The EMBO Journal vol.7 no.3 pp.639-648, 1988

Import of honeybee prepromelittin into the endoplasmic reticulum: energy requirements for membrane insertion

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Communicated by W.Neupert

The import of small precursor proteins, derived from the honeybee secretory protein prepromelittin, into dog pancreas microsomes is independent of signal recognition particle (SRP) and docking protein, but requires that charged amino acids at the amino terminus of the mature part are counterbalanced by amino acids with the opposite charge at the carboxy terminus. The import pathway of such precursor proteins was resolved into two sequential steps: (i) binding of precursors to microsomes, and (ii) insertion of precursors into the membrane. Formation of an intramolecular disulfide bridge within the mature part of these precursor proteins allowed association of the oxidized precursors with the microsomal membrane but reversibly inhibited their membrane insertion. Furthermore, membrane insertion was inhibited by ATP depletion. Different prepromelittin derivatives were found to depend on ATP to varying degrees. We conclude that insertion of prepromelittin-derived precursor proteins into microsomal membranes involves a competent conformation of the precursor proteins and that, in general, this is accomplished with the help of both a cytoplasmic component and ATP.

Key words: endoplasmic reticulum/ATP/disulfide bonds/prepromelittin derivatives/protein import

Introduction

The import of secretory and plasma membrane proteins into the endoplasmic reticulum (ER) can be considered to consist of three sequential, but distinct steps (Zimmermann and Meyer, 1986): (i) specific association of the precursor with the membrane; (ii) insertion of the precursor into the membrane; and (iii) partial or complete transfer of the mature polypeptide across the membrane. The first two of these steps depend on a signal sequence and either the signal recognition particle (SRP)/docking protein- and ribosome/ribosome receptor-systems for precursors with more than 80 amino acids or certain structural features within the mature part of the precursor protein for precursors with less than 80 amino acids (Müller and Zimmermann, 1987). All precursors of secretory and plasma membrane proteins imported into microsomes in the absence of protein synthesis have shown a dependence on nucleoside triphosphates (Caulfield *et al.*, 1986; Conolly and Gilmore, 1986; Hansen *et al.*, 1986; Mueckler and Lodish, 1986a,b; Perara *et al.*, 1986; Rothblatt and Meyer, 1986a; Waters and Blobel, 1986; Schlenstedt and Zimmermann, 1987; Wiech *et al.*, 1987). For the precursor of M13 coat protein, a small membrane protein, an ATP-requiring reaction was assigned a role in the maintenance of an import competent state of the precursor protein, possibly through induction of a conformational change (Wiech *et al.*, 1987).

We have previously studied the structural basis for import competence of the precursor of a small eukaryotic secretory protein, prepromelittin (70 amino acids), by a series of amino acid substitutions within its mature part (Müller and Zimmermann, 1987). We concluded that import of this protein, and of related precursor proteins with about the same size, depends on either the compensation of a single (or a cluster of) charged amino acid(s) at the amino terminus of the mature part with a single (or a cluster of) amino acid(s) with the opposite charge at the carboxy terminus, or the absence of charged amino acids from both ends of the mature part altogether. We therefore proposed a loop structure within the mature part of these precursor proteins as a prerequisite for import into microsomes. Since prepromelittin (Zimmermann and Mollay, 1986) and its derivatives (Müller and Zimmermann, 1987) are able to bypass the SRP/docking protein-system, we suggested that the loop structure substitutes for this system.

Here we have attempted to resolve the apparent contradiction that on the one hand (in the case of SRP/docking protein-independent precursors) a certain conformation was found to be a prerequisite, while on the other hand, a looseness of conformation or 'unfoldedness' appears to be a prerequisite for a protein to be imported (Zimmermann and Meyer, 1986). We observed that the postulated conformation was compatible with, and possibly required for, the first step in import: membrane association. It then had to be altered, however, to enable the second step: membrane insertion. Furthermore, we found that different prepromelittin derivatives that had the signal sequence in common but differed from one another mainly by the presence or absence of oppositely charged amino acids at the amino and carboxy termini of their mature part, showed a dependence on ATP to a different extent. These data support the view that at least SRP/docking-independent precursor proteins undergo conformational changes prior to or during insertion into the membrane of the ER, and that these changes are typically accomplished with the aid of a cytoplasmic component and ATP.

Results

Different prepromelittin-derived precursor proteins show quantitative differences in their ATP-requirements for import into microsomes

Construction of plasmids coding for SRP- and docking protein-independent, small precursor proteins (mol. wt < 9 kd) derived from honeybee prepromelittin and mouse dihydrofolate reductase (Figure 1) have been described (Müller and Zimmermann, 1987). The precursor proteins ppΔ-DHFR/3 and pΔ-DHFR/3Thr, that are distinguished

from one another mainly by the presence or absence of a pair of oppositely charged amino acids located at both termini of the mature part, were processed and sequestered by microsomes in the absence of ongoing protein synthesis (Figure 2, lanes 1 and 2). The ATP-requirements for import of these precursor proteins into dog pancreas microsomes were assayed by preincubating reticulocyte lysates containing the relevant precursors with the ATP-hydrolyzing enzyme potato apyrase (Waters and Blobel, 1986). Apyrase treatment completely inhibited subsequent processing and sequestration of precursor protein ppΔ-DHFR/3 (Figure 2, lanes 3 and 4), both of which were restored by addition of ATP (Figure 2, lanes 5 and 6). Other docking protein-independent precursor proteins with single or clustered amino acids of opposite charge at both termini of their mature part also required ATP for import into microsomes (data not shown). Since there are phosphate transferring enzymes in the rabbit reticulocyte lysate, it was not possible to determine whether depletion of ATP or another nucleoside triphosphate was responsible for inhibition of import. Import of precursor protein pΔ-DHFR/3Thr, however, was hardly affected at all by apyrase treatment (Figure 2, lanes 3 and 4). We conclude that small precursor proteins derived from prepromelittin and dihydrofolate reductase are imported into microsomes post-translationally, and that this process depends on ATP or another nucleoside triphosphate to varying degrees depending on characteristics within the mature part. Since the main difference between the ATP-dependent precursor protein ppΔ-DHFR/3 and the 'ATP-independent' precursor protein pΔ-DHFR/3Thr is in the mature part and not in the signal sequence, the ATP-consuming step is probably related to the overall conformation of the precursor proteins.

Conformational stabilization of small precursor proteins by disulfide bonds within the mature part inhibits their import into microsomes

To introduce a second cysteine residue into the precursor proteins described above, oligonucleotide-directed mutagenesis with the gapped duplex method (Kramer *et al.*, 1984) was performed, resulting in precursor proteins ppΔ-DHFR/3^{C} and pΔ-DHFR/3Thr^{C}. The amino acid substitutions were confirmed by DNA sequencing (Figure 1). The import of the newly designed precursor proteins into dog pancreas microsomes under standard conditions (reducing conditions) fulfilled all criteria established for the parental precursor proteins (data not shown). The existence of an intramolecular disulfide bridge between the two cysteine residues of the mature part, formed after oxidation of reticulocyte lysates containing [^{35}S]cysteine-labeled precursor proteins, was proved by gel electrophoresis in the absence of reducing agents and by HPLC-analysis of tryptic peptides (Nicholson *et al.*, 1987) generated under oxidizing or reducing conditions (see Materials and methods).

Oxidation of reticulocyte lysates containing these precursor proteins caused total inhibition of import (Figure 3, lanes 4 and 5), whereas the import of precursor proteins having only one cysteine residue (ppΔ-DHFR/3 and pΔ-DHFR/3Thr) was hardly affected (Figure 3, lanes 4 and 5). We conclude that the inhibitory effect of oxidation was exerted on the precursor proteins via formation of disulfide

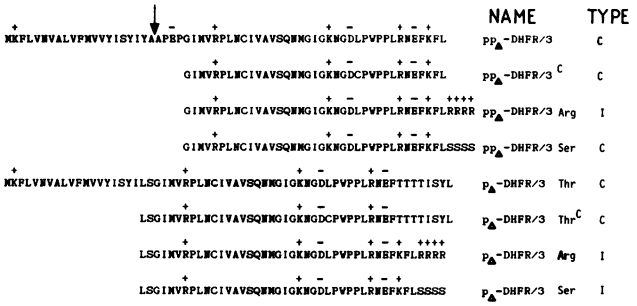

Fig. 1. Amino acid sequences of precursor proteins, derived from honeybee prepromelittin and mouse dihydrofolate reductase. Amino acid sequences, given in single letter code, were derived from DNA sequencing of the corresponding plasmids using the chain termination method for dideoxynucleotides. The proposed cleavage site of the precursor proteins, indicated by an arrow, and the names of the precursor proteins have been described previously (Müller and Zimmermann, 1987) with the exception of precursor proteins derived by oligonucleotide directed mutagenesis. Precursors with newly introduced cysteine residues are designated by a superscribed 'C' in their 'Name'. 'Type' refers to the competence (C) or incompetence (I) of the corresponding precursor proteins for import into dog pancreas microsomes according to our previous publication (Müller and Zimmermann, 1987).

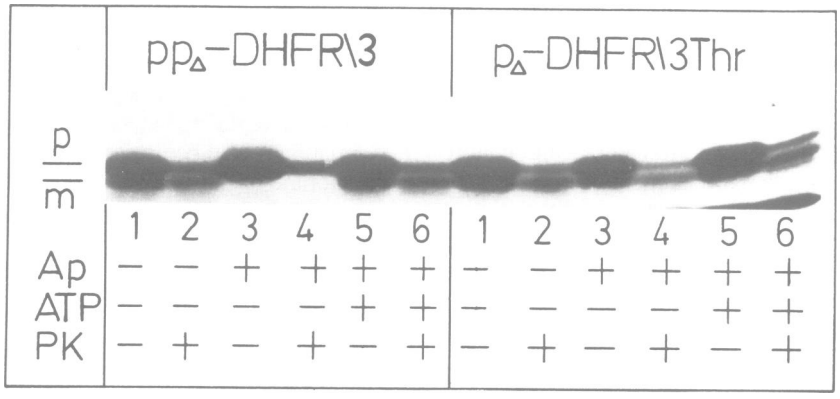

Fig. 2. Effect of depleting reticulocyte lysates of ATP on import of precursor proteins into dog pancreas microsomes. Translation of precursor proteins was carried out in rabbit reticulocyte lysates for 10 min at 37°C. The translation reactions were supplemented with a combination of cycloheximide and RNase A. Following further incubation for 5 min at 37°C the samples were divided into three aliquots. Two aliquots were supplemented with apyrase (6 U/ml) (**lanes 3–6**), the other aliquot with water (**lanes 1** and **2**). After incubation for 5 min at 37°C, one sample, containing apyrase, received ATP (8 mM) (lanes 5 and 6), the other one water (lanes 3 and 4). Microsomes were then added to all samples and the incubation was continued for 15 min at 37°C. Each reaction was subsequently divided into two halves. One half was not treated with Proteinase K (lanes 1, 3 and 5), the other half was treated with Proteinase K (lanes 2, 4 and 6). The samples were subjected to precipitation with ammonium sulfate (final conc. 66%) and analysed by gel electrophoresis. p, precursor; m, mature form; Ap, apyrase; PK, Proteinase K.

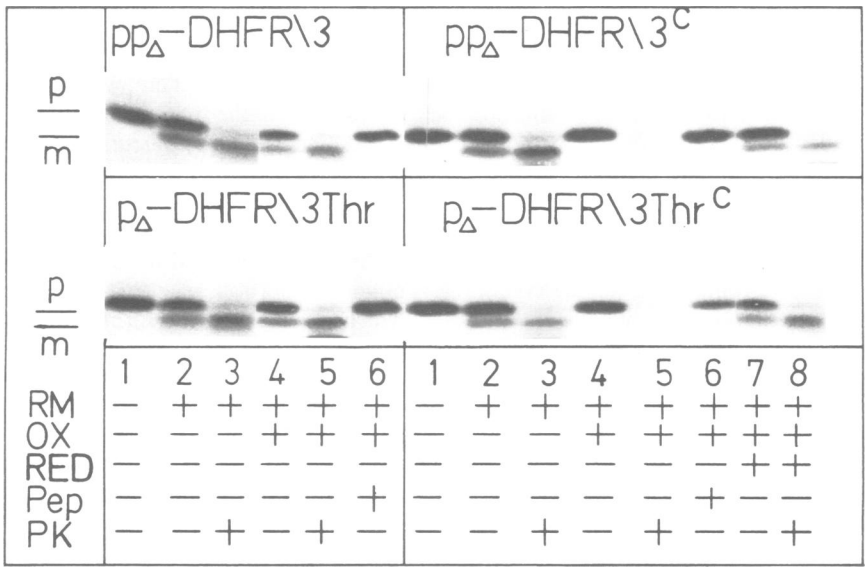

Fig. 3. Effect of oxidation on import of precursor proteins into microsomes. Precursor proteins were synthesized in rabbit reticulocyte lysate for 10 min at 37°C. After addition of cycloheximide and RNase A, the samples were divided into three aliquots. One aliquot was supplemented with water (**lanes 1–3**), the second aliquot with $K_3Fe(CN)_6$ (final conc. 10 mM) (**lanes 4, 5, 7** and **8**) and the third aliquot with $K_3Fe(CN)_6$ (final conc. 10 mM) together with a small cysteine containing peptide (16mer), derived from precursor protein pp_Δ-DHFR/3 (**lane 6**). After incubation for 2 min at 20°C the sample lacking $K_3Fe(CN)_6$ was divided into two aliquots. One aliquot received water (lane 1), the other aliquot as well as the oxidized samples received microsomes (lanes 2–8). After incubation for 5 min at 37°C, the samples containing $K_3Fe(CN)_6$ were divided into two aliquots. One aliquot was supplemented with DTT (final conc. 20 mM) (lanes 7 and 8), the other aliquot with water (lanes 4 and 5) and the incubation was continued for 10 min at 37°C. Each sample was then divided into two halves. One half was treated with Proteinase K (lanes 3, 5 and 8), the other half was not treated (lanes 1, 2, 4, 6 and 7). After precipitation with ammonium sulfate, the samples were analysed by gel electrophoresis. p, precursor; m, mature form; RM, microsomes; OX, oxidation; RED, reduction; PEP, peptide; PK, Proteinase K.

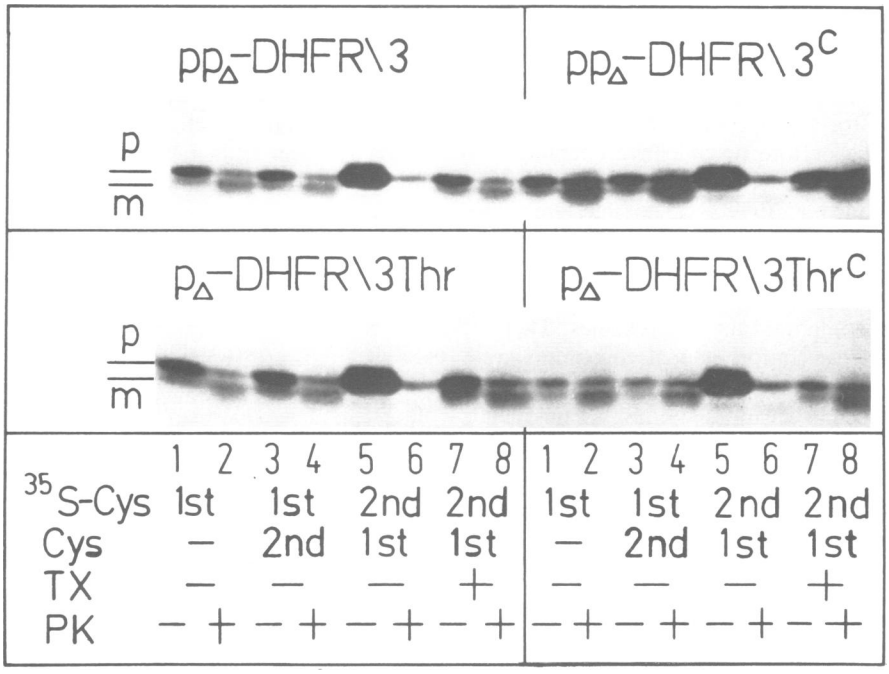

Fig. 4. Effect of oxidative coupling of cysteine residues to precursor proteins on import into microsomes. Unlabeled precursor proteins were synthesized in rabbit reticulocyte lysates for 10 min at 37°C. After addition of cycloheximide and RNase A, the samples were incubated with $K_3Fe(CN)_6$ (final conc. 10 mM) for 2 min at 20°C in the presence of either [^{35}S]cysteine (**lanes 1–4**) or unlabeled cysteine (**lanes 5–8**). After this first coupling reaction, samples containing radioactivity were divided into two aliquots. One aliquot received water (lanes 1 and 2), the second aliquot unlabeled cysteine (final conc. 12.5 mM) (lanes 3 and 4). All samples were supplemented with microsomes and incubated for 15 min at 37°C. The unlabeled samples were then layered onto a two step sucrose gradient and subjected to centrifugation as described in Materials and methods. The interfaces were divided into two aliquots and incubated with [^{35}S]cysteine and DTT (final conc. 10 mM) in the absence of Triton X-100 (lanes 5 and 6) or presence of Triton X-100 (final conc. 0.5%) (lanes 7 and 8) for 2 min at 37°C. For the second coupling reaction, all samples were subsequently supplemented with $K_3Fe(CN)_6$ (final conc. 25 mM) and further incubated for 5 min at 20°C. All samples were then divided into two aliquots. One aliquot was treated with Proteinase K (lanes 2, 4, 6 and 8), the other aliquot was not treated with Proteinase K (lanes 1, 3, 5 and 7). After precipitation with ammonium sulfate, all samples were analysed by gel electrophoresis in the absence of reducing agents. p, precursor; m, mature form; Cys, cysteine; TX, Triton X-100; PK, Proteinase K; 1st, first coupling reaction; 2nd, second coupling reaction.

bonds within the mature part, and not by inactivation of the import apparatus. The inhibition of import by oxidation was reversed by the addition of reducing agents to the oxidized precursor proteins (Figure 3, lanes 7 and 8). Therefore, docking protein-independent precursor proteins containing a disulfide bond in their mature part cannot be imported into microsomes.

The inhibitory effect of disulfide bonds within the mature part of precursor proteins on import could be due to either the disulfide bridge *per se* or certain effects on the conformation of the precursor proteins. To differentiate between these alternatives, unlabeled cysteine-containing precursor proteins were synthesized in reticulocyte lysates and coupled to [^{35}S]cysteine by oxidation. These post-translationally labeled precursor proteins were still imported into microsomes (Figure 4, lanes 1 and 2). Import was also observed when an excess of free, unlabeled cysteine was added after the coupling reaction but prior to the import assay (Figure 4, lanes 3 and 4). Since the mature forms were still labeled, the [^{35}S]cysteine, coupled to the precursor proteins, could not have been released during the membrane transfer. This was confirmed by an experiment where unlabeled cysteine was coupled to unlabeled precursor proteins by oxidation. After import into microsomes the unlabeled cysteine was exchanged for [^{35}S]cysteine by reisolation of the microsomes, reduction and subsequent addition of [^{35}S]cysteine in the presence of oxidizing agents, either in the presence or absence of detergents. Only the precursor protein was labeled when the second coupling reaction was carried out in the absence of Triton X-100 (Figure 4, lanes 5 and 6), whereas both the precursor and mature form were labeled in the presence of Triton X-100 during the second coupling reaction (Figure 4, lanes 7 and 8). Thus either the oxidizing agent or the cysteine (or both) could not penetrate into intact microsomes and therefore no oxidative coupling of [^{35}S]cysteine to proteins inside microsomes could occur. We conclude that disulfide bonds *per se* do not interfere with import into microsomes, because precursor proteins with one (ppΔ-DHFR/3 and pΔ-DHFR/3Thr) and even two (ppΔ-DHFR/3^{c} and pΔ-DHFR/3Thr^{c}) cysteine residues linked to their mature parts were imported into microsomes. Therefore, it is more likely that conformational constraints are responsible for the import incompetence of precursor proteins having intramolecular disulfide bonds within their mature part. This may also be true for the inhibitory effect on import of oxidative coupling of small cysteine-containing peptides (16 amino acids) by disulfide linkage to precursor proteins having either two or only one cysteine (Figure 3, lane 6).

Small precursor proteins can associate with microsomes at low temperature and be subsequently imported into microsomes at elevated temperature

The data above show that the import of precursor proteins was reversibly blocked by either formation of disulfide bonds within the mature part, or by ATP depletion. On the basis of these observations, and a recently described system for membrane association of SRP and docking protein-dependent precursor proteins (Gilmore and Blobel, 1985), we tried to uncouple different steps in the import pathway by accumulating intermediates at low temperature.

Docking protein-independent precursor proteins were synthesized in reticulocyte lysates and were then incubated with dog pancreas microsomes at 4°C. The microsomes were subsequently recovered from the interface of a two step sucrose gradient and thereby separated from soluble precursor proteins in the supernatant and from aggregated as well as ribsome-associated precursor proteins in the pellet. When microsomes were present during the incubation of precursor proteins ppΔ-DHFR/3 or pΔ-DHFR/3Thr at low temperature, a significant proportion of the total precursor protein was recovered from the interface (Table I). Only a minor proportion of precursor protein pΔ-DHFR/3Ser, which was incompetent for import into microsomes (Figure 1), was detectable in the interface under the same conditions (Table I). Most of the precursor protein remained soluble in the supernatant and a small amount of precursor protein sedimented through the two sucrose cushions. The omission of microsomes during the incubation at low temperature drastically diminished the proportion of precursor proteins ppΔ-DHFR/3 and pΔ-DHFR/3Thr recovered from the interface (Table I). Precursor proteins with two cysteine residues behaved similarly to their parental precursors (see below). It appears, therefore, that docking protein-independent precursor proteins that are competent for import into microsomes associate with microsomal membranes under these conditions. The proportion of precursor protein associated with microsomes lies in the range of the observed efficiencies for import of these precursor proteins into microsomes (Müller and Zimmermann, 1987).

To determine whether the precursors associated with microsomes at low temperature were true intermediates in the import pathway, we reisolated precursor proteins associated with microsomes after incubation at low temperature and assayed import into microsomes during a second incubation at elevated temperature. In addition, we studied the sensitivity of this reaction to dilution. Dilution of the association reaction with a 'precursor-free' translation mixture drastically diminished the amount of precursor protein re-

Table I. Association of precursor proteins with microsomal membranes at low temperature

	Associated precursor (% of total precursor)					
	ppΔ-DHFR/3		pΔ-DHFR/3Thr		pΔ-DHFR/3Ser	
RM	−	+	−	+	−	+
Supernatant	80.3	75.5	92.4	71.2	65.2	93.9
Interface	5.3	16.7	4.5	25.8	6.0	1.0
Pellet	2.5	3.0	1.5	1.0	1.0	4.5
Sum	88.1	95.2	98.4	98.0	72.2	99.4

Precursor proteins were synthesized in rabbit reticulocyte lysates for 10 min at 37°C. After addition of cycloheximide and RNase A, the samples were divided into two aliquots. One aliquot was incubated with water (−RM), the other aliquot with microsomes (+RM) for 5 min at 4°C. The samples were again divided into two aliquots. One aliquot was diluted with an equal volume of double strength sample buffer for electrophoresis, the other aliquot was layered onto a two step sucrose gradient, subjected to centrifugation and subsequently fractionated as described in Materials and methods. The supernatants and the interfaces were diluted with an equal volume of double strength sample buffer and the pellets were resuspended in sample buffer. All samples were analysed by gel electrophoresis and fluorography. Densitometric analysis of the resulting X-ray films was carried out on an LKB densitometer and the amount of precursor protein, recovered in the various fractions, was calculated as a percentage of the total precursor protein used in the assay.

covered in the interface: at a 10-fold dilution, less than 4% of the precursor protein associated with microsomes compared with the undiluted reaction mixture (Table II). Dilution of the complete import reaction with a 'precursor-free' translation mixture also impaired processing and sequestration of both precursor proteins: even a 5-fold dilution completely inhibited import into microsomes. Incubation at 37°C of reisolated microsomes, which had been preincubated with precursor proteins (without dilution) at 4°C allowed processing and sequestration of both precursor proteins ppΔ-DHFR/3^c and pΔ-DHFR/3Thr^c. Dilution of this reaction mixture during the 37°C incubation with several volumes of 'precursor-free' translation mixture did not diminish import of either precursor protein as drastically as dilution of the association reaction or of the complete import reaction: after 5-fold dilution ~60% of precursor protein was still imported into microsomes and even 10-fold dilution allowed import with more than 20% efficiency. We conclude that precursor proteins, recovered from the interface of the two step sucrose gradient, are associated with microsomes in a way that allows them to be chased to their mature and sequestered forms inside the microsomal vesicles when the temperature is raised from 4 to 37°C. Since the chase reaction, which reflects the membrane insertion and transfer events, is not affected by dilution to the same degree as is the association reaction or total import, a significant proportion of the precursor proteins seems to be inserted into the microsomal membrane without release from their site of membrane association into the aqueous environment. Thus at least 60% of the associated precursor protein is a real intermediate in a functional location on the import pathway of docking protein-independent precursor proteins.

Table II. Import of precursor proteins associated with microsomes at low temperature into microsomes at elevated temperature

	Dilution	ppΔ-DHFR/3^c (%)	pΔ-DHFR/3Thr^c (%)
Precursor associated with microsomes at 4°C	–	100	100
	1:5	9.1	21.3
	1:10	1.1	3.1
Mature protein imported after shift from 4 to 37°C	–	100	100
	1:5	61.3	70.8
	1:10	22.5	45.5
Mature protein imported at 37°C	–	100	100
	1:5	<1.0	1.8
	1:10	<1.0	<1.0

Precursor proteins were synthesized in rabbit reticulocyte lysates for 10 min at 37°C. After addition of cycloheximide and RNase A, the samples were divided into three aliquots. These aliquots were diluted (5- or 10-fold) or were not diluted with translation mixtures, lacking labeled amino acids and transcript. All samples were again divided. One third of each sample was incubated with microsomes for 15 min at 37°C, diluted with an equal volume of double strength sample buffer and analysed by gel electrophoresis. The remaining two-thirds of each sample was supplemented with microsomes and incubated for 5 min at 4°C, layered onto the two step sucrose gradient and subjected to centrifugation as described in Materials and methods. After fractionation of the gradients, one-eighth of each recovered interface was diluted with an equal volume of double strength sample buffer and analysed by gel electrophoresis. Three quarters of each sample were supplemented with either 1 volume, 5 volumes, or 10 volumes of translation mixture, lacking labeled amino acids and transcripts. All samples were incubated for 10 min at 37°C and then divided into two aliquots. One aliquot was treated with Proteinase K, the other aliquot was not treated. After precipitation with ammonium sulfate, the samples were analysed by gel electrophoresis and fluorography. Densitometric analysis of the X-ray films was carried out on an LKB densitometer and the amount of mature protein sequestered inside the microsomes in the various diluted import reactions was calculated as a percentage of the total mature protein formed during the undiluted import reaction.

ATP is not necessary for association of small precursor proteins with, but is required for insertion into, microsomal membranes

The uncoupling of a membrane association step, occurring at 4°C, from a membrane insertion step, occurring at 37°C, enabled us to study which of the steps require ATP. Precursor proteins were incubated with microsomes at 4°C in the presence or absence of ATP (to allow association) then reisolated and incubated a second time at 37°C in the presence or absence of ATP. Precursor protein ppΔ-DHFR/3^c, which was imported in the total reaction only in the presence of ATP, was processed and sequestered in this two step reaction only if the second incubation at 37°C, the insertion reaction, was supplemented with ATP (Figure 5, lanes 1–6). The presence of ATP during the association reaction at 4°C did not appear to be required for the subsequent insertion reaction, since in the absence of ATP during the first in-

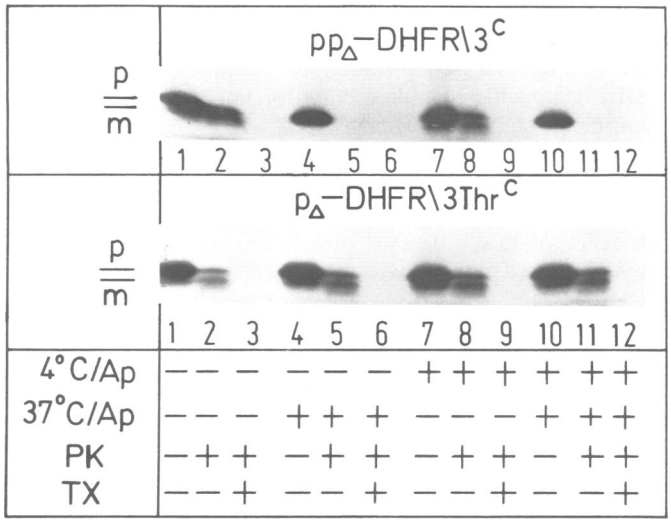

Fig. 5. Effect of depleting reticulocyte lysates of ATP on association of precursor proteins with and insertion into microsomal membranes. Precursors proteins were synthesized in rabbit reticulocyte lysates for 10 min at 37°C. After addition of cycloheximide and RNase A, the samples were divided into two aliquots. One aliquot was incubated with apyrase (final conc. 80 U/ml) (**lanes 7–12**), the other aliquot with water (**lanes 1–6**) for 5 min at 37°C. All samples were supplemented with microsomes, incubated for 5 min at 4°C (first incubation), and subjected to the two step sucrose gradient centrifugation as described in Materials and methods. After fractionation of the gradients, the recovered interfaces were supplemented with one volume of translation mixture, lacking labeled amino acids and *in vitro* transcripts and either treated with apyrase (final conc. 80 U/ml) for 5 min at 37°C (lanes 4–6 and 10–12) or not treated with apyrase (lanes 1–3 and 7–9). After incubation for 15 min at 37°C (second incubation) each sample was divided into three aliquots. One aliquot was subjected to protease treatment in the absence of Trixon X-100 (lanes 2, 5, 8 and 11), the second aliquot in the presence of Triton X-100 (final conc. 0.5%) (lanes 3, 6, 9 and 12). The third aliquot was not treated with Proteinase K (lanes 1, 4, 7 and 10). All samples were subsequently analysed by gel electrophoresis and fluorography. p, precursor; m, mature form;. 4°C/Ap, 1st incubation at 4°C in the presence or absence of apyrase; 37°C/Ap, 2nd incubation at 37°C in the presence or absence of apyrase; PK, Proteinase K; TX, Triton X-100.

cubation processing and sequestration of this precursor protein was detected when ATP was present during the second incubation (Figure 5, lanes 7–9). Precursor protein pΔ-DHFR/3Thr^c, which did not depend on ATP in the total import reaction, also showed no requirement for ATP during the two step reaction since the first incubation at 4°C, as well as the second incubation at 37°C, could be performed in the presence of apyrase without any significant loss of processing and sequestration (Figure 5, lanes 1–12). The association of docking protein-independent precursor proteins with microsomes, therefore, does not depend on ATP, whereas the ATP-requirement observed for the import reaction of most precursor proteins has to be ascribed to the subsequent membrane insertion step. Since we are not yet able to separate membrane insertion of prepromelittin derivatives, as defined by cleavage of the signal peptide, from membrane transfer, as defined by protection of the mature protein by the microsomal membrane from externally added proteases, it cannot be excluded that membrane transfer also depends on ATP.

The dependence on ATP for membrane insertion should correlate with the requirement for rabbit reticulocyte lysate (Wiech *et al.*, 1987). To test this, precursor proteins were synthesized and incubated with microsomes at 4°C. Then the precursor proteins, associated with microsomes, were separated by the two step gradient centrifugation as above. Processing and sequestration of precursor protein ppΔ-DHFR/3^c, which required ATP for import, was observed during the chase incubation at 37°C when reticulocyte lysate and ATP were present (Figure 6, lanes 1 and 2). However, a buffer which was supplemented with ATP (Figure 6, lanes 5 and 6) or not (Figure 6, lanes 3 and 4) did not promote transfer of this precursor protein across the microsomal membrane. In contrast, the precursor protein pΔ-DHFR/3Thr^c, which did not depend on ATP for import into microsomes, was chased to the mature and sequestered protein in the absence of reticulocyte lysate, i.e. in the presence of buffer, regardless of whether ATP had been added (Figure 6, lanes 5 and 6) or not (Figure 6, lanes 3 and 4). It appears that import of the ATP-dependent precursor proteins depends on both ATP and rabbit reticulocyte lysate, whereas import of the ATP-independent precursor protein requires neither ATP nor reticulocyte lysate. Furthermore, it seems that ATP has no direct effect on the precursors, but interacts with a component of the reticulocyte lysate in order to affect precursors.

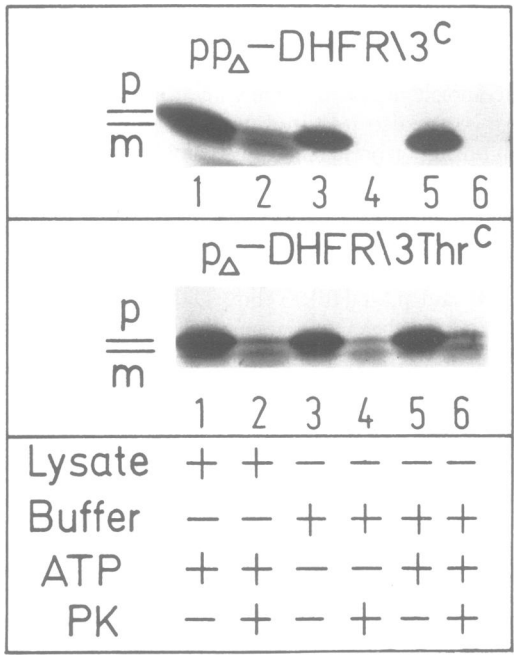

Fig. 6. Insertion of membrane-associated precursor proteins into the microsomal membrane in the presence or absence of rabbit reticulocyte lysate. Precursor proteins were synthesized in rabbit reticulocyte lysates for 10 min at 37°C. After incubation with cycloheximide and RNase A and treatment with apyrase for 5 min at 37°C, the samples were supplemented with microsomes and incubated at 4°C for 5 min. The samples were then applied to a two step sucrose gradient and subjected to centrifugation as described in Materials and methods. The gradients were fractionated and the interfaces, containing microsomes with associated precursor proteins, were divided into three aliquots. One aliquot was supplemented with ATP (final conc. 8 mM) in 50 mM Hepes–KOH (pH 7.4) and the original volume of translation mixture, lacking labeled amino acids and transcripts (**lanes 1** and **2**). The second aliquot was supplemented with the same volume of the buffer used for the two step sucrose gradient (50 mM Hepes–KOH, pH 7.4, 50 mM KAc, 2 mM MgAc, 1 mM DTT) (**lanes 3** and **4**), and the third aliquot received ATP (final conc. 8 mM) in addition to this buffer (**lanes 5** and **6**). After incubation at 37°C for 15 min, each sample was divided into two aliquots. One aliquot was incubated with Proteinase K (lanes 2, 4 and 6) and the other aliquot was incubated with water (lanes 1, 3 and 5). All samples were analysed by gel electrophoresis. p, precursor; m, mature form; lysate, translation mixture containing rabbit reticulocyte lysate, energy regenerating system and unlabeled amino acids; PK, Proteinase K; TX, Triton X-100.

Disulfide bonds within the mature part of small precursor proteins do not impair association with, but inhibit insertion into, microsomal membranes

Oxidized or reduced precursor proteins ppΔ-DHFR/3^c and pΔ-DHFR/3Thr^c were incubated with microsomes at 4°C in the absence of ATP, reisolated and again incubated at 37°C in the presence of ATP under oxidizing or reducing conditions. These precursor proteins were processed and sequestered by microsomes when the second incubation, the insertion reaction, was carried out under reducing conditions, regardless of whether oxidized or reduced precursor proteins had been used for the preceding association reaction (Figure 7, lanes 3, 4, 7 and 8). Oxidizing conditions during the insertion step inhibited import into microsomes in every case (Figure 7, lanes 1, 2, 5 and 6). The two step import of precursor proteins ppΔ-DHFR/3 and pΔ-DHFR/3Thr (having only one cysteine residue), however, was not inhibited by oxidizing conditions during either the first (Figure 7, lanes 7 and 8) or the second (Figure 7, lanes 1 and 2) or both incubations (Figure 7, lanes 5 and 6). Thus, oxidation during the second incubation did not inactivate the import apparatus but rather acted on precursor proteins ppΔ-DHFR/3^c and pΔ-DHFR/3Thr^c directly, probably by new formation of disulfide bonds or maintenance of the disulfide bonds that had already been introduced during the first incubation. We conclude that disulfide bonds within the mature part of the docking protein-independent precursor proteins interfere with membrane insertion but do not interfere with membrane association. Since disulfide bonds *per se* can be transported across microsomal membranes (as shown above), the disulfide stabilized structure of the mature part of prepromelittin derivatives must be incompatible with insertion into the microsomal membrane.

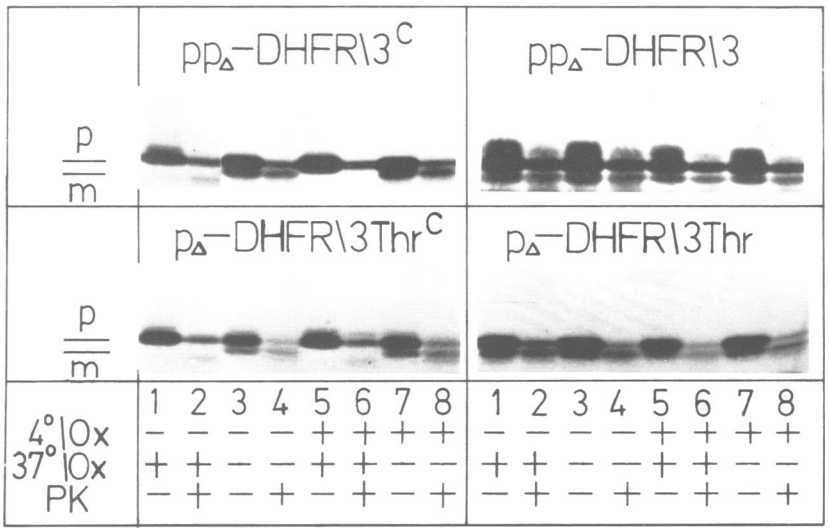

Fig. 7. Effect of oxidation on association of precursor proteins with and insertion into microsomal membranes. Precursor proteins were synthesized in rabbit reticulocyte lysates for 10 min at 37°C. After addition of cycloheximide and RNase A and treatment with apyrase (final conc. 80 U/ml) for 5 min at 37°C, the samples were divided into two aliquots. One aliquot was incubated with $K_3Fe(CN)_6$ (final conc. 10 mM) (**lanes 5–8**), the other aliquot with water (**lanes 1–4**), for 2 min at 20°C. All samples were then supplemented with microsomes, incubated for 5 min at 4°C (first incubation) and subjected to the two step sucrose gradient centrifugation as described in Materials and methods. The gradient buffer in the case of the oxidized sample contained $K_3Fe(CN)_6$ (final conc. 5 mM) instead of DTT. After fractionation of the gradients, the interfaces were supplemented with one volume of translation mixture, lacking labeled amino acids and transcripts and either pre-treated wtih $K_3Fe(CN)_6$ (final conc. 10 mM) (lanes 1, 2, 5 and 6) or with DTT (final conc. 10 mM) (lanes 3, 4, 7 and 8) for 2 min at 20°C. After incubation for 15 min at 37°C (second incubation), one aliquot of each sample was treated with Proteinase K (lanes 2, 4, 6 and 8), the other aliquot was not treated (lanes 1, 3, 5 and 7). All samples were then analysed by gel electrophoresis and fluorography. p, precursor; m, mature form; 4°C/Ox, 1st incubation at 4°C under oxidizing conditions; 37°C/Ox, 2nd incubation at 37°C under oxidizing conditions; PK, Proteinase K.

Discussion

The analysis of the mechanism for import of proteins into the ER had been hampered for a long time by the fact that at least some of the steps involved in import (membrane association, insertion and transfer) did not seem to occur after completion of protein synthesis. This apparent obligate coupling of translation and import prevented the elucidation of the mechanism of import, particularly with respect to intermediates, energy requirements, and the positive or negative role of certain conformations. We are focusing on the import of prokaryotic and eukaryotic membrane and secretory proteins of low mol. wt into dog pancreas microsomes (Zimmermann and Meyer, 1986). The precursor of a prokaryotic membrane protein, M13 procoat protein (Watts *et al.*, 1983; Wiech *et al.*, 1987), and the precursors of two eukaryotic secretory proteins, frog prepropeptide GLa (Schlenstedt and Zimmermann, 1987) and honeybee prepromelittin (Zimmermann and Mollay, 1986), were found to be imported into microsomes in the absence of protein synthesis. Here we show that this is generally true for small precursors derived from prepromelittin (Müller and Zimmermann, 1987).

The 'competent conformation'

From mutation analysis of prepromelittin we have suggested that a competent structure of the precursor protein must exist as a prerequisite for import into microsomes. A loop may be formed within the mature part of the prepromelittin derivatives whereby the oppositely charged amino acids at the amino and carboxy termini of the mature part come closely together and possibly interact via electrostatic bonds (Müller and Zimmermann, 1987). A disulfide bond within the mature part of prepromelittin derivatives, however, inhibits their import into microsomes. The inhibitory effect of disulfide bonds is caused by stabilization of the loop structure within the mature part of the precursor protein rather than by interference of disulfide bonds *per se* since precursor proteins, linked via a disulfide bridge to cysteine residues, are competent for import.

Effects of disulfide bonds and ATP-depletion

Incubation at low temperature has been used successfully to study the association of precursor proteins with mitochondria (Pfaller *et al.*, 1985; Pfaller and Neupert, 1987; Pfanner and Neupert, 1987), peroxisomes (Small *et al.*, 1987) and chloroplasts (Cline *et al.*, 1985). By incubating small precursor proteins with dog pancreas microsomes at low temperature, membrane association of precursor proteins can be separated from membrane insertion and subsequent transfer (shown here). The prepromelittin derivatives are inserted and transferred into the microsomal vesicles from their sites of association by raising the temperature. We do not know the nature of the association of the prepromelittin derivatives with the microsomal membrane. It may involve either a protein–lipid or a protein–protein interaction (or both). The prepromelittin derivatives can associate with microsomes even in the absence of ATP or in the presence of a disulfide bond within their mature part. However, the subsequent membrane insertion and transfer of the associated precursor proteins, resulting in mature protein sequestered inside the microsomal vesicles, requires the cleavage of the disulfide bond and depends on ATP if there are amino acids with opposite charges at both ends of the mature part of the precursors. We speculate that the loop structure of the mature part of the prepromelittin derivatives has to be changed into a less folded structure to be compatible with insertion into, and eventually transfer across, the microsomal membrane. We assume that ATP and a component from the reticulocyte lysate are required for this conformational change.

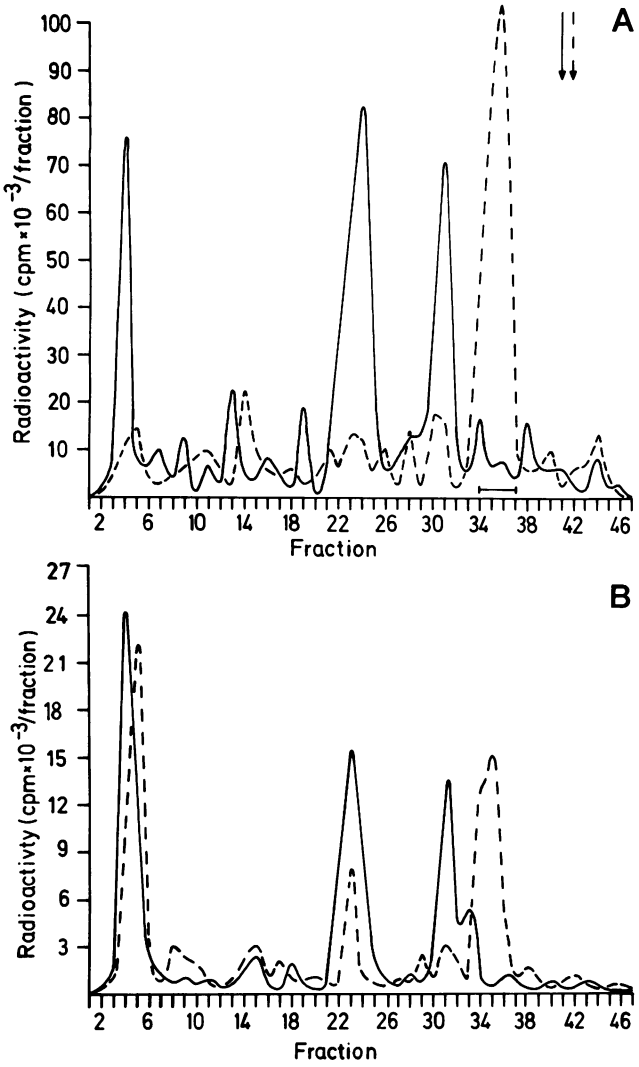

Fig. 8. HPLC analysis of oxidized and reduced precursor proteins containing two cysteine residues. (**A**) (----) HPLC run of oxidized undigested or digested precursor protein; (—) HPLC run of reduced undigested or digested precursor protein. (**B**) (----) second HPLC run of oxidixed sample, recovered from the first run under oxidizing conditions; (—) second HPLC run of the reduced sample, recovered from the first run under oxidizing conditions.

Role of ATP

Previous studies indicate that nucleoside triphosphates are required for import of proteins into mitochondria (Pfanner and Neupert, 1986; Eilers *et al.*, 1987; Chen and Douglas, 1987; Pfanner *et al.*, 1987), chloroplasts (Grossman *et al.*, 1980; Flügge and Hinz, 1986; Pain and Blobel, 1987), peroxisomes (Bellion and Goodman, 1987), *Escherichia coli* inner membrane vesicles (Chen and Tai, 1985; Geller *et al.*, 1986), yeast ER-derived membranes (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986a,b; Waters and Blobel, 1986) and into dog pancreas microsomes (Caulfield *et al.*, 1986; Conolly and Gilmore, 1986; Mueckler and Lodish, 1986a,b; Perara *et al.*, 1986; Schlenstedt and Zimmermann, 1987; Wiech *et al.*, 1987). Strikingly, import into mitochondria of hybrid proteins between *Neurospora crassa* preproteolipid and mouse dihydrofolate reductase, differing only in their mature part, required varying levels of nucleoside triphosphates (Pfanner *et al.*, 1987). Prepropeptide GLa and M13 procoat protein were also shown to depend on ATP for import into dog pancreas microsomes to a different extent (Schlenstedt and Zimmermann, 1987; Wiech *et al.*, 1987). Here we demonstrate that precursor proteins derived from prepromelittin have different requirements for ATP. Apparently it is the mature part of the precursor proteins and, specifically, the position of charged amino acids within the mature part which is critical for the ATP-requirement rather than the total number of charged amino acids wich have to be inserted into microsomal membranes. In reticulocyte lysate, an equilibrium may exist between the precursor proteins containing the loop structure and precursor proteins in a less folded conformation. The ATP-dependent component may be able to influence this equilibrium, independently of whether the precursor is free or already membrane associated. The differences between various precursor proteins and their requirements for ATP may reflect differences in the equilibrium distribution of the particular protein between a conformation with, and another one without, a loop.

We assume that the ATP-requiring system observed here is identical to the ATP-dependent component present in rabbit reticulocyte lysates, which has been shown to be involved in the import of M13 procoat protein into microsomes (Wiech *et al.*, 1987). Furthermore, it may be identical to the ATP-dependent system required for the import of prepropeptide GLa, another SRP/docking protein- and ribosome/ribosome-receptor-independent precursor protein (Schlenstedt and Zimmermann, 1987), and similar to the system involved in import of prepro-α-factor in a homologous yeast system (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986a,b; Waters and Blobel, 1986; Waters *et al.*, 1986). Strikingly, import of the latter precursor protein is also independent of ribosomes (Hansen *et al.*, 1986). In contrast, SRP-arrested docking protein-dependent precursor proteins depend on GTP for association with the microsomal membrane (Conolly and Gilmore, 1986). This, however, may reflect the functional interaction between ribosomes and the ribosome receptor. It may also be a dependence on GTP in the other cases where precursor proteins with attached tRNA were employed and ribosome- as well as nucleoside triphosphate-dependence were observed (Mueckler and Lodish, 1986a,b; Perara *et al.*, 1986).

A working hypothesis

We propose the following model for the import of small precursor proteins into the ER: precursor proteins with a content of less than 80 amino acids, apparently, are released from the ribosome before an interaction between either the signal peptide and signal recognition particle or the ribosome and ribosome receptor can occur (Zimmermann and Mollay, 1986; Schlenstedt and Zimmermann, 1987; Müller and Zimmermann, 1987). Their primary structures, however, have evolved in a way which allows them to stay competent for membrane association without the aid of these components. Possibly, a loop is formed by the mature part. Even disulfide bridges are allowed at this point, although they probably never occur *in vivo* (Obha *et al.*, 1981; Peters and Davidson, 1982). This structure can associate with the microsomal membrane. It is presently unclear whether a signal receptor is involved at this step (Robinson *et al.*, 1987; Wiedmann *et al.*, 1987). We assume that an unfolding, i.e. destabilization of this structure, has to take place before membrane insertion can occur. Typically, this is catalysed by a cytoplasmic protein and ATP. Strikingly, the protein without any charged amino acid residues at the mature ter-

mini does not depend on this activity. Insertion may occur in the form of a hairpin structure between the signal peptide and the mature amino terminus. This event leads to exposure of the cleavage site to the luminal side of the membrane and, typically, results in processing by signal peptidase. The subsequent membrane transfer of the mature protein may occur in one or more steps and may or may not involve proteinaceous membrane components or nucleoside triphosphates.

Materials and methods

Materials

[α-^{32}P]ATP (2000 Ci/mmol) was purchased from Dupont New England Nuclear. [^{35}S]Methionine (1000 Ci/mmol) and [^{35}S]cysteine (1100–1400 Ci/mmol) were purchased from Amersham Corp. Trypsin (type XIII from bovine pancreas, 10 000 U/mg), potato apyrase (grade VIII) and cycloheximide were from Sigma. Soy bean trypsin inhibitor, Proteinase K, restriction endonucleases, calf intestinal phosphatase, ribonuclease A and SP6 RNA polymerase were from Boehringer Mannheim. T4 DNA ligase, T4 polynucleotide kinase were from New England Biolabs. Cloned Klenow fragment of *E.coli* DNA polymerase I, dideoxy-, deoxy- and ribonucleotides, the cap analogue 7mG(5')ppp(5')G, Sephadex G-25 and PEG 8000 were from Pharmacia P-L Biochemicals. Human placental ribonuclease inhibitor (RNasin) was from Promega Biotec. All other chemicals were from Merck unless otherwise stated. X-ray films (X-Omat AR) were from Kodak.

DNA manipulations

Isolation of DNA fragments and oligonucleotides, plasmid preparation on large or small scales, ligation, phosphorylation and dephosphorylation of DNA, transformation of *E.coli* and sequence analysis were carried out as described previously (Maniatias *et al.*, 1982; Müller and Zimmermann, 1987).

For construction of plasmids coding for precursor proteins ppΔ-DHFR/3^c and pΔ-DHFR/3Thr^c, which contain a second cysteine residue instead of leucine as compared with the parental precursors ppΔ-DHFR/3 and pΔ-DHFR/3Thr (Müller and Zimmermann, 1987), the small *Eco*RI/*Hin*dIII fragment of plasmids coding for the latter proteins were cloned into the polylinker of plasmid pMa5-8 (Fritz *et al.*, 1986). Gapped duplex DNA was generated by mixing purified ssDNA of these plasmids, prepared by helper phage (M13K07) infection of transformed male *E.coli* cells (WK6) (Levinson *et al.*, 1984), with the purified large fragment of plasmid pMc5-8 which had been cleaved by *Eco*RI and *Hin*dIII, followed by subsequent heat denaturation and cooling. The purified mutagenic oligonucleotide d(5' AGGCCAGGGGCAGTCTCC3'), phosphorylated by T4 polynucleotide kinase, was hybridized to the gapped plasmids. For the gap-filling and sealing reactions, a combination of cloned Klenow fragment of *E.coli* DNA polymerase I and T4 DNA ligase was used. The reaction mixture was subjected to alkaline sucrose gradient centrifugation (5–20%) and the closed circular DNA was recovered and used for transformation of repair-deficient *E.coli* strain WK6mutS. Mixed plasmid DNA from transformants, resistant against chloramphenicol (25 μg/ml), was prepared on a small scale and segregated by transformation of su$^-$-strain WK6. Chloramphenicol-resistant and ampicillin-sensitive clones were screened for incorporation of the mutagenic oligonucleotide by two cycles of colony hybridization with ^{32}P-labeled oligonucleotide under high stringency. The small *Eco*RI/*Hin*dIII fragments of plasmids, isolated from positive transformants, were cloned into plasmids pUC19, for DNA-sequencing, and pSP65 for *in vitro* transcription.

In vitro transcription and translation

Plasmids having an SP6 promoter (Melton *et al.*, 1984) located before the cDNA and coding for precursor proteins were transcribed with SP6 RNA polymerase as described previously (Müller and Zimmermann, 1987). Translation in nuclease-treated rabbit reticulocyte lysates (Pelham and Jackson, 1976; Zimmermann *et al.*, 1979) was performed at reticulocyte concentrations of 40% (v/v) in a total volume of 12.5 μl at 37°C for times indicated in the presence of *in vitro* transcripts and either [^{35}S]methionine (final conc. 1.4 mCi/ml) or [^{35}S]cysteine (final conc. 1.7 mCi/ml).

A cysteine containing peptide (16mer) was derived from precursor pp$_\Delta$-DHFR/3 in the following way: unlabeled precursor protein was synthesized in a reticulocyte lysate in the presence of microsomes for 30 min at 37°C (total vol. 250 μl). Microsomes were reisolated by centrifugation (Beckman airfuge, A-100/30 rotor, 25 p.s.i., 5 min), resuspended in 20 mM Tris–HCl (pH 7.4), precipitated by ammonium sulfate (final conc. 66%), dissolved in 20 mM Tris–HCl (pH 7.4), and passed through a Sephadex G-25 column that had been equilibrated with the same buffer. Fractions 3–5 were combined, precipitated with TCA (final conc. 5%) and subsequently dissolved in 60 mM NH_4HCO_3 (pH 8.0). After addition of trypsin (final conc. 150 μg/ml) the sample was incubated for 90 min at 37°C and then supplemented with a 2-fold molar excess of soybean trypsin inhibitor.

Post-translational protein import

Translations were carried out in rabbit reticulocyte lysates in the presence of *in vitro* transcripts coding for the corresponding precursor proteins. After incubation for 10 min at 37°C, the translation reactions were supplemented with a combination of cycloheximide (final conc. 100 μg/ml) and RNase A (final conc. 80 μg/ml). Following further incubation for 5 min at 37°C, dog pancreas microsomes, prepared and treated with nucleases and EDTA as described previously (Watts *et al.*, 1983), were added and the incubation was continued for 15 min at 37°C. The absorbance at 280 nm [measured in 2% (w/v) SDS] of microsomes in any particular post-translational import reaction was 5.

Fractionation of import reactions

Post-translational import reactions (36 μl) were layered onto a two step sucrose gradient consisting of a 50 μl cushion of 0.5 M sucrose (in 50 mM Hepes–KOH, pH 7.4, 50 mM KAc, 2 mM MgAc, 1 mM DTT) layered above 50 μl of 2 M sucrose in the same buffer. The gradient was subjected to centrifugation for 5 min in a A-100/30 rotor at 25 p.s.i. in a Beckman airfuge. Five fractions were subsequently removed with an Eppendorf pipette from the top of the gradient in the following order: supernatant (40 μl); first sucrose layer (40 μl), which was discarded; interface (5 μl) between the first and second sucrose layer; second sucrose layer (50 μl), which was discarded; and pellet. Under these conditions the sum of precursor proteins recovered in pellet, interface and supernatant comprised about 80–90% of the total precursor protein used in the association assay.

Analytical procedures

Sequestration of the processed precursor proteins by dog pancreas microsomes was analysed by incubation of the complete import reaction with Proteinase K (100 μg/ml) (Zimmermann and Mollay, 1986; Müller and Zimmermann, 1987). Samples containing *in vitro* synthesized proteins were either analysed directly or after precipitation with ammonium sulfate (Müller and Zimmermann, 1987) on urea-containing SDS–polyacrylamide gels (19%) (Ito *et al.*, 1980). For fluorography, the gels were soaked in sodium salicylate (Chamberlain, 1979), dried and exposed to X-ray films at −80°C for 12–48 h.

Analysis of intramolecular disulfide bridges

The formation of a disulfide bond within the mature part of the precursor proteins ppΔ-DHFR/3^c and pΔ-DHFR/3Thr^c changed their electrophoretic migration behaviour in the absence of reducing agents. The oxidized forms always had a somewhat higher apparent mol. wt than their reduced counterparts (data not shown). With this assay we excluded that formation of dimers or multimers between cysteine-containing proteins via intermolecular disulfide bonds had occurred, since no labeled material in the mol. wt range of two or more times 8 kd was detected on gels in the absence of reducing agents. When trypsin digestion was carried out under reducing conditions, the precursor proteins ppΔ-DHFR/3^c and pΔ-DHFR/3Thr^c were cleaved into two [^{35}S]cysteine-labeled peptides having 16 and 10 amino acids. The smaller peptide, derived from precursor pp$_\Delta$-DHFR/3^c, eluted from the column in fraction 24 and the larger one in fraction 31 (Figure 8A, solid line). When the digestion was carried out after oxidation, however, one peak was observed, eluting in fraction 36, which should contain both peptides linked by a disulfide bridge (Figure 8A, broken line). The identity of the material in fraction 36 after analysis of the oxidized and digested sample was confirmed by rechromatography with or without prior reduction. Under oxidizing conditions the radioactivity eluted in fraction 35, i.e. almost identical to the elution during the first run (Figure 8B, broken line). Since the elution buffer used for HPLC did not contain any oxidizing agents, a small proportion of the single peptide was apparently cleaved into two peptides. After reduction of the originally oxidized material, however, only two peaks were observed (Figure 8B, solid line). The position of the peaks in fractions 23 and 31 was in close agreement with those of tryptic peptides generated from the reduced precursor (Figure 8, A versus B). These results were consistent with the interpretation that fraction 36 of the run with the oxidized and digested precursor contained a dimeric peptide, consisting of the larger and smaller peptide linked by a disulfide bridge, and that reduction of the disulfide bridge cleaved this dimeric peptide into the two original peptides. Analysis of precursor pΔ-DHFR/3Thr^c led to similar conclusions (data not shown).

The following conditions were used for the HPLC analysis shown in Figure 8.

(A) Precursor protein ppΔ-DHFR/3^c was synthesized in a rabbit reticulocyte lysate (500 μl translation volume) for 10 min at 37°C. After addition of cycloheximide and RNase A, the translation reaction was divided into two aliquots. One aliquot received $K_3Fe(CN)_6$ (final conc. 10 mM), the other aliquot water. After incubation for 2 min at 20°C, the samples were precipitated twice with ammonium sulfate (final conc. 66 and 55%) and dissolved in 60 mM NH_4HCO_3 (pH 8.0) which had been supplemented with $K_3Fe(CN)_6$ (final conc. 10 mM) in the case of the oxidized sample. Both samples were subsequently passed through a Sephadex G-25 column pre-equilibrated with the same buffers. Fractions of 250 μl were collected and analysed for radioactivity. Fractions 4 and 5 of the first peak, containing the labeled precursor protein, were combined and divided into two aliquots. One aliquot was incubated with trypsin (165 μg/ml), pre-treated with tosyl phenylalanine chloromethyl ketone, for 90 min at 37°C, the other aliquot with water. After addition of a 2-fold molar excess of soybean trypsin inhibitor and of PMSF (final conc. 2 mM) the oxidized sample was supplemented with $K_3Fe(CN)_6$ (final conc. 10 mM) and both samples received SDS (final conc. 2%). After incubation for 2 h at 37°C, trifluoroacetic acid (final conc. 0.1%) was added and the samples were subjected to analysis on HPLC (reverse-phase, μBondapak-phenyl column 3.9 mm × 30 cm, 10 μm particle size) (Yuan *et al.*, 1982). The column was equilibrated at a flow-rate of 0.8 ml/min in solvent A (0.1% v/v trifluoroacetic acid). Following sample injection, the column was flushed with solvent A for 20 min and the peptides were then eluted with a linear gradient of 0–50% solvent B (0.1% trifluoroacetic acid, 9.9% water, 90% acetonitrile, v/v/v) (Nicholson *et al.*, 1987). Fractions were collected at 2 min intervals and analysed for radioactivity. The total run time, including column equilibration, was 105 min. All HPLC runs were performed at a constant flow-rate of 0.8 ml/min at room temperature. The positions of the main peaks of the runs with undigested precursor protein are indicated by arrows. The peak in fraction 4 represents material which did not bind to the column. In a control experiment, the oxidation of the precursor protein prior to the tryptic digestion was carried out in the presence of an excess of free cysteine (final conc. 5 mM). The elution behaviour of the respective tryptic peptides from the HPLC column closely resembled that of tryptic peptides generated under reducing conditions. Preincubation of cysteine with the oxidizing agent prior to mixing with the precursor protein, followed by tryptic digestion, caused the appearance of a single peak at a position identical to that observed after HPLC analysis of tryptic peptides generated under oxidizing conditions (data not shown).

(B) Fractions 34–37 (as indicated by a horizontal line in Figure 8A) of the HPLC run with tryptic peptides of oxidized precursor protein pp$_\Delta$-DHFR/3^c were combined and subjected to a second HPLC run. The sample was dried under vacuum, dissolved in 1 ml of 60 mM NH_4HCO_3 (pH 8.0) and divided into two aliquots. One aliquot was supplemented with $K_3Fe(CN)_6$ (final conc. 10 mM) and the other aliquot with DTT (final conc. 25 mM). After incubation for 2 min at 37°C, SDS (final conc. 2%) was added and the incubation was continued for 2 h. After addition of trifluoroacetic acid (final conc. 0.1%) the samples were analysed by HPLC as described above. About 85% of the radioactivity in fractions 34–37 of the first run were recovered in the second runs under both oxidizing and reducing conditions. A larger proportion of material (fraction 3–6), however, did not bind to the column during the second HPLC run.

Acknowledgements

We want to thank Dr H.-J. Fritz (Genzentrum, München, FRG) for generously providing plasmids pMa5-8 and pMc5-8, Dr Ronald Mertz (Genzentrum, München, FRG) for synthesizing oligonucleotides, Christian Magold for his help during the oligonucleotide-directed mutagenesis, and Dr Donald W.Nicholson for his valuable advice and support in performing the HPLC runs. We are grateful to Birgitta Kasseckert and Maria Sagstetter for excellent technical assistance and to Drs Donald W.Nicholson and Nikolaus Pfanner for critical comments on the manuscript. This work was supported by grant B10 from the Sonderforschungsbereich 184: 'Molekulare Grundlagen der Biogenese von Zellorganellen'.

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Received on August 24, 1987; revised on December 16, 1987