The EMBO Journal vol.6 no.9 pp.2627–2633, 1987

Mitochondrial porin of *Neurospora crassa*: cDNA cloning, *in vitro* expression and import into mitochondria

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Communicated by W.Neupert

cDNA encoding porin of *Neurospora crassa*, the major protein component of the outer mitochondrial membrane, was isolated and the nucleotide sequence was determined. The deduced protein sequence consists of 283 amino acids (29 979 daltons) and shows sequence homology of around 43% to yeast porin; however, no significant homology to bacterial porins was apparent. According to secondary structure predictions, mitochondrial porin consists mainly of membrane-spanning sided β-sheets. Porin was efficiently synthesized *in vitro* from the cDNA; this allowed us to study in detail its import into mitochondria. Thereby, three characteristics of import were defined: (i) import depended on the presence of nucleoside triphosphates; (ii) involvement of a proteinaceous receptor-like component on the surface of the mitochondria was demonstrated; (iii) insertion into the outer membrane was resolved into at least two distinct steps: specific binding to high-affinity sites and subsequent assembly to the mature form.

Key words: mitochondria/porin/import receptors/ATP/β-sheet structure

Introduction

Porin is the major protein component of the outer mitochondrial membrane forming diffusion pores for molecules with mol. wts lower than 4000–6000 daltons (Colombini, 1979; Zalman *et al.*, 1980; Manella *et al.*, 1983; Benz, 1985). It is nuclear-coded, synthesized in the cytoplasm and posttranslationally inserted into the outer membrane of mitochondria (Freitag *et al.*, 1982b; Mihara *et al.*, 1982; Gasser and Schatz, 1983). Like all proteins of the outer mitochondrial membrane studied so far, porin is synthesized without a transient presequence and import is independent of a membrane potential. In contrast, the majority of mitochondrial proteins are synthesized as larger precursors and import into mitochondria depends on an energized inner membrane (for review see Harmey and Neupert, 1985; Zimmermann, 1986; Nicholson and Neupert, 1987).

The import pathway of porin appears to be relatively simple and thus offers a good possibility to study the initial steps in translocation of proteins into mitochondria. In particular we wish to understand how porin is targeted specifically to mitochondria. Thus the targeting signal of the porin precursor and the decoding structures on the mitochondria have to be identified. Recently, we have described specific proteinaceous binding sites on the outer mitochondrial membrane which appear to serve as receptors for precursors (Zwizinski *et al.*, 1984; Pfaller *et al.*, 1985; Schmidt *et al.*, 1985; Pfanner and Neupert, 1987).

We report here on the isolation and sequencing of full-length cDNA clones for porin of *Neurospora crassa*. Porin is composed of largely polar amino acids; it is homologous to yeast porin (Mihara and Sato, 1985) but shows no significant homology to bacterial porins. Despite this fact, the secondary structure (predominantly β-sheet) and membrane arrangement of mitochondrial porins seem to be very similar to those of bacterial porins. An amphiphilic α-helical structure at the very amino-terminus is suggested to be the mitochondrial targeting signal. Porin was expressed *in vitro* from the cloned cDNA and was imported into isolated mitochondria. Insertion was found to depend on a proteinaceous receptor-like component on the mitochondrial surface and to require nucleoside triphosphates.

Results

Screening for porin cDNA clones by expression cloning

dC-tailed cDNAs from *N. crassa* (see Materials and methods) were annealed with a mixture of *Pst*I-cut and dG-tailed expression vectors pEX1-3 possessing polylinkers in all reading frames at the 3′-end of the *cro–lacZ* gene (Stanley and Luzio, 1984). Transformation into *Escherichia coli* strain pop2136 carrying the c*Its*857 repressor was performed. Using polyclonal antibodies against porin, six immune-positive clones were obtained from approximately 15 000 clones. In order to confirm the identity of these clones, hybridization-selected translation (Viebrock *et al.*, 1982; Parnes *et al.*, 1981) was performed. In this assay three plasmids (ppor5, ppor10 and ppor23) selected mRNA which directed the synthesis of a translation product with the size of porin and which was recognized by antibodies against porin (data not shown). Northern blot analysis indicated that the *Pst*I-inserts did not represent full-length cDNAs (data not shown). In order to obtain full-length cDNA clones, a cDNA library in pBR322 was screened by colony hybridization using ppor10 insert as a probe. Positive clones were found with a frequency of 0.1%. Northern blot analysis demonstrated that two of these clones contained inserts (ppor31 and ppor42) which displayed the same mobility as mRNA to which the labelled ppor10 insert hybridized, suggesting that these inserts represented full-length cDNAs (Figure 1).

Fig. 1. Northern blot analysis. Poly(A)$^+$ RNA and ppor31 and ppor42 cDNA inserts were resolved on a denaturing 2% agarose gel, blotted onto Biodyne A filter and finally hybridized against a nick-translated, ^{32}P-labelled ppor10 insert. 50 μg (**lane 1**), 25 μg (**lane 2**), 5 μg (**lane 3**) of poly(A)$^+$ RNA; 1 ng and 10 ng of ppor31 insert (**lanes 4** and **5**); 1 ng and 10 ng of ppor42 insert (**lanes 6** and **7**).

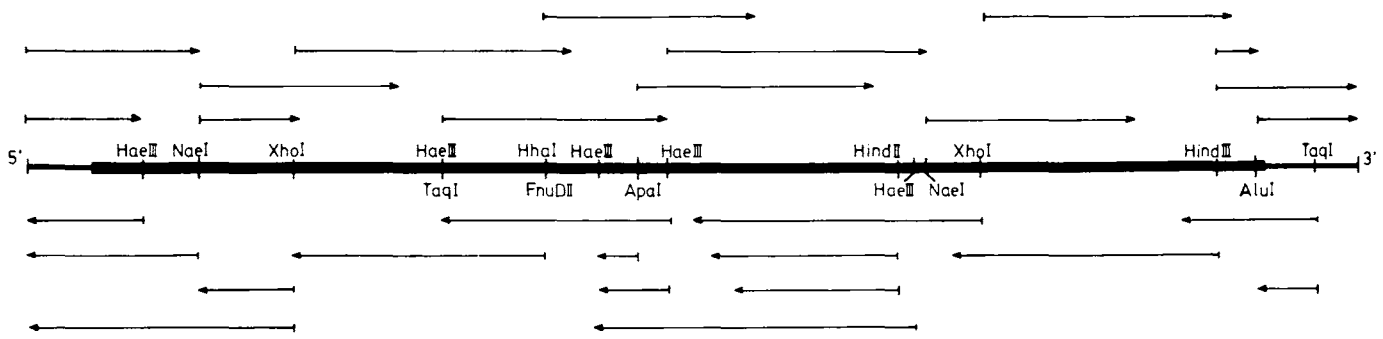

Fig. 2. Strategy for sequencing full-length cDNA for *N. crassa* porin. The inserts of ppor10, 31 and 42 were digested with restriction enzymes as indicated and subcloned into pUC19. Sequencing was carried out by the dideoxy-chain termination method (Sanger *et al.*, 1977) using primers and [^{35}S]dATP as radioactive label. Extent and direction of sequencing are indicated by arrows. The coding region is shown as thickened bar.

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                                                                  -30
                                                  CA TTC ACC ATC ATA CCC TGT CGC CCT CAC ACA ACA TCT TTC ACA

                                  30                                  60                                  90
ATG GCT GTT CCC GCT TTC TCT GAC ATC GCC AAG TCG GCC AAC GAC CTC CTC AAC AAG GAC TTC TAC CAC CTC GCT GCC GGC ACC ATC GAG
Met Ala Val Pro Ala Phe Ser Asp Ile Ala Lys Ser Ala Asn Asp Leu Leu Asn Lys Asp Phe Tyr His Leu Ala Ala Gly Thr Ile Glu

                                 120                                 150                                 180
GTC AAG TCC AAC ACC CCC AAC AAT GTC GCC TTC AAG GTT ACC GGC AAG TCC ACA CAC GAC AAG GTC ACC AGC GGT GCT CTC GAG GGC AAA
Val Lys Ser Asn Thr Pro Asn Asn Val Ala Phe Lys Val Thr Gly Lys Ser Thr His Asp Lys Val Thr Ser Gly Ala Leu Glu Gly Lys

                                 210                                 240                                 270
TTC ACC GAC AAG CCC AAT GGC TTG ACC GTC ACC CAG ACC TGG AAC ACT GCC AAC GCC CTT GAG ACC AAG GTC GAG ATG GCC GAC AAC CTC
Phe Thr Asp Lys Pro Asn Gly Leu Thr Val Thr Gln Thr Trp Asn Thr Ala Asn Ala Leu Glu Thr Lys Val Glu Met Ala Asp Asn Leu

                                 300                                 330                                 360
GCC AAG GGT CTC AAG GCT GAG GGT ATC TTC TCT TTC CTC CCC GCC ACC AAC GCT CGC GGC GCC AAG TTC AAC CTC CAC TTC AAG CAG AGC
Ala Lys Gly Leu Lys Ala Glu Gly Ile Phe Ser Phe Leu Pro Ala Thr Asn Ala Arg Gly Ala Lys Phe Asn Leu His Phe Lys Gln Ser

                                 390                                 420                                 450
AAC TTC CAC GGC CGT GCT TTC TTC GAC CTC CTC AAG GGC CCC ACC GCC AAC ATT GAC GCC ATT GTC GGC CAC GAG GGT TTC CTC GCT GGT
Asn Phe His Gly Arg Ala Phe Phe Asp Leu Leu Lys Gly Pro Thr Ala Asn Ile Asp Ala Ile Val Gly His Glu Gly Phe Leu Ala Gly

                                 480                                 510                                 540
GCC TCC GCT GGC TAC GAT GTC CAG AAG GCT GCC ATC ACT GGT TAC AGC GCC GCT GTC GGC TAC CAC GCC CCT ACC TAC AGC GCT GCC ATC
Ala Ser Ala Gly Tyr Asp Val Gln Lys Ala Ala Ile Thr Gly Tyr Ser Ala Ala Val Gly Tyr His Ala Pro Thr Tyr Ser Ala Ala Ile

                                 570                                 600                                 630
ACC GCT ACT GAC AAC CTG AGC GTC TTC TCC GCT TCT TAC TAC CAC AAG GTC AAC TCC CAG GTT GAG GCC GGC TCC AAG GCC ACC TGG AAC
Thr Ala Thr Asp Asn Leu Ser Val Phe Ser Ala Ser Tyr Tyr His Lys Val Asn Ser Gln Val Glu Ala Gly Ser Lys Ala Thr Trp Asn

                                 660                                 690                                 720
TCC AAG ACC GGT AAC ACC GTC GGC CTC GAG GTC GCC ACC AAG TAC CGC ATT GAC CCC GTC TCT TTC GTC AAG GGC AAG ATC AAC GAC CGT
Ser Lys Thr Gly Asn Thr Val Gly Leu Glu Val Ala Thr Lys Tyr Arg Ile Asp Pro Val Ser Phe Val Lys Gly Lys Ile Asn Asp Arg

                                 750                                 780                                 810
GGT GTC GCT GCC ATT GCC TAC AAT GTT CTC CTC CGT GAG GGC GTC ACC CTC GGT GTT GGT GCC TCT TTC GAT ACC CAG AAG CTT GAC CAG
Gly Val Ala Ala Ile Ala Tyr Asn Val Leu Leu Arg Glu Gly Val Thr Leu Gly Val Gly Ala Ser Phe Asp Thr Gln Lys Leu Asp Gln

                                 840                                 870                                 900
GCC ACC CAC AAG GTC GGC ACC AGC TTC ACC TTC GAG TCT TAG AGT AAT CGC AGG ACA CAT GTG AAG CAG CGG CGT AAT AGT ATT CCT TCG
Ala Thr His Lys Val Gly Thr Ser Phe Thr Phe Glu Ser End
                                      ↓      930↓          ↓
AAC AAA TTC TCA ACT GGA ATA GAT AAC CCC CCT TTG CTG GCA
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Fig. 3. Nucleotide sequence of *N. crassa* porin cDNA and the deduced amino acid sequence. Nucleotides are numbered beginning with the first nucleotide of the first ATG triplet. Putative polyadenylation signals are underlined. Arrows indicate the polyadenylation sites of different clones.

Nucleotide sequence of N. crassa porin cDNA

The complete nucleotide sequence of the cDNA encoding porin was determined by double strand sequencing (Chen and Seeburg, 1985) of subcloned restriction fragments in the pUC19 plasmid as outlined in Figure 2. The coding region contains 849 nucleotides (Figure 3). Both the 5′- and 3′-non-coding region are relatively short (44 nucleotides and 75–93 nucleotides, respectively). All of the sequenced cDNAs (ppor10, ppor31 and ppor42) had a poly(A)-tail, but at different positions and of variable lengths (Figure 3). Possible polyadenylation signals may be AACAAA and AAUAGA (nucleotide position 901–906 and 918–923, respectively). The sequence preceding the first ATG (CTTTCACA) resembles the corresponding sequence in other *N. crassa* mRNAs (Viebrock *et al.*, 1982; Alton *et al.*, 1982; Wondt *et al.*, 1983; Schlechtman and Yanofsky, 1983; Römisch *et al.*, 1987; Tropschug and Neupert, unpublished data).

Primary structure and comparison of the protein sequence of N. crassa porin with yeast porin, bacterial porins and mitochondrial carrier proteins

The open reading frame of *N. crassa* porin cDNA corresponds to a protein consisting of 283 amino acids. The deduced mol. wt of 29 976 daltons is in good agreement with that determined by SDS–polyacrylamide gel electrophoresis (~31 kd). The deduced amino acid composition agrees with that determined by Freitag *et al.* (1982c). Comparison with the sequence of yeast

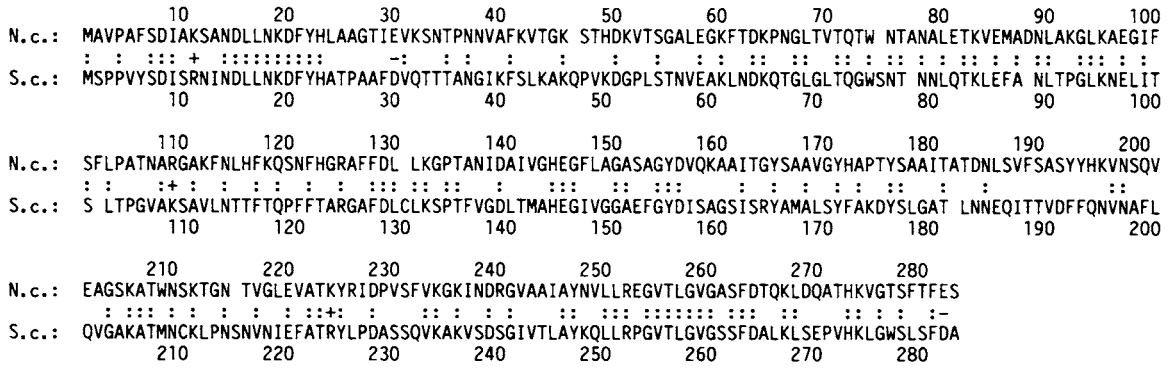

Fig. 4. Comparison of the deduced amino acid sequences between yeast and *N. crassa* porin, showing identities (double point) and conservation of charged (+,−) amino acids. Gaps were introduced to maximize homologies.

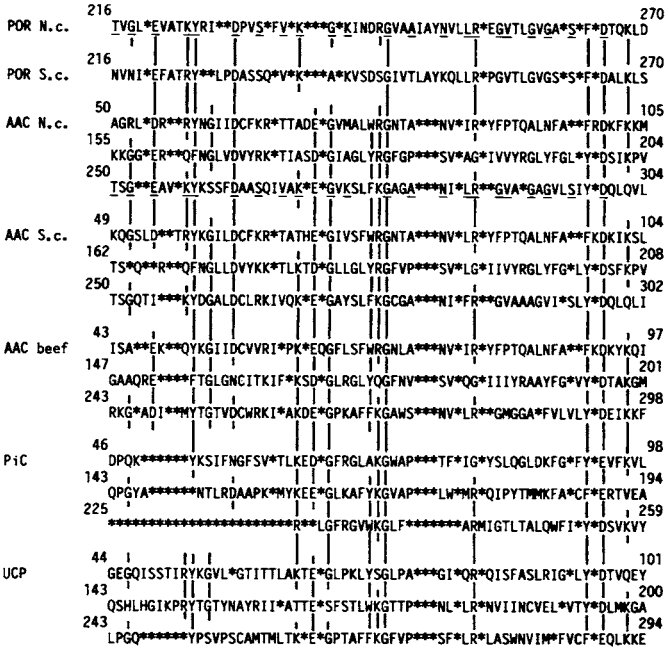

Fig. 5. Sequence comparison between mitochondrial porins and carriers. *N. crassa* porin (Por N.c.) and yeast porin (Por S.c.; Mihara and Sato, 1985) were aligned with the 'repeats' of the tripartite structure of ADP/ATP carrier of *Neurospora* (AAC N.c.; Arends and Sebald, 1984), of yeast (AAC S.c.; Adrian *et al.*, 1986) and of beef heart mitochondria (AAC beef; Aquila *et al.*, 1982) and with the uncoupler protein from mitochondria of brown adipose tissue (UCP; Aquila *et al.*, 1985) and phosphate carrier of beef heart mitochondria (PiC; Aquila *et al.*, 1987). Identical and isofunctional residues are marked by vertical lines. Identical residues at the C-termini of *N. crassa* porin and ADP/ATP carrier are underlined. Numbers indicate position of the residue in the sequence. Gaps (*) are introduced to maximize similarities.

porin (Mihara and Sato, 1985) shows that both proteins have the same length and that ~43% of the residues are conserved (Figure 4), whereas 60% of the charged amino acids are conserved. Both porins possess a high number of charged residues (*N. crassa*: 28 pos./24 neg., and yeast: 24 pos./23 neg.) and polar residues (*N. crassa*: 121, and yeast: 118). Both proteins have the same overall polarity index (Capaldi and Vanderkooi, 1972) of 46.3%, a value which is in the range of that observed for soluble proteins.

Sequence comparison of the mitochondrial porins with bacterial porins (OmpC, OmpF and PhoE), which are closely related to each other (Mizuno *et al.*, 1983) shows no apparent homology (data not shown). On the other hand, *N. crassa* porin shows a significant local similarity to the *N. crassa* ADP/ATP carrier (Arends and Sebald, 1984) (residues 216−270 and 250−304, respectively). Moreover, the distribution of certain distinct residues is remarkably similar between the mitochondrial porins and various mitochondrial carriers (ADP/ATP carrier, uncoupler protein and phosphate carrier; Figure 5). These critical residues were reported to be conserved within the three similar sequences ('repeats') of the tripartite structure of ADP/ATP carrier, uncoupler protein and phosphate carrier (Aquila *et al.*, 1987).

Secondary structure predictions and membrane arrangement of mitochondrial porins

The hydropathy profile (Kyte and Doolittle, 1982) of *N. crassa* porin suggests that the amino-terminal third of the protein is strongly hydrophilic in character and that the rest is moderately hydrophilic in comparison (Figure 6). Neither a long stretch of uncharged and hydrophobic amino acids, nor a significant clustering of charges can be seen. The plot for yeast porin (Mihara and Sato, 1985) is very similar to that of the *N. crassa* porin, with the exception of the regions around amino acid position 105−125 and 180−205.

According to Kyte and Doolittle (1982), a membrane spanning α-helix is postulated to require a mean hydropathy value of >1.6 for a span of >19 amino acids. No such stretch can be detected in the hydropathy plot for porin (Figure 6). By using a more refined analysis which takes into account different degrees of hydrophobicity on opposite sides of α-helices (for details see Aquila *et al.*, 1985), one long amphiphilic α-helical structure was observed. In both proteins the first 18 amino acids are able to form an amphiphilic α-helix; however, no further α-helical structure long enough to traverse the membrane was found. In a search for amphiphilic β-sheets, distinct regions of oscillations with a length of two residues were obtained.

Following the proposal of Paul and Rosenbusch (1985) that a minimum of six residues is sufficient to span the membrane in β-configuration, about 12−16 membrane spanning segments may be present in the porin molecules. Both *N. crassa* and yeast porin thus appear to consist predominantly of stretches of sided β-sheets. The hydrophilic sides of the β-sheets may be involved in forming the aqueous channel and the hydrophobic ones may interact with the membrane lipids. A dense network of hydrogen bonds and/or ion pairs between neighbouring β-sheets may stabilize the molecule, which would explain the extreme resistance against protease treatment (see below). For bacterial porins a β-sheet structure has been previously demonstrated (Paul and Rosenbusch, 1985; Kleffel *et al.*, 1985; Weckesser *et al.*, 1984; Yoshimura *et al.*, 1983).

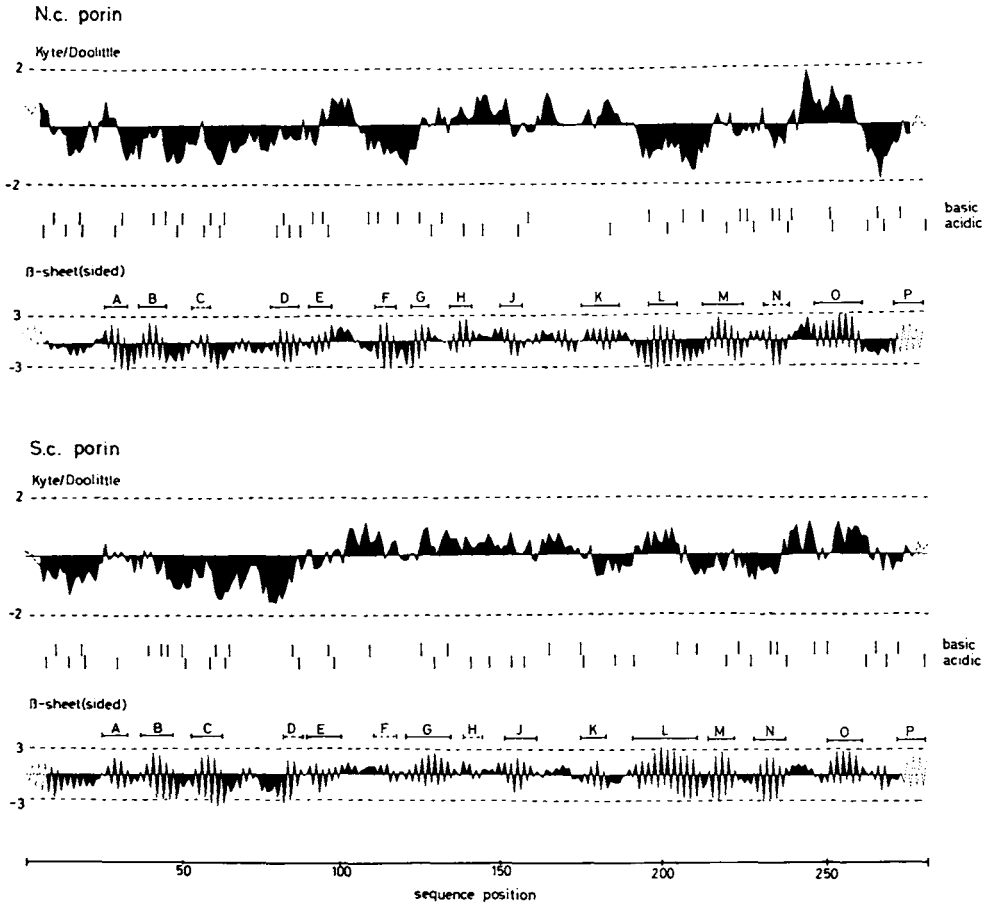

Fig. 6. Hydropathy plots and distribution of charges in the primary structure of *N. crassa* (N.c.) and yeast (S.c.) porin. Hydropathy was evaluated according to Kyte and Doolittle (1982) with a span setting of eleven residues (hydrophilic: negative values; hydrophobic: positive values). Sided β-sheet hydropathy plots were obtained by adding the hydropathy indices of the respective residues using the weight in parentheses: residue n (1.0), n + 2 (0.5), n + 4 (0.5) and n + 6 (0.5). Sided β-sheets can be seen as oscillations between hydrophobic and hydrophilic values. Tentative prediction of possible membrane spanning β-sheets is indicated by bars (A–P). All programs are written in BASIC or FORTRAN and run on a Kontron PSI 80 microcomputer linked to a GRAPHTEC MP 1000 plotter.

Binding and membrane insertion of porin depend on a trypsin-sensitive protein component on the mitochondrial surface

Porin cDNA was cloned into the transcription vector pDS5 (Stüber *et al.*, 1984) and porin precursor synthesized in a transcription–translation system in the presence of [^{35}S]-methionine. Subsequent SDS–PAGE and fluorography revealed a single major labelled polypeptide. This polypeptide comigrated with both precursor and purified mature porin and could be precipitated by antiserum against porin (data not shown).

Specific binding of porin precursor and insertion into the outer membrane can be monitored by differential protease sensitivity (Pfaller and Neupert, 1987, accompanying paper). Unspecifically bound precursor was found to be very sensitive to treatment with proteinase K, 5 μg/ml being sufficient to digest it completely. Specifically bound precursor, i.e. precursor which is bound to saturable high affinity sites, was largely resistant to 5 μg/ml proteinase K but completely sensitive to 100 μg/ml. In contrast, mature, assembled porin was found to be largely resistant to 100 μg/ml proteinase K.

Porin, synthesized from the cDNA, was incubated with mitochondria and mitochondria were subsequently treated with different concentrations of proteinase K (Figure 7). After incubation at 0°C for 5 min part of the precursor was resistant against 5 μg/ml proteinase K (lane 2); this represents specifically bound porin. Only a negligible fraction was resistant against 100 μg/ml proteinase K (lane 3); apparently at low temperature and short incubation periods, only a very small fraction of the precursor completely inserted into the membrane. The levels of specifically bound (lane 5) and inserted porin (lane 6) were higher when the incubation was performed for 30 min. A small but significant part of the specifically bound porin could be chased into a protease-resistant membrane location after re-isolating mitochondria and incubating them at 25°C (lane 8 versus 3). Insertion into the membrane was much more expressed when incubation of mitochondria and porin was carried out at 25°C (lane 10).

Mitochondria which had been pretreated with trypsin displayed a strongly reduced capacity to bind the porin precursor in a specific manner (lanes 12 and 15). In contrast, unspecific binding was only slightly reduced (lanes 11, 14, 17 and 19). Integration into the outer membrane was almost completely abolished in these trypsin-pretreated mitochondria (lanes 13, 16, 18 and 20). These findings support the view that both specific binding and membrane insertion require a protease-sensitive component on the outer membrane.

Import of porin depends on nucleoside triphosphates

Import of several precursor proteins into mitochondria has been shown to require nucleoside triphosphates (NTPs, e.g. ATP or GTP). Examples include the precursor proteins of F_1-ATPase subunit β (Pfanner and Neupert, 1986), ADP/ATP carrier and

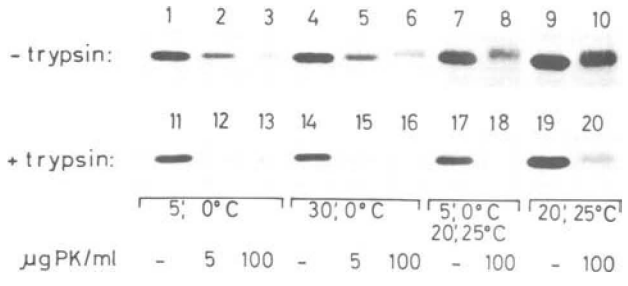

Fig. 7. Effect of trypsin pretreatment on import of porin into mitochondria. Mitochondria were pretreated either without (**lanes 1–10**) or with (**lanes 11–20**) 1 μg trypsin per 1 mg/ml mitochondrial protein for 15 min at 25°C, re-isolated by centrifugation and incubated with postribosomal supernatant of plasmid-programmed reticulocyte lysate. Incubations were performed at 0°C for 5 min (**lanes 1–3** and **11–13**), for 30 min (**lanes 4–6** and **14–16**) or at 25°C for 20 min (**lanes 9, 10** and **19, 20**). Samples 7, 8, 17 and 18 were first incubated at 0°C for 5 min, re-isolated and then incubated at 25°C. Mitochondria re-isolated from the various import assays were incubated without (lanes 1, 4, 7, 9, 11, 14, 17 and 19) or with 5 μg/ml (lanes 2, 5, 12 and 15) or 100 μg/ml (lanes 3, 6, 8, 10, 13, 16, 18 and 20) proteinase K (PK). A fluorograph of the dried SDS–polyacrylamide gel is shown.

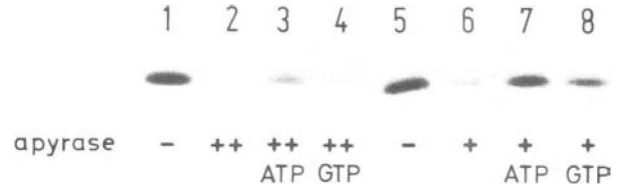

Fig. 8. Import of porin requires nucleoside triphosphates. Mitochondria (2.5 mg mitochondrial protein/ml) were incubated for 15 min at 25°C and post-ribosomal supernatant of reticulocyte lysate (75 μl, containing the porin precursor) was incubated for 15 min at 30°C and 15 min at 25°C in the presence of 1 U (**lanes 2–4**) or 0.05 U apyrase (**lanes 6–8**). Controls received an apyrase preparation which had been heated before use (10 min at 95°C) corresponding to 1 U (**lane 1**) and 0.05 U (**lane 5**). The mitochondria were added to the reticulocyte lysate and the import reactions were performed as described (Pfanner and Neupert, 1986) including 20 μM oligomycin and a buffer containing 3% (w/v) bovine serum albumin. Reaction 3 and 7 received 8 mM ATP and 4 and 8 received 8 mM GTP. Samples were incubated for 25 min at 25°C and then were cooled to 0°C and treated with proteinase K (300 μg/ml final concentration). Mitochondria were re-isolated, washed in SEM medium (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) plus 1 mM PMSF, transferred to new cups and re-isolated again. Samples were analyzed by SDS–polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown.

fusion proteins consisting of different parts of F_0-ATPase subunit 9 precursor and mouse dihydrofolate reductase (Pfanner *et al.*, 1987). We tested whether import of porin also required NTPs. Both postribosomal supernatants of reticulocyte lysates (containing radiolabelled porin precursor derived from transcription–translation of porin cDNA) and mitochondria were depleted of ATP by incubation with apyrase (an adenosine-5′-triphosphatase and adenosine-5′-diphosphatase) as previously described (Pfanner and Neupert, 1986). Oligomycin was included to inhibit formation of ATP by the F_0F_1-ATPase. Reticulocyte lysates and mitochondria were mixed and incubated at 25°C. Import of porin, tested by resistance to high concentrations of proteinase K (see above), was strongly reduced following apyrase treatment (Figure 8, lane 6 versus 5). When higher amounts of apyrase were used, the import was completely inhibited (lane 2 versus 1). Addition of ATP or GTP after pretreatment with apyrase restored the import to a large extent (lanes 7 and 8). When high amounts of apyrase had been used, addition of ATP or GTP

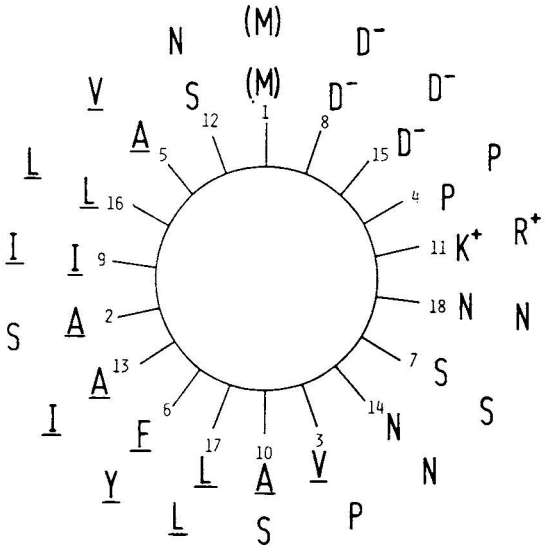

Fig. 9. Helical wheel projection of the amino-terminal region of porin. The first 18 amino acids of *N. crassa* (inner circle) and yeast (outer circle) are drawn in a helical wheel. Hydrophobic residues are underlined and charges are indicated. The numbers refer to the residue positions in the sequence.

only led to partial restoration of import (lanes 3 and 4); this is apparently due to the continued presence and action of apyrase during the import reaction. The amounts of precursor protein in the reticulocyte lysate (Pfanner and Neupert, 1986) and the protease resistance of mature endogenous porin were not affected by pretreatment with apyrase (data not shown). In conclusion, NTPs are required for the import of porin into the outer mitochondrial membrane. The efficiency of import seems to depend on the levels of NTPs present.

Discussion

In this report we describe the identification, sequencing and expression of porin cDNA from *N. crassa*. The deduced amino acid sequence reveals that the primary structure of porin is not strongly conserved between *N. crassa* and *Saccharomyces cerevisiae*, in contrast to that of other mitochondrial proteins, such as ADP/ATP carrier (Arends and Sebald, 1984; Adrian *et al.*, 1986), cytochrome c_1 (Römisch *et al.*, 1987; Sadler *et al.*, 1984), cytochrome *c* (Heller and Smith, 1966; Lederer and Simon, 1974; Stuart *et al.*, 1987; Boss *et al.*, 1981) and β-subunit of F_1-ATPase (Rassow, Neupert and Tropschug, in preparation; Takeda *et al.*, 1985). Despite this fact, the secondary structure of the porins from *N. crassa* and *S. cerevisiae* seem to be significantly conserved as sided β-sheets which traverse the membrane.

Areas of similarity have been identified between sequences of mitochondrial porins and various mitochondrial carrier proteins. These areas of similarity include critical amino acid residues which are thought to be involved in maintaining structural features, like folding (in particular glycines) and formation of ion pairs within the molecule or with phospholipids (charged amino acids) (Aquila *et al.*, 1987). These similarities may be due to phylogenetic relationships between the various proteins. Deletions and insertions may reflect differences in the secondary structure of the membrane-spanning segments. Structural analysis suggests that the first 18 amino acids of *N. crassa* porin are able to form an amphiphilic α-helix. This is more apparent when these amino acids are drawn in a helical wheel projection (von Heijne, 1986). An almost identical structure is seen with the yeast

porin (Figure 9). According to von Heijne (1986) most of the mitochondrial targeting sequences studied to date form amphiphilic α-helices with a high hydrophobic moment. The cleavable presequences of mitochondrial precursor proteins in general are highly positively charged. In contrast, the amphiphilic α-helical structure at the amino-terminus of the porins is negatively charged. The reason for this may be that positively-charged amphiphilic presequences of proteins that are targeted to the inner membrane or the matrix respond to the membrane potential across the inner membrane, which is negative inside. Porin which does not require a membrane potential for insertion may only require an amphiphilic structure for insertion into the outer membrane irrespective of the overall charge of this structure. This may indicate a divergence of import pathways for outer and inner membrane proteins beyond the level of receptor-mediated initial contact with the membrane, but before a response of the target signal to the membrane potential.

Import of porin into mitochondria was studied *in vitro* with the precursor protein synthesized efficiently from the cDNA. Pretrypsinization of mitochondria abolished import of porin. These data corroborate our proposal that the porin precursor has to interact with a receptor-like protein on the mitochondrial surface (Zwizinski *et al.*, 1984; Pfaller *et al.*, 1985; Pfaller and Neupert, submitted). In this respect porin behaves like other mitochondrial precursor proteins. It is not yet clear whether the initial reaction of the precursor with the outer membrane represents insertion into the lipid bilayer followed by interaction with a receptor-like protein, or whether in fact, the order of events is reversed.

Import of porin requires nucleoside triphosphates (NTPs). Obviously, proteolytic processing or membrane potential-dependent translocation across the inner membrane are not the primary reasons for the requirement of NTPs in the import of porin into mitochondria. The exact role of NTPs, however, remains to be elucidated. At least four possibilities can be postulated: (i) NTPs are required to unfold the precursor or stabilize it in a distinct import-competent conformation; (ii) the receptor-like protein has to be phosphorylated in order to bind the precursor; (iii) if the integration of the precursor into the lipid bilayer is the first step, this insertion could require energy in the form of NTPs (possibly a mediating protein being phosphorylated); (iv) the assembly is NTP-dependent. Studies on the biogenesis of ADP/ATP carrier, F_1-ATPase subunit β, F_0-ATPase subunit 9 and derivatives thereof suggest that the first possibility is the most likely one (Pfanner *et al.*, 1987). A strong hint in this direction is that a water-soluble porin which in many respects behaves like the precursor form of porin does not require ATP for import. A possible explanation for this is that water-soluble porin is already unfolded due to the isolation, denaturation and renaturation conditions employed (Pfaller and Neupert, submitted).

Materials and methods

Bacterial strains and plasmids

E. coli strain pop2136 was obtained by integrating into the chromosome of MM294 (F⁻*endA thi hsdR*) a 2.4 kb fragment of bacteriophage lambda carrying the c*Its*857 allele (Vidal-Ingigliardi and Raibaud, 1985). This strain was used as host for expression vectors pEX1,2,3 (Stanley and Luzio, 1984). Colony hybridization (Grunstein and Hogness, 1975) was performed using *E. coli* 5K (Hubacek and Glover, 1970) transformed with the cDNA library (see below) cloned into the *Pst*I site of pBR322. *E. coli* strains HB101 (Kedes *et al.*, 1975) and DH1 (Low, 1968) were used as hosts for pDS5 (Stüber *et al.*, 1984) and pUC19 (Viera and Messing, 1982) derived plasmids.

Construction of a N. crassa cDNA library

Total RNA and poly(A)$^+$ RNA were isolated from *N. crassa* hyphae as described by Michel *et al.* (1979) and Sheldon *et al.*, (1972), respectively. cDNA synthesis was performed as described by Gubler and Hoffman (1983) with the following modifications:

Synthesis of the first strand. 40 μg poly(A)$^+$ RNA were dissolved in 20 μl 10 mM CH_3HgOH and incubated for 10 min at 20°C. 4 μl 700 mM β-mercaptoethanol were added and incubation continued for 5 min. 6 μl RNasin (16 U/μl), 10 μl 10× first strand buffer (Maniatis *et al.*, 1982), 20 μl oligo dT_{12-18} (1 mg/ml), 10 μl dATP, dGTP, dCTP, dTTP (10 mM each), 10 μCi [α-^{32}P]dATP and 200 U AMV reverse transcriptase (Life Science) were added in a total volume of 100 μl. Incubation was at 42°C for 2 h. The reaction was stopped by addition of 0.1% SDS, 50 mM EDTA pH 8, phenol-extraction and separation of the RNA/DNA hybrid over a Sephadex G-50 column (bed volume 7 ml). The yield was 4.5 μg single-stranded cDNA (9% efficiency). Second strand synthesis was performed essentially as described by Gubler and Hoffman (1983) starting with 1 μg first strand. Again the reaction was stopped by addition of SDS/EDTA, phenol-extraction and separation over a Sephadex G-50 column.

dG/dC-Tailing and annealing

pBR322 was cut with *Pst*I and purified over a 0.8% agarose gel. Tailing of this vector with 15–20 residues of dG was performed as described by Hoeijmakers *et al.* (1980). Tailing of double stranded cDNA with dC turned out to be critical and was performed as follows: 300 ng cDNA were tailed in a buffer containing 140 mM cacodylate, 60 mM Tris/HCl pH 7.6, 1 mM $CoCl_2$, 0.2 mM dCTP in a total volume of 100 μl. After preincubation for 5 min at 37°C, 33 U terminal deoxynucleotidyl transferase (Pharmacia) was added, the reaction was performed for 8 min at 37°C, terminated by addition of 25 mM EDTA pH 8, heated to 65°C for 10 min and used without further purification. 50 ng dG-tailed pBR322 was annealed to 4.5 ng dC-tailed cDNA in 100 μl 100 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 7.8 by heating to 68°C for 5 min and cooling slowly to room temperature.

Preparation of mitochondria

Neurospora crassa (wild type 74A) was grown as described (Schleyer *et al.*, 1982). Disruption of cells and isolation of mitochondria were carried out as reported by Pfanner and Neupert (1985).

Isolation of mitochondrial porin and preparation of antibodies

Porin was isolated from a mitochondrial pellet by a procedure previously described (Freitag *et al.*, 1982a). For raising antibodies, TCA-precipitated purified porin (100 μg) was denatured in 4% SDS, 120 mM Tris–HCl, pH 6.8, mixed with an equal volume of incomplete Freund's adjuvant and was injected intradermally into the neck region of a rabbit. After two further subcutaneous injections, at 3 and 5 weeks, the rabbit was bled. The antiserum was used in the immunological screening of bacterial colonies. For immunoprecipitation an antiserum prepared according to Freitag *et al.* (1982c) was used.

Screening procedures

cDNA clones were screened using immunostaining as described by Stanley (1983). Transformants containing full-length cDNAs were selected by colony hybridization (Grunstein and Hogness, 1975).

DNA manipulations and sequencing

DNA manipulations were essentially done as described by Maniatis *et al.* (1982). *E. coli* strains were transformed as described by Hanahan (1983). Small and large scale plasmid preparations were carried out according to Birnboim and Doly (1979); large scale isolation included a CsCl/ethidium bromide equilibrium centrifugation step (Radloff *et al.*, 1967). cDNA restriction fragments subcloned into the polylinker of pUC19 were sequenced by extension of a primer hybridized to alkali-denatured plasmid DNA (Chen and Seeburg, 1985). [^{35}S]dATP labelled DNA molecules of different lengths were separated on a 6% acrylamide/8 M urea-gel. Using universal and reverse primers both strands were sequenced with the exception of a short segment (~30 bp) from one strand at the extreme 3'-end.

Transcription–translation and import of porin into mitochondria

Transcription of porin cDNA cloned into pDS5 was carried out as described by Stüber *et al.* (1984). The transcription mixture was used without further purification for translation in a nuclease-treated, rabbit reticulocyte lysate prepared according to Pelham and Jackson (1976). Translation was performed as described (Zimmermann *et al.*, 1979) for 60 min at 30°C and in the presence of 50 μCi [^{35}S]methionine (1000 Ci/mmol). Transfer into isolated mitochondria was carried out as reported (Zimmermann and Neupert, 1980), experiments with apyrase (Sigma A6160, Grade VIII) were performed according to Pfanner and Neupert (1986), and trypsin pretreatment was carried out as described (Zwizinski *et al.*, 1984) with the following modification: usually a mitochondrial suspension (corresponding to 20 μg mitochondrial protein) was incubated with 5–10 μl of postribosomal supernatant of plasmid programmed reticulocyte lysate in a final volume of 100 μl. For protease digestion, samples were incubated with 100 μg/ml proteinase K for 30 min at 0°C and proteolysis was stopped by addition of 0.5 mM (final concentration) of phenylmethylsulfonyl fluoride (PMSF).

Acknowledgements

We would like to thank Dr K.Stanley (EMBL, Heidelberg) for introducing us to expression cloning and for the gift of the expression vectors, pEX1,2,3; Dr O.Raibaud (Institut Pasteur, Paris) for the *E. coli* strain pop2136; Drs D.Stüber (Hoffmann-La Roche, Basel) and H.Bujard (ZMBH, Heidelberg) for the plasmid pDS5 and G.Müller and Dr R.Zimmermann for help with the *in vitro* transcription system; Drs H.Weich and G.Riemann (Gesellschaft für Biotechnologische Forschung, Braunschweig) for stimulating discussions concerning cDNA synthesis and Drs G.R.Hartmann and P.Heinrich (Institut für Biochemie der Universität München) for initial help with DNA double strand sequencing and computer programs. We are grateful to C.Forster for expert technical assistance and to R.Stuart for critically reading the manuscript. This work was supported by the Genzentrum München (Projekt 7) and the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 184).

References

Adrian,G.S., McCammon,M.T., Montgomery,D.L. and Douglas,M.G. (1986) *Mol. Cell. Biol.*, **6**, 626–634.
Alton,N.K., Buxton,F., Patel,V., Giles,N.H. and Vapnek,D. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1955–1959.
Aquila,H., Misra,D., Eulitz,M. and Klingenberg,M. (1982) *Hoppe-Seyler's Z. Physiol. Chem.*, **363**, 345–349.
Aquila,H., Link,T.A. and Klingenberg,M. (1985) *EMBO J.*, **4**, 2369–2376.
Aquila,H., Link,T.A. and Klingenberg,M. (1987) *FEBS Lett.*, **212**, 1–9.
Arends,H. and Sebald,W. (1984) *EMBO J.*, **3**, 377–382.
Benz,R. (1985) *Crit. Rev. Biochem.*, **19**, 145–190.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **11**, 4077–4092.
Boss,J.M., Gillam,S., Zitomer,R.S. and Smith,M. (1981) *J. Biol. Chem.*, **256**, 12958–12961.
Capaldi,R.A. and Vanderkooi,G. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 930–932.
Chen,E.J. and Seeburg,P.H. (1985) *DNA*, **4**, 165–170.
Colombini,M. (1979) *Nature*, **279**, 643–645.
Freitag,H., Genchi,G., Benz,R. Palmieri,F. and Neupert,W. (1982a) *FEBS Lett.*, **145**, 72–76.
Freitag,H., Janes,M. and Neupert,W. (1982b) *Eur. J. Biochem.*, **126**, 197–202.
Freitag,H., Neupert,W. and Benz,R. (1982c) *Eur. J. Biochem.*, **123**, 629–636.
Grasser,S.M. and Schatz,G. (1983) *J. Biol. Chem.*, **258**, 3427–3430.
Grunstein,M. and Hogness,D. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 3961–3965.
Gubler,U. and Hoffman,B.J. (1983) *Gene*, **27**, 263–269.
Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557–580.
Harmey,M.A. and Neupert,W. (1985) In Martonosi,A. (ed.), *The Enzymes of Biological Membranes*. Plenum Press, New York, Vol. 4, 431–464.
Heller,J. and Smith,E.L. (1966) *J. Biol. Chem.*, **241**, 3165–3180.
Hoeijmakers,J.H.H., Borst,P., Van den Burg,J., Weissmann,C. and Cross,G.A.M. (1980) *Gene*, **8**, 391–417.
Hubacek,J. and Glover,S.W. (1970) *J. Mol. Biol.*, **50**, 111–127.
Kedes,L.H., Chang,A.C.Y., Hanseman,D. and Cohen,S.N. (1975) *Nature*, **255**, 533–538.
Kleffel,B., Garavito,R.M., Baumeister,W. and Rosenbusch,J.P. (1985) *EMBO J.*, **4**, 1589–1592.
Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.
Lederer,F. and Simon,A.M. (1974) *Biochim. Biophys. Res. Commun.*, **56**, 317–328.
Low,B. (1968) *Proc. Natl. Acad. Sci. USA*, **60**, 160–167.
Manella,C.A., Colombini,M. and Frank,J. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2243–2247.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Michel,R., Wachter,E. and Sebald,W. (1979) *FEBS Lett.*, **101**, 373–376.
Mihara,K. and Sato,R. (1985) *EMBO J.*, **4**, 769–774.
Mihara,K., Blobel,G. and Sato,R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7102–7106.
Mizuno,T., Chou,M.-Y., Inouye,M. (1983) *J. Biol. Chem.*, **258**, 6932–6940.
Nicholson,D.W. and Neupert,W. (1987) In Das,R.C. and Robbins,P.W. (eds), *Protein Transfer and Organelle Biogenesis*. Academic Press, New York (in press).
Parnes,J.R., Velan,B., Felsenfeld,A., Ramanathan,L., Ferrini,U., Appela,E. and Seidman,J.G. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2253–2257.
Paul,C. and Rosenbusch,J.P. (1985) *EMBO J.*, **4**, 1593–1597.
Pelham,H.R.B. and Jackson,R.J. (1976) *Eur. J. Biochem.*, **67**, 247–256.
Pfaller,R. and Neupert,W. (1987) *EMBO J.*, **6**, 2635–2642.
Pfaller,R., Freitag,H., Harmey,M.A., Benz,R. and Neupert,W. (1985) *J. Biol. Chem.* **260**, 8188–8193.
Pfanner,N. and Neupert,W. (1985) *EMBO J.*, **4**, 2819–2825.
Pfanner,N. and Neupert,W. (1986) *FEBS Lett.*, **209**, 152–156.
Pfanner,N. and Neupert,W. (1987) *J. Biol. Chem.*, **262**, 7528–7536.
Pfanner,N., Tropschug,M. and Neupert,W. (1987) *Cell*, **49**, 815–823.
Radloff,R., Bauer,W. and Vinograd,J. (1967) *Proc. Natl. Acad. Sci. USA*, **57**, 1514–1521.
Römisch,J., Tropschug,M., Sebald,W. and Weiss,H. (1987) *Eur. J. Biochem.*, **164**, 111–115.
Sadler,J., Suda,K., Schatz,G., Kaudewitz,F. and Haid,A. (1984) *EMBO J.*, **3**, 2137–2143.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Schlechtman,M.G. and Yanofsky,C. (1983) *J. Mol. Appl. Genet.*, **2**, 83–99.
Schleyer,M., Schmidt,B. and Neupert,W. (1982) *Eur. J. Biochem.*, **125**, 109–116.
Schmidt,B., Pfaller,R., Pfanner,N., Schleyer,M. and Neupert,W. (1985) In Quagliariello,E. (ed.), *Achievements and Perspectives of Mitochondrial Research. Vol. II. Biogenesis*. Elsevier Science, BV, pp. 389–396.
Sheldon,R., Jurale,C. and Kates,J. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 417–421.
Stanley,K.K. (1983) *Nucleic Acids Res.*, **11**, 4077–4092.
Stanley,K.K. and Luzio,J.P. (1984) *EMBO J.*, **3**, 1429–1434.
Stuart,R., Neupert,W. and Tropschug,M. (1987) *EMBO J.*, **6**, 2131–2137.
Stüber,D., Ibrahimi,J., Cutler,D., Dobberstein,B. and Bujard,H. (1984) *EMBO J.*, **3**, 3143–3148.
Takeda,M., Vassarotti,A. and Douglas,M.G. (1985) *J. Biol. Chem.*, **260**, 15458–15465.
Vidal-Ingigliardi,D. and Raibaud,O. (1985) *Nucleic Acids Res.*, **13**, 5919–5926.
Viebrock,A., Perz,A. and Sebald,W. (1982) *EMBO J.*, **1**, 565–571.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259–268.
von Heijne,G. (1986) *EMBO J.*, **5**, 1335–1342.
Weckesser,J., Zalman,L.S. and Nikaido,H. (1984) *J. Bacteriol.*, **159**, 199–205.
Wondt,P.L., Pastink,A., Kempers-Veenstra,A., Jansen,A.E.M., Mayer,W.H. and Planta,R.J. (1983) *Nucleic Acids Res.*, **11**, 5347–5360.
Yoshimura,F., Zalman,L.S. and Nikaido,H. (1983) *J. Biol. Chem.*, **258**, 2308–2314.
Zalman,L.S., Nikaido,H. and Kagawa,Y. (1980) *J. Biol. Chem.*, **255**, 1771–1774.
Zimmermann,R. (1986) In Strauss,A.W., Boime,I. and Kreil,G. (eds), *Protein Compartmentalization*. Springer Verlag, NY, 119–136.
Zimmermann,R., Paluch,U., Sprinzl,M. and Neupert,W. (1979) *Eur. J. Biochem.*, **99**, 247–252.
Zimmermann,R. and Neupert,W. (1980) *Eur. J. Biochem.*, **109**, 217–229.
Zwizinski,C., Schleyer,M. and Neupert,W. (1984) *J. Biol. Chem.*, **259**, 7850–7856.

Received on May 19, 1987; revised on June 11, 1987