 μM (○), 2 μM (●), 20 μM (■), or 50 μM (×) synthetic presequence of mouse mitochondrial malate dehydrogenase (**A**) or that of pig mitochondrial aspartate aminotransferase (**B**). Sucrose gradient centrifugation and radioactivity measurement were performed as described for Figure 2.

Purification of PBF from rat liver and heart

Since protein transport into mitochondria is apparently more active in various types of cells rather than in reticulocytes, PBF must be present in the cytosol of cells other than

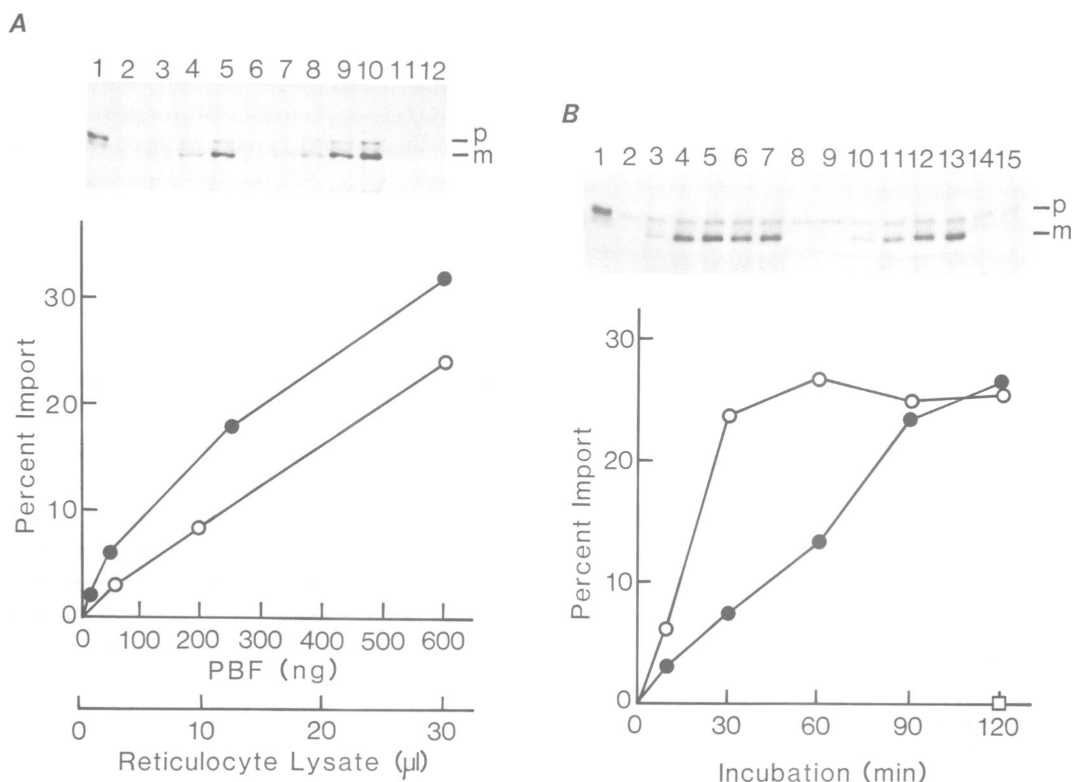


Fig. 6. Effect of rabbit reticulocyte lysate and purified PBF on import of purified pOTC into isolated mitochondria. (**A**) pOTC (40 ng in 1 μl of 8 M urea, 2×10^4 d.p.m.) was mixed with 50 μl of the import assay mixture containing isolated rat liver mitochondria (100 μg of protein) plus none (lanes 2, 6 and 11), 3 μl (lane 3), 10 μl (lane 4) or 30 μl (lane 5) of dialyzed rabbit reticulocyte lysate, 10 ng (lane 7), 50 ng (lane 8), 250 ng (lane 9) or 600 ng (lanes 10 and 12) of the purified PBF and 0.1 mM dinitrophenol (lanes 11 and 12). After incubation at 25°C for 90 min, the mitochondria were reisolated and subjected to 10% SDS−PAGE and fluorography. The relevant portion of the fluorogram is shown. Lane 1, 20% of pOTC added to the import assays in lanes 2−12. p, pOTC; m, mature OTC. The results were quantified by densitometric tracing of the fluorogram and are shown in the lower part. ○, reticulocyte lysate; ●, PBF. (**B**) pOTC (40 ng in 1 μl of 8 M urea, 2×10^4 d.p.m.) was mixed with 50 μl of the import assay mixture containing isolated rat liver mitochondria (100 μg of protein) plus none (lanes 14 and 15), 30 μl of dialyzed rabbit reticulocyte lysate (lanes 2−7) or 400 ng of purified PBF (lanes 8−13). After incubation at 25°C for 0 min (lanes 2, 8 and 14), 10 min (lanes 3 and 9), 30 min (lanes 4 and 10), 60 min (lanes 5 and 11), 90 min (lanes 6 and 12) or 120 min (lanes 7, 13 and 15), the mitochondria were reisolated and subjected to SDS−PAGE and fluorography. The relevant portion of the fluorogram is shown. Lane 1, 20% of pOTC added to the import assays in lanes 2−15. The results were quantified by densitometric tracing of the fluorogram and are shown in the lower part. ○, reticulocyte lysate; ●, PBF.

reticulocytes. PBF was purified from the cytosol fraction of rat liver and heart by affinity chromatography on pOTC−Sepharose. The PBF activity was absorbed by and eluted from the affinity gel. The affinity purified PBFs of rat liver and heart formed a soluble complex with the purified recombinant pOTC. The liver and heart PBFs were further purified by DEAE−5PW chromatography and sucrose gradient centrifugation. The purified preparations gave a major polypeptide on SDS−PAGE which comigrated with rabbit reticulocyte PBF (data not shown). These results indicate that PBF is present in the cytosol of rat liver and heart, as well as in rabbit reticulocyte lysate.

Discussion

The competence of transport of mitochondrial precursor proteins is a major problem awaiting a solution. Several lines of evidence show that the precursor proteins must be unfolded or be maintained in a transport-competent loose conformation before being imported into the mitochondria (Eilers and Schatz, 1986; Chen and Douglas, 1987; Schleyer and Neupert, 1987; Eilers *et al.*, 1988; Vestweber and Schatz, 1988a,b). The possibility that the presequence itself prevents folding of the mature portion of the precursor proteins was ruled out by the following results. First, Endo and Schatz (1988) showed that an artificial precursor protein in which the presequence of yeast cytochrome oxidase subunit IV is fused to mouse dehydrofolate reductase is folded as the corresponding presequence-free protein. Second, Altieri *et al.* (1989) obtained the precursor of rat mitochondrial aspartate aminotransferase in *E.coli* and purified it in an enzymatically active dimeric form. Third, we showed that the guanidine-denatured pOTC as well as mature OTC could be converted into an enzymatically active trimeric form (Murakami *et al.*, 1990).

We reported that a protein factor(s) in the reticulocyte lysate stimulates the uptake of *in vitro* synthesized pOTC by isolated mitochondria (Miura *et al.*, 1983). However, purification of this factor(s) was hampered by the fact that the reticulocyte lysate used in the synthesis of the precursor protein was present in the *in vitro* import assay. Ohta and Schatz (1984) reconstituted an *in vitro* import system which included the purified precursor for yeast mitochondrial ATPase β-subunit, and showed that a yeast cytosolic protein

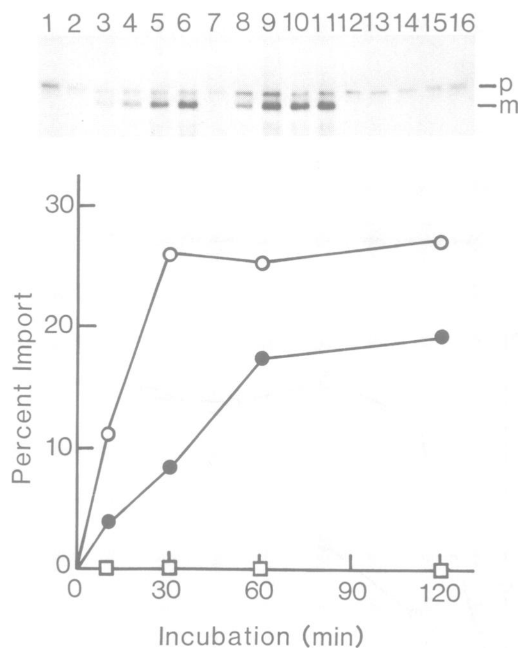


Fig. 7. Effect of hsp70 on import of purified pOTC into isolated mitochondria. pOTC (40 ng in 1 μl of 8 M urea, 2×10^4 d.p.m.) was mixed with 50 μl of the import assay mixture containing isolated rat liver mitochondria (100 μg of protein) plus 400 ng of purified PBF (lanes 2−6), 12 μg of purified yeast hsp70 (lanes 12−16) or PBF plus hsp70 (lanes 7−11) and incubated at 25°C for 0 min (lanes 2, 7 and 12), 10 min (lanes 3, 8 and 13), 30 min (lanes 4, 9 and 14), 60 min (lanes 5, 10 and 15) or 120 min (lanes 6, 11 and 16). The mitochondria were reisolated and subjected to SDS−PAGE and fluorography. The relevant portion of the fluorogram is shown. Lane 1, 10% of pOTC added to the import assays in lanes 2−16. p, pOTC; m, mature OTC. The results were quantified by densitometric tracing of the fluorogram and are shown in the lower part. ●, PBF; ○, PBF plus hsp70; □, hsp70.

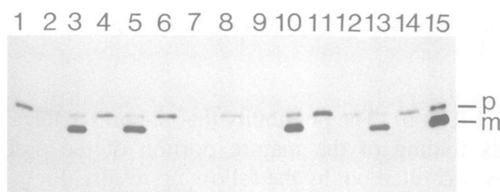


Fig. 8. Effect of PBF depletion on import of *in vitro* synthesized pOTC into mitochondria. pOTC was synthesized in untreated (lanes 1−5), pOTC−Sepharose treated (lanes 6−10) or OTC−Sepharose treated (lanes 11−5) reticulocyte lysate. The translation mixtures (50 μl) containing $\sim 4 \times 10^4$ d.p.m. of pOTC were incubated with isolated rat liver mitochondria (100 μg of protein) at 25°C for 0 min (lanes, 2, 4, 7, 9, 12 and 14) or 60 min (lanes 3, 5, 8, 10, 13 and 15) in the absence (lanes 2, 3, 7, 8, 12 and 13) or presence (lanes 4, 5, 9, 10, 14 and 15) of 400 ng of purified PBF. The mitochondria were reisolated and subjected to SDS−PAGE and fluorography. Lanes 1, 6 and 11, 10% of the translated mixtures used for the import assay in lanes 2−5, 7−10 and 12−15, respectively.

factor of ~40 kd is required for import. We purified a large amount of recombinant pOTC and reconstituted the *in vitro* mitochondrial import with the purified precursor protein (Murakami *et al.*, 1988a). This made way for purification of the cytosolic factor which associates with pOTC.

PBF, which specifically binds to pOTC, was purified to homogeneity from rabbit reticulocyte lysate by pOTC−Sepharose affinity chromatography, DEAE−5PW HPLC and sucrose gradient centrifugation. PBF binds to the presequence portion of pOTC, as indicated by the following observations. PBF bound to pOTC−Sepharose but not to mature OTC−Sepharose. PBF formed a soluble complex with purified pOTC but not with purified mature OTC. Formation of the PBF−[^{35}S]pOTC complex was inhibited by cold pOTC or synthetic presequence of pOTC but not by mature OTC. Furthermore, the concentration of the synthetic presequence required for the inhibition (2−50 μM) is similar to that of pOTC (10−50 μM) required for the inhibition (Murakami *et al.*, 1990). Ono and Tuboi (1988) reported purification of a factor from reticulocyte lysate that binds to the synthetic presequence of rat ornithine aminotransferase and stimulates import of the presequence peptide into the mitochondria. M_r of this factor was estimated by gel filtration to be 200 000. It remains to be elucidated whether this factor is identical to PBF.

PBF is apparently a general factor involved in protein import into the mitochondria. The purified PBF stimulated import of the purified pOTC into isolated mitochondria. Binding competition experiments showed that PBF also binds to presequences of other mitochondrial precursor proteins with a similar affinity (Figures 4 and 5). PBF is present not only in rabbit reticulocyte lysate but in the cytosol of rat liver and heart where mitochondrial protein import is active.

Deshaies *et al.* (1988) showed that hsp70 is involved in the post-translational import of precursor proteins into both mitochondria and lumen of endoplasmic reticulum in yeast. Murakami *et al.* (1988b) reported that the stimulatory activity of mitochondrial protein import consists of at least two distinct factors. One is hsp70 and the other is an *N*-ethylmaleimide-sensitive protein(s). Our present results demonstrate by using a purified precursor protein and purified cytosolic factors that both PBF and hsp70 are involved in pOTC transport into the mitochondria. The results show that PBF alone can stimulate pOTC import and that hsp70 further enhances the PBF-stimulated import. Hsp70 alone is ineffective. Our results also show that PBF is required for mitochondrial import of native (*in vitro* synthesized) pOTC as well as of purified and urea-denatured pOTC.

We propose that PBF plays an important role in an early step of mitochondrial protein import. PBF binds to the presequence portion of the nascent or completed precursor protein, prevents it from folding, and holds it in an import-competent form. Hsp70 may also be involved in conferring or maintaining import-competent conformation of the precursor proteins. The precursor−PBF(−hsp70) complex may then bind to the mitochondrial surface, and the presequence portion of the precursor protein may be displaced from PBF to a 'receptor' on the mitochondrial surface, after which the precursor is imported into the mitochondria. Isolation of the homogeneous PBF is expected to facilitate studies on protein targeting to the mitochondria and aid in the identification of other components involved in mitochondrial protein import.

Materials and methods

Preparation of ³⁵S-labeled pOTC

pOTC was labeled in *E.coli* with Tran^{35}S-label (mixture of [^{35}S]methionine and [^{35}S]cysteine, ICN Laboratories), and purified as described (Murakami *et al.*, 1988a). Specific radioactivity of the labeled pOTC was 5×10^8 d.p.m./mg protein.

Assay of PBF activity

PBF was assayed for its ability to form a soluble complex with radiolabeled and purified pOTC. ^{35}S-labeled and purified pOTC (40 ng in 1 μl of 8 M urea, 2 × 10^4 d.p.m.) was mixed with 50 μl of 20 mM potassium HEPES (pH 7.6) containing PBF. The mixture was layered on a linear 5−20% sucrose gradient (4.9 ml) containing 20 mM potassium HEPES (pH 7.6) and centrifuged for 5 h at 50 000 r.p.m. (225 000 *g*) at 20°C in a Hitachi 55P ultracentrifuge using an RPS55T rotor. Fractions (0.35 ml) were collected by means of a needle inserted through the bottom of the centrifuge tube, and 0.1 ml portions were counted for radioactivity. One unit of PBF is defined as the activity that holds half the amount of the added pOTC in a soluble complex.

Preparation of pOTC−Sepharose

pOTC was expressed in *E.coli* and inclusion bodies consisting of pOTC were isolated and washed with 1% Triton X-100 as described (Murakami *et al.*, 1988a). The washed inclusion bodies were dissolved in 2 ml of 10% (w/v) SDS and heated at 65°C for 30 min. The solubilized pOTC (~2 mg) was covalently attached to 10 ml of CNBr-activated Sepharose CL-4B as described by Kadonaga and Tjian (1986).

Purification of PBF from rabbit reticulocyte lysate

Affinity chromatography with pOTC−Sepharose. Rabbit reticulocyte lysate was prepared as described (Schimke *et al.*, 1973). The lysate (200 ml) was centrifuged at 105 000 *g* for 60 min, and the supernatant (post-ribosomal lysate) was dialyzed for 20 h at 4°C against 20 mM potassium HEPES buffer (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride. The dialyzed lysate was mixed with 10 ml of packed pOTC−Sepharose resin which had been equilibrated with 20 mM potassium HEPES (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride. The lysate and the resin slurry were incubated at 25°C for 30 min with gentle mixing. The resin was then collected by centrifugation at 200 *g* for 10 min, resuspended in 100 ml of 20 mM potassium HEPES (pH 7.6) and poured into a 1.5 × 5 cm column. The column was washed with 500 ml of 20 mM potassium HEPES (pH 7.6), and bound proteins were eluted with 20 mM potassium HEPES (pH 7.6) containing 0.5 M KCl. Protein was monitored at A_{280} and 10 ml with the highest absorbance were pooled.

HPLC with DEAE−5PW. Affinity purified fraction was dialyzed at 4°C against two 1 l portions of 20 mM Tris−HCl, pH 7.6. The dialyzed fractions were applied to an HPLC DEAE−5PW column (0.75 × 7.5 cm, TOSOH, Tokyo) equilibrated with 20 mM Tris−HCl, pH 7.6. The column was washed with 30 ml of the same buffer. Bound proteins were eluted with a linear gradient of 0−0.5 M NaCl (30 ml). PBF activity was eluted at ~0.4 M NaCl and the active fractions (1.0 ml in total) were collected.

Sucrose gradient centrifugation. The DEAE−5PW fraction (5 × 0.2 ml portion) was layered on linear 5−20% sucrose gradients (4.8 ml) containing 20 mM Tris−HCl, pH 7.6, and centrifuged at 20°C at 50 000 r.p.m. (226 400 *g*) in a Hitachi 55P ultracentrifuge, using an RPS55T rotor. Fractions were collected through the bottom of the centrifuge tubes. Purification was checked by measuring the PBF activity and by SDS−PAGE.

Gel filtration analysis

Purified PBF (10 μg, 20 μl) was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and applied to an HPLC G3000S_{XL} (0.78 × 30.0 cm, TOSOH) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. Elution was performed with the same buffer at a flow rate of 0.5 ml/min. The column had been calibrated with 10 μg each of the following proteins: bovine thyroglobulin (M_r = 670 000), bovine γ globulin (M_r = 158 000), chicken ovalbumin (M_r = 44 000) and horse myoglobin (M_r = 17 000).

Transport of purified pOTC into isolated mitochondria

^{32}S-labeled pOTC (40 ng in 1 μl of 8 M urea, 2 × 10^4 d.p.m.) was mixed with 50 μl of the import assay mixture that contained 27.5 mM potassium HEPES (pH 7.6), 120 mM potassium acetate, 1.2 mM magnesium acetate, 2.2 mM dithiothreitol, 1 mM ATP, 10 mM potassium succinate, 10 mM potassium L-malate, 2.6 units of creatine kinase, 10 mM creatine phosphate, 5 mM NADH, 100 μg of isolated rat liver mitochondria, 200 μg/ml of bovine serum albumin, and dialyzed rat reticulocyte lysate, purified PBF and/or purified yeast hsp70. The mixture was incubated at 25°C for 90 min unless otherwise stated. The transport mixture was mixed with 150 μl of 0.25 M sucrose containing 0.1 mM dinitrophenol, layered on 100 μl of 0.34 M sucrose, and then centrifuged at 15 000 r.p.m. for 30 min in a refrigerated microcentrifuge to remove the aggregated pOTC. The supernatant was then centrifuged at 15 000 r.p.m. for 10 min and reisolated mitochondria were subjected to SDS−PAGE and fluorography. Rat liver mitochondria were prepared from male Wistar rats as described (Schnaitman and Greenawalt, 1968).

Depletion of PBF from reticulocyte lysate and in vitro translation

mRNA of rat liver pOTC was synthesized by *in vitro* transcription of the plasmid pSPT18/pOTC (Murakami *et al.*, 1988). To deplete PBF from rabbit reticulocyte lysate, 100 μl of pOTC−Sepharose were added to 1 ml of nuclease treated lysate. OTC−Sepharose was used as a control. After incubation at 25°C for 10 min, the resin was removed by centrifugation. The supernatant was used for *in vitro* translaton which was performed in the presence of [^{35}S]methionine (1000 Ci/mmol, New England Nuclear) as described (Mori *et al.*, 1981c).

Purification of PBF from rat liver and heart

Rat liver and heart were homogenized in 9 vol of 0.25 M sucrose containing 5 mM potassium HEPES (pH 7.6) and the homogenates were centrifuged at 105 000 *g* for 60 min at 4°C. PBF was purified from the supernatant (cytosolic fraction) in the same manner as for the reticulocyte lysate. One gram of rat liver and heart yielded ~8 μg and 2.5 μg of nearly homogeneous PBF, respectively.

Materials

Synthetic presequence peptide of pOTC was synthesized by Applied Biosystems Japan (Tokyo, Japan). Those of mitochondrial aspartate aminotransferase and malate dehydrogenase were provided by Drs S.Tanase and Y.Morino (Kumamoto University). Yeast hsp70 which was purified as described by Chirico *et al.* (1988) was provided by Dr T.Endo (Nagoya University, Japan). The hsp70 preparation was ~80% homogeneous as judged by SDS−PAGE.

Acknowledgements

We thank Drs S.Tanase and Y.Morino for providing the synthetic peptides, Dr T.Endo for providing the purified yeast hsp70, Drs F.Goto, K.Goto, S.Ohkawara and S.Mori (Kumamoto University) for valuable suggestions and M.Ohara for comments.

References

Altieri,F., Mattingly,J.R., Rodriguez-Berrocal,F.J., Youssef,J., Iriarte,A., Wu,T. and Martinez-Carrion,M. (1989) *J. Biol. Chem.*, **264**, 4782−4786.

Argan,C. and Shore,C. (1985) *Biochem. Biophys. Res. Commun.*, **134**, 21−28.

Chen,W.-J. and Douglas,M.G. (1987) *J. Biol. Chem.*, **262**, 15598−15604.

Chirico,W.J., Waters,G. and Blobel,G. (1988) *Nature*, **322**, 805−810.

Conboy,J.G., Kalousek,F. and Rosenberg,L.E. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5724−5729.

Douglas,M.G., McCammon,M.T. and Vassarotti,A. (1986) *Microbiol. Rev.*, **50**, 166−178.

Deshaies,R.J., Koch,B.D., Werner-Washburne,M., Craig,E.A. and Schekman,R. (1988) *Nature*, **322**, 800−805.

Eilers,M. and Schatz,G. (1986) *Nature*, **322**, 228−232.

Eilers,M., Hwang,S. and Schatz,G. (1988) *EMBO J.*, **7**, 1139−1143.

Endo,T. and Schatz,G. (1988) *EMBO J.*, **7**, 1153−1158.

Horwich,A.L., Fenton,W.A., Williams,K.R., Kalousek,F., Kraus,J.P., Doolittle,R.F., Konigsberg,W. and Rosenberg,L.E. (1984) *Science*, **224**, 1068−1074.

Joh,T., Nomiyama,H., Maeda,S., Shimada,K. and Morino,Y. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6065−6069.

Joh,T., Takeshima,H., Tsuzuki,T., Shimada,K., Tanase,S. and Morino,Y. (1987) *Biochemistry*, **26**, 2515−2520.

Kadonaga,J.T. and Tjian,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5889−5893.

Miura,S., Mori,M., Amaya,Y. and Tatibana,M. (1982) *Eur. J. Biochem.*, **122**, 641−647.

Miura,S., Mori,M. and Tatibana,M. (1983) *J. Biol. Chem.*, **258**, 6671−6674.

Mori,M., Miura,S., Tatibana,M. and Cohen,P.P. (1980) *J. Biochem. (Tokyo)*, **88**, 1829−1836.

Mori,M., Morita,T., Miura,S. and Tatibana,M. (1981a) *J. Biol. Chem.*, **256**, 8263−8266.

Mori,M., Morita,T., Ikeda,F., Amaya,Y., Tatibana,M. and Cohen,P.P. (1981b) *Proc. Natl. Acad. Sci. USA*, **78**, 6056−6060.

Mori,M., Miura,S., Tatibana,T. and Cohen,P.P. (1981c) *J. Biol. Chem.*, **256**, 4127−4132.

Morita,T., Miura,S., Mori,M. and Tatibana,M. (1982) *Eur. J. Biochem.*, **122**, 501–509.
Murakami,K., Amaya,Y., Takiguchi,M., Ebina,Y. and Mori,M. (1988a) *J. Biol. Chem.*, **263**, 18437–18442.
Murakami,H., Pain,P. and Blobel,G. (1988b) *J. Cell Biol.*, **107**, 2051–2057.
Murakami,K., Tokunaga,F., Iwanaga,S. and Mori,M. (1990) *J. Biochem. (Tokyo)*, **108**, 207–214.
Ohta,S. and Schatz,G. (1984) *EMBO J.*, **3**, 651–657.
Ono,H. and Tuboi,S. (1988) *J. Biol. Chem.*, **263**, 3188–3193.
Pfanner,N., Muller,H.K., Harmey,M.A. and Neupert,W. (1987a) *EMBO J.*, **6**, 3449–3454.
Pfanner,N., Tropschug,M. and Neupert,W. (1987b) *Cell*, **49**, 815–823.
Pfanner,N., Hartl,F.-U. and Neupert,W. (1988) *Eur. J. Biochem.*, **175**, 205–212.
Schimke,R.T., Rhoads,R.E. and McKnight,G.S. (1973) *Methods Enzymol.*, **30**, 694–701.
Schleyer,L.L. and Neupert,W. (1987) *EMBO J.*, **6**, 2635–2642.
Schnaitman,C. and Greenawalt,J.W. (1968) *J. Cell Biol.*, **38**, 158–175.
Takiguchi,M., Miura,S., Mori,M., Tatibana,M., Nagata,S. and Kaziro,Y. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7412–7416.
Vener,K. and Schatz,G. (1987) *EMBO J.*, **6**, 2449–2456.
Vener,K. and Schatz,G. (1988) *Science*, **241**, 1307–1313.
Vestweber,D. and Schatz,G. (1988a) *J. Cell Biol.*, **107**, 2037–2044.
Vestweber,D. and Schatz,G. (1988b) *EMBO J.*, **7**, 1147–1151.

Received on May 14, 1990; revised on July 9, 1990