

# Translocation Arrest by Reversible Folding of a Precursor Protein Imported into Mitochondria. A Means to Quantitate Translocation Contact Sites

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**Abstract.** Passage of precursor proteins through translocation contact sites of mitochondria was investigated by studying the import of a fusion protein consisting of the NH<sub>2</sub>-terminal 167 amino acids of yeast cytochrome *b*<sub>2</sub> precursor and the complete mouse dihydrofolate reductase. Isolated mitochondria of *Neurospora crassa* readily imported the fusion protein. In the presence of methotrexate import was halted and a stable intermediate spanning both mitochondrial membranes at translocation contact sites accumulated. The complete dihydrofolate reductase moiety in this intermediate was external to the outer membrane, and the 136 amino acid residues of the cytochrome *b*<sub>2</sub> moiety remaining after cleavage by the matrix processing peptidase spanned both outer and inner membranes. Removal of methotrexate led to import of the intermediate retained at the contact site into the matrix.

Thus unfolding at the surface of the outer mitochondrial membrane is a prerequisite for passage through translocation contact sites. The membrane-spanning intermediate was used to estimate the number of translocation sites. Saturation was reached at 70 pmol intermediate per milligram of mitochondrial protein. This amount of translocation intermediates was calculated to occupy ~1% of the total surface of the outer membrane. The morphometrically determined area of close contact between outer and inner membranes corresponded to ~7% of the total outer membrane surface. Accumulation of the intermediate inhibited the import of other precursor proteins suggesting that different precursor proteins are using common translocation contact sites. We conclude that the machinery for protein translocation into mitochondria is present at contact sites in limited number.

THE two surrounding membranes, outer and inner membranes of mitochondria and chloroplasts, are in close proximity to each other at distinct "contact sites" (Hackenbrock, 1968; Douce and Joyard, 1979; Cline et al., 1985; Cremers et al., 1988). Cytosolic ribosomes were observed to be attached at these sites and therefore a role for contact sites in the transport of nuclear encoded proteins into mitochondria was proposed (Kellems et al., 1975). Biochemical evidence for the role of contact sites was obtained by the characterization of translocation intermediates on the import pathway, namely precursor proteins spanning both outer and inner mitochondrial membranes (Schleyer and Neupert, 1985; Schwaiger et al., 1987; Pfanner et al., 1987a).

Three different methods for accumulating contact site intermediates in vitro were developed: (a) The import reaction was performed at low temperature to retard the translocation process; (b) Antibodies were bound to carboxy-terminal portions of the precursors before import; and (c) Reduction of nucleoside triphosphates in the import reaction (for review see Pfanner et al., 1988; Hartl et al., 1989). We propose that

these procedures render the mature protein part of the precursor incompetent for translocation by conferring a more stably folded structure. The topology of the intermediates formed was characterized, on one hand as accessible to the processing peptidase in the matrix, cleaving off the amino-terminal presequence of the precursor protein, while on the other hand as being exposed to externally added proteases, digesting the part of the precursor which had remained outside the mitochondrion. It was concluded that at sites of import both mitochondrial membranes are sufficiently close together to be spanned by a single polypeptide chain. Decoration of antibody bound precursor proteins trapped in contact sites with protein A-gold particles demonstrated the identity of morphologically described and biochemically defined contact sites (Schwaiger et al., 1987).

Contact sites involved in mitochondrial protein import appear to be stable structures since they survive subfractionation of mitochondria and can be enriched by subsequent sucrose gradient centrifugation (Schwaiger et al., 1987). On the other hand, translocation contact site intermediates span-

ning the membranes are apparently embedded in a hydrophilic environment suggesting the possibility that protein components might be directly involved in the translocation process (Pfanner et al., 1987a).

To determine the number of translocation contact sites, we made use of the following observation. Import and processing of a fusion protein between a mitochondrial presequence (amino acid residues 1–22 of cytochrome oxidase subunit IV) and dihydrofolate reductase (DHFR)<sup>1</sup> was found to be inhibited after binding of the folate antagonist methotrexate to the DHFR moiety (Eilers and Schatz, 1986). It was concluded that binding of the antagonist prevented unfolding and thereby membrane translocation of the fusion protein.

We constructed a hybrid protein containing a longer stretch of a mitochondrial precursor protein. The amino-terminal 167 amino acid residues of cytochrome *b*<sub>2</sub> precursor (Guiard, 1985), an intermembrane space protein, were fused to DHFR (Stueber et al., 1984). Upon import into mitochondria in the presence of methotrexate, this construct formed a stable intermediate spanning contact sites. The cytochrome *b*<sub>2</sub> part penetrated far enough into the matrix for the presequence to be correctly cleaved by the processing peptidase while the stably folded DHFR remained outside the outer membrane. Saturation of contact sites and a complete block of import of authentic precursor proteins was reached at  $70 \pm 20$  pmol accumulated intermediate per milligram of mitochondrial protein. The 136 amino acid residues of the cytochrome *b*<sub>2</sub> part remaining after cleavage by the matrix processing peptidase sufficed to span the two membranes at contact sites.

## Materials and Methods

### Synthesis of Precursor Proteins

Precursor proteins were synthesized in vitro in rabbit reticulocyte lysates (Pelham and Jackson, 1976), which were programmed with specific RNA transcribed by SP6-RNA-polymerase (Melton et al., 1984) from pGEM3 plasmids (Promega Biotec, Madison, WI). Full-length cDNAs coding for the Fe-S-protein of complex III (Harnisch et al., 1985) and *F*<sub>1</sub> $\beta$  were isolated from a *Neurospora crassa* library and prepared for in vitro transcription as described previously (Pfaller et al., 1988).

### Import of Precursor Proteins into Isolated Mitochondria

Growth of *Neurospora crassa* (wild-type 74A) and isolation of mitochondria by differential centrifugation were carried out as described (Pfanner and Neupert, 1985), except that the time for grinding of hyphae with sand was reduced to 30 s. Isolated mitochondria were resuspended at a concentration of 1 mg/ml in SEM buffer (0.25 M sucrose, 1 mM EDTA, 10 mM morpholino propane sulfonic acid [MOPS], adjusted to pH 7.2 with KOH).

Import reactions contained 80% reticulocyte lysate to which 2 mM DTT, 2 mM NADH and 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added. Methotrexate (Sigma Chemical Co., St. Louis, MO) was used at a final concentration of 100 nM. For accumulation of contact site intermediates methotrexate was added to the reticulocyte lysate containing pb<sub>2</sub>DHFR precursor and the mixture (usually 50  $\mu$ l) was incubated for 5 min at 0°C. Then mitochondria were added in 5  $\mu$ l of SEM buffer to a final concentration of 1  $\mu$ g/ml. Incubation for import was for up to 60 min at 25°C. To establish identical import conditions when applying increasing amounts of precursor protein in reticulocyte lysate, untranslated lysates were prepared. These were treated essentially as translated reticulocyte lysates, but [<sup>35</sup>S]methionine and mRNA were omitted.

1. *Abbreviations used in this paper:* DHFR, dihydrofolate reductase; *F*<sub>1</sub> $\beta$ , *F*<sub>1</sub>-ATPase subunit  $\beta$ .

For inhibition of the matrix-localized processing peptidase, 7.5 mM EDTA and 0.5 mM 1,10-phenanthroline (Schmidt et al., 1984; Hartl et al., 1986) were added from 250 and 50 mM stock solutions, respectively. When indicated, import of precursors was prevented by addition of 1  $\mu$ M valinomycin from a 100-fold concentrated stock solution in ethanol. Control samples received the same volume of inhibitor-free solutions.

## Miscellaneous

Published procedures were used for DNA manipulations (Maniatis et al., 1982; Kleene et al., 1987), protease treatment and reisolation of mitochondria after import (Hartl et al., 1986), TCA-precipitation of proteins (Hartl et al., 1987a), protein determination (Bradford, 1976), SDS-PAGE (Laemmli, 1970), and fluorography (Chamberlain, 1979). <sup>35</sup>S-labeled proteins were quantified by liquid scintillation counting after excising the corresponding bands from polyacrylamide gels and extraction with H<sub>2</sub>O<sub>2</sub> (Bonner, 1983). Electron microscopy of isolated mitochondria was performed as published (Desel et al., 1982; Schwaiger et al., 1987). Morphometric analyses were performed using the semiautomatic image-analyzing system MOP/AMO 3 with an incorporated Z80 microprocessor (Kontron Analytical, Everett, MA).

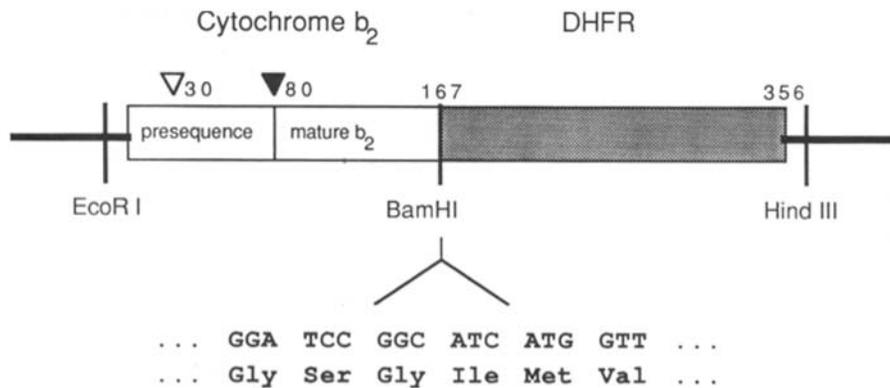
## Results

### Synthesis of pb<sub>2</sub>DHFR Fusion Protein

A hybrid gene was constructed encoding the 167 NH<sub>2</sub>-terminal amino acids of the precursor of cytochrome *b*<sub>2</sub> fused to the complete sequence of cytosolic mouse DHFR. For this purpose the Bam HI–Hind III fragment coding for the COOH-terminal part of yeast cytochrome *b*<sub>2</sub> (Guiard, 1985), starting at residue 168, was substituted in the plasmid pDS-6226 (Hartl et al., 1987a) by the Bam HI–Hind III fragment of the plasmid pDSS/3 (Stueber et al., 1984) containing the DHFR gene. For in vitro transcription the fused pb<sub>2</sub>DHFR gene was cut out with Eco RI and Hind III and ligated to the corresponding sites of the expression vector pGEM3. The protein which is encoded by the fused gene contains four regions (Fig. 1). Starting at the amino terminus these are: (a) the presequence of cytochrome *b*<sub>2</sub> (80 residues) followed by (b) 87 residues of the NH<sub>2</sub>-terminal part of the mature protein, (c) a linker of two amino acids (Gly, Ile), and (d) the entire sequence of the DHFR (187 residues). The product obtained by coupled transcription/translation in a reticulocyte lysate in the presence of [<sup>35</sup>S]methionine had an apparent relative molecular mass of 40,000. This agrees well with the calculated molecular mass of the construct of 40,385 D.

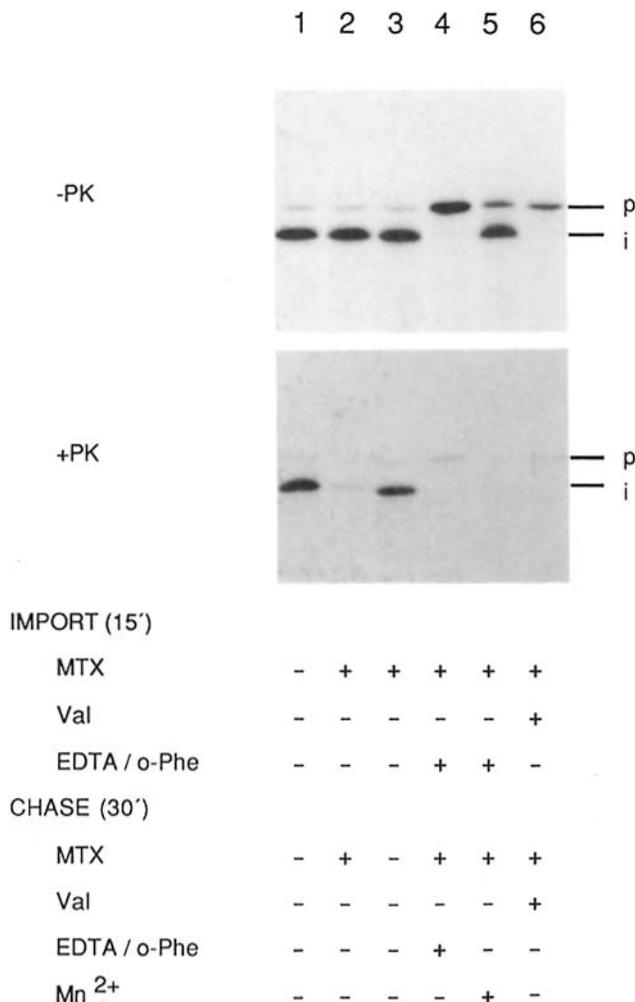
### Import of the Fusion Protein into Isolated Mitochondria

Radiolabeled precursor of pb<sub>2</sub>DHFR protein was incubated with isolated mitochondria of *N. crassa*. The precursor was imported into mitochondria and proteolytically processed (Fig. 2). Import into mitochondria was demonstrated by the resistance of processed pb<sub>2</sub>DHFR to externally added proteinase K (Fig. 2, lane *I*). Dissipation of the mitochondrial membrane potential inhibited processing of the precursor (Fig. 2, lane *6*). Upon import into yeast mitochondria both authentic cytochrome *b*<sub>2</sub> and pb<sub>2</sub>DHFR fusion protein were proteolytically processed in two steps (not shown). The first cleavage is performed in the matrix by the metal dependent processing peptidase resulting in the formation of an intermediate-sized form. The second cleavage occurs at the outer surface of the inner membrane after retranslocation of the intermediate (Hartl et al., 1987a). In mitochondria of *N. crassa* only the first cleavage to the intermediate sized form is ob-



(Hawlitsek et al., 1989). The second cleavage site (at position 80) has been determined by sequence analysis of the amino terminus of the mature-sized protein (Guiard et al., 1975). At the site of fusion between the cytochrome  $b_2$  part and the DHFR two residues (Gly, Ile) are inserted.

**Figure 1.** Fusion protein between residues 1-167 of cytochrome  $b_2$  and DHFR. Fusion protein  $pb_2$ DHFR consisting of the first 167 amino acid residues of cytochrome  $b_2$  precursor (Guiard, 1985) and the complete sequence of mouse DHFR (Stueber et al., 1984) was constructed as described in Materials and Methods. The site of the first cleavage (between residues 31 and 32) is suggested by the difference in apparent molecular weight between precursor and processed form and by comparison to the sequences of known cleavage sites of the mitochondrial processing enzyme



**Figure 2.** Import of  $pb_2$ DHFR fusion protein into isolated mitochondria. Six reactions of reticulocyte lysate (50  $\mu$ l each) containing radiolabeled  $pb_2$ DHFR were brought to 2 mM NADH, 2 mM DTT, and 15 mM ammonium sulfate. They were incubated for 5 min at 0°C either in the absence (reaction 1) or presence of 100 nM methotrexate (MTX) (reactions 2-6). In addition reaction 4 and 5 contained 7.5 mM EDTA and 0.5 mM 1,10-phenanthroline (*o-Phe*) to inhibit the mitochondrial processing peptidase, and reaction 6 contained 1  $\mu$ M valinomycin (*Val*). Isolated mitochondria of *N. crassa* (10  $\mu$ g per reaction) were added and incubation for import was

performed (Ostermann, J., F. U. Hartl, and W. Neupert, unpublished data).

Addition of methotrexate to the import reaction inhibited completion of translocation of the  $pb_2$ DHFR precursor into mitochondria. The fusion protein associated with mitochondria remained sensitive to added proteinase K but the presequence reached the matrix compartment where cleavage by the metal-dependent processing peptidase occurred (Fig. 2, lane 2). Apparently, in the presence of methotrexate, processed  $b_2$ DHFR accumulated spanning both membranes at contact sites. Addition of EDTA and 1,10-phenanthroline during import inhibited the processing enzyme. Under these conditions unprocessed  $pb_2$ DHFR accumulated spanning contact sites (Fig. 2, lane 4). Proteolytic cleavage could then be achieved by reactivation of the processing peptidase with Mn<sup>2+</sup> (Fig. 2, lane 5).

To make sure that  $pb_2$ DHFR in contact sites was on the authentic import pathway, mitochondria which had accumulated the translocation intermediate in the presence of methotrexate were reisolated from the import reactions and resuspended in twice the original volume in the absence of methotrexate. During a subsequent incubation in the presence of 2 mM ATP,  $b_2$ DHFR was chased into a protease protected position in the interior of mitochondria (Fig. 2, lane 3 vs. lane 2). This result demonstrated that the contact

carried out for 15 min at 25°C. Afterwards mitochondria were reisolated at 4°C by centrifugation and resuspended on ice in 80  $\mu$ l of BSA-buffer (3% BSA, 0.25 M sucrose, 80 mM KCl, 10 mM MOPS, pH 7.2) again containing 2 mM NADH, 2 mM DTT, and 15 mM ammonium sulfate. Mitochondria of reactions 2 and 4-6 were resuspended in the presence of 100 nM methotrexate. Reactions 4 and 6 again contained EDTA/1,10-phenanthroline and valinomycin, respectively. Reaction 5 received 1 mM MnCl<sub>2</sub> to reactivate the processing peptidase. Before a chase for 30 min at 25°C, 20  $\mu$ l of cold reticulocyte lysate was added to all reactions. After cooling on ice half of each reaction received proteinase K (PK) (20  $\mu$ g/ml, final concentration). Protease treatment was performed for 25 min at 0°C and stopped by the addition of 1 mM PMSF from a 100-fold concentrated stock solution in ethanol. Mitochondria were reisolated, dissociated in SDS containing buffer, and analyzed by SDS-PAGE and fluorography. *p*, precursor form of  $b_2$ DHFR ( $pb_2$ DHFR); *i*, intermediate-sized form (*i*- $b_2$ DHFR).

site intermediate had not moved away from the import machinery and resumed its passage via the contact site when the methotrexate was removed.

### Length of the Polypeptide Chain Spanning Contact Sites

Binding of methotrexate stabilizes the folded structure of DHFR and thus renders the protein highly resistant towards digestion by proteases. This behavior was also observed with the pb<sub>2</sub>DHFR fusion protein. On incubation of a reticulocyte lysate containing newly synthesized pb<sub>2</sub>DHFR with proteinase K in the presence of methotrexate, the DHFR moiety of the construct remained intact (Fig. 3 A, lanes 2 and 3 vs. lane 1). Only the cytochrome b<sub>2</sub> part was digested indicating that the DHFR part of the fusion protein folded independently. We tested whether the intact DHFR moiety could also be produced by proteolytic cleavage from the b<sub>2</sub>DHFR molecules spanning contact sites. Isolated mitochondria which had accumulated the translocation intermediate in the presence of methotrexate were reisolated from the import reactions and washed with SEM buffer to remove unspecifically associated pb<sub>2</sub>DHFR. They contained essentially only the proteolytically processed contact site intermediate which was removed by added proteinase K (Fig. 3 A, lanes 2 and 3 vs. lane 1). If the processing enzyme was inhibited by chelators, unprocessed pb<sub>2</sub>DHFR accumulated as translocation intermediate (Fig. 3 B, lanes 7-9). The supernatants of the protease reactions were precipitated and analyzed by SDS electrophoresis. A single protease-resistant

fragment was detected which migrated with an apparent molecular weight slightly higher than authentic DHFR (Fig. 3, lanes 4-6 and 10-12).

We conclude that the complete DHFR moiety of the translocation intermediate had remained outside the mitochondrion leaving at most the 167 amino acid residues of the cytochrome b<sub>2</sub> part of the construct to span the two membranes. Since cleavage by the matrix processing enzyme removes ~30 amino acids from the NH<sub>2</sub> terminus of the precursor, a polypeptide chain consisting of ~135 amino acids is sufficient to span the mitochondrial membranes at contact sites.

### Titration of Contact Sites

Since the concentration of pb<sub>2</sub>DHFR protein in the reticulocyte lysate did not exceed 2 pmol/ml, low amounts of mitochondria (50 ng) had to be used in titration experiments to reach saturating concentrations of precursor. Isolated mitochondria were incubated for 60 min in reticulocyte lysates containing increasing amounts of <sup>35</sup>S-labeled pb<sub>2</sub>DHFR and 100 nM methotrexate. Then mitochondria were reisolated from the import reactions and washed once with SEM buffer to remove unspecifically associated unprocessed pb<sub>2</sub>DHFR protein. Mitochondria and aliquots of the combined supernatants were subjected to SDS gel electrophoresis and fluorography. The amounts of radioactivity contained in the bands corresponding to the translocation intermediate and to the free pb<sub>2</sub>DHFR were determined. The respective amounts of protein were calculated. Analysis of the data in analogy to

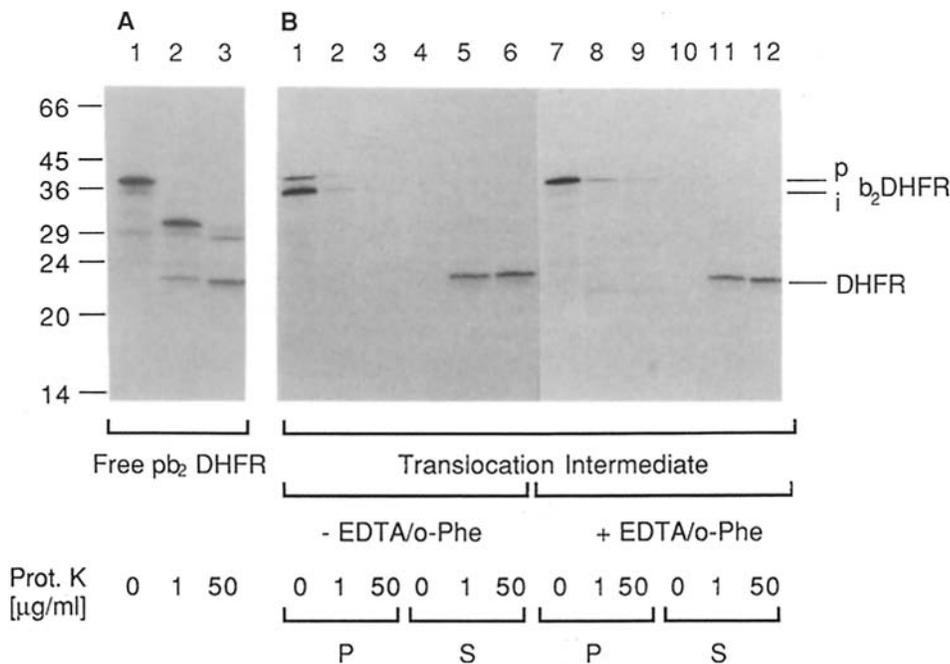
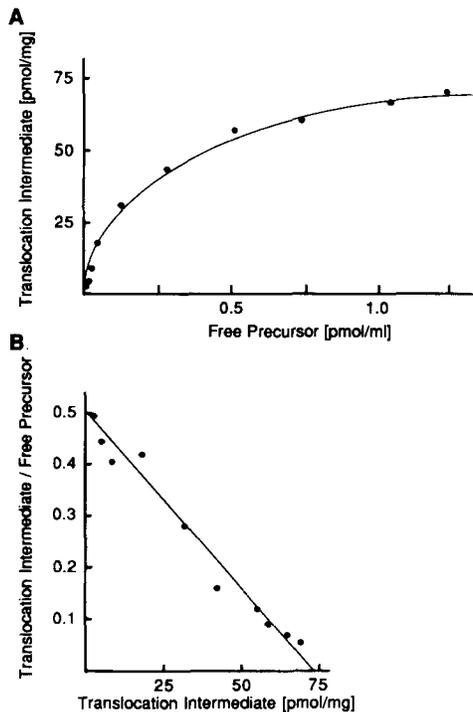


Figure 3. Fragments of free pb<sub>2</sub>DHFR and of translocation intermediates produced by proteinase K. (A) Radiolabeled pb<sub>2</sub>DHFR was precipitated from a reticulocyte lysate by ammonium sulfate (33% saturation). The precipitate was dissolved in SEM buffer and desalted by Sephadex G25 gel-filtration. Methotrexate was added to 100 nM final concentration and the sample was divided into three 50-μl reactions, each corresponding to 3 μl of original reticulocyte lysate. Reactions 2 and 3 received 1 μg/ml and 50 μg/ml proteinase K (final concentrations), respectively. Reaction 1 served as control. After incubation for 25 min at 0°C protease activity was stopped by addition of 1 mM PMSF. TCA precipitates were analyzed. (B) b<sub>2</sub>DHFR translocation intermediate was accumulated in the presence of methotrexate essentially as described in

the legend to Fig. 2 in two parallel reactions. One of them contained 7.5 mM EDTA and 0.5 mM 1,10 phenanthroline. Mitochondria (30 μg) were reisolated from the two import reactions and washed once with SEM buffer containing 100 nM methotrexate. The mitochondrial pellets obtained after recentrifugation were resuspended in 150 μl SEM/100 nM methotrexate and divided into three reactions each. Treatment with proteinase K was performed as described above. Then the reactions were separated into mitochondrial pellets and supernatants. Pellets (P, lanes 1-3 and 7-9) and supernatants (S, lanes 4-6 and 10-12) were precipitated with TCA and were analyzed by SDS electrophoresis and fluorography. The positions in the gel of pb<sub>2</sub>DHFR, i-b<sub>2</sub>DHFR (produced by the action of matrix processing peptidase), DHFR, and of molecular weight markers are indicated.



**Figure 4.** Titration of translocation contact sites. Isolated mitochondria (50 ng mitochondrial protein per reaction) were incubated for 60 min at 25°C in the presence of increasing amounts of radiolabeled pb<sub>2</sub>DHFR contained in reticulocyte lysates. The total volume of each reaction was brought to 50  $\mu$ l by the addition of untranslated reticulocyte lysate. Methotrexate was added to 100 nM (see legend to Fig. 2). Mitochondria were reisolated and washed by resuspension in 50  $\mu$ l of SEM buffer/100 nM methotrexate and recentrifugation. The supernatants obtained were combined with the supernatants of the first centrifugation. The mitochondrial pellets and aliquots of the combined supernatants were separated by SDS electrophoresis. Amounts of contact site intermediate (processed pb<sub>2</sub>DHFR) and of free pb<sub>2</sub>DHFR, respectively, were determined as described in Materials and Methods. (A) Amounts of free precursor (pmol/ml) plotted vs. amounts of translocation intermediate (pmol/mg of mitochondrial protein). (B) Scatchard analysis of the same data for determination of the saturating amounts of contact site intermediate.

Scatchard resulted in a straight line demonstrating that there were no cooperative effects during the accumulation of translocation intermediates (Scatchard, 1949). As shown in Fig. 4, saturation of contact sites was reached when  $70 \pm 20$  pmol of translocation intermediate were accumulated per milligram of mitochondrial protein ( $n = 6$ ). In these experiments the level of the membrane potential across the inner membrane was not limiting, since lowering the membrane potential by different concentrations of carbonylcyanide *m*-chlorophenylhydrazone did not change the characteristics of saturation with pb<sub>2</sub>DHFR intermediate (not shown).

#### **Block of Import of Authentic Precursor Proteins by Translocation Intermediate**

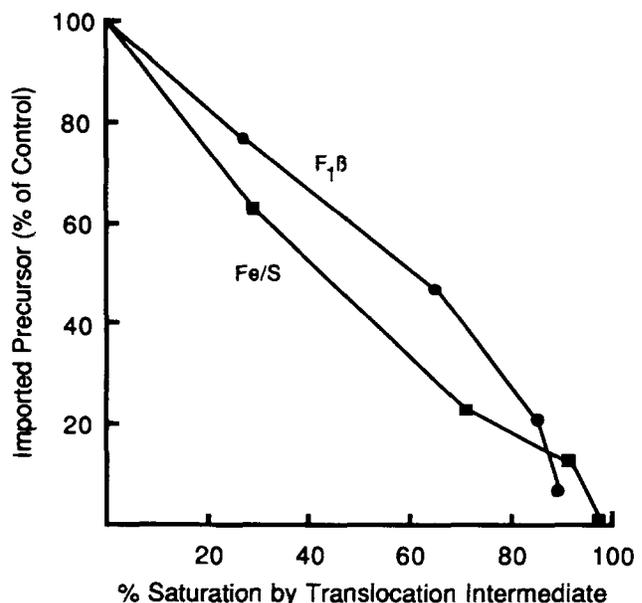
Did the saturation of contact sites with translocation intermediate affect the import of authentic mitochondrial precursor proteins? To answer this question, competition experiments were carried out. The Fe/S protein of complex III and

the  $\beta$  subunit of F<sub>1</sub>ATPase (F<sub>1</sub> $\beta$ ) were synthesized in a reticulocyte lysate and added to mitochondria which had accumulated increasing amounts of pb<sub>2</sub>DHFR contact site intermediate as described above. Rates of import of both precursor proteins decreased in relation to the amounts of contact site intermediate present. Saturation of contact sites caused a complete block of import of Fe/S protein and F<sub>1</sub> $\beta$  (Fig. 5).

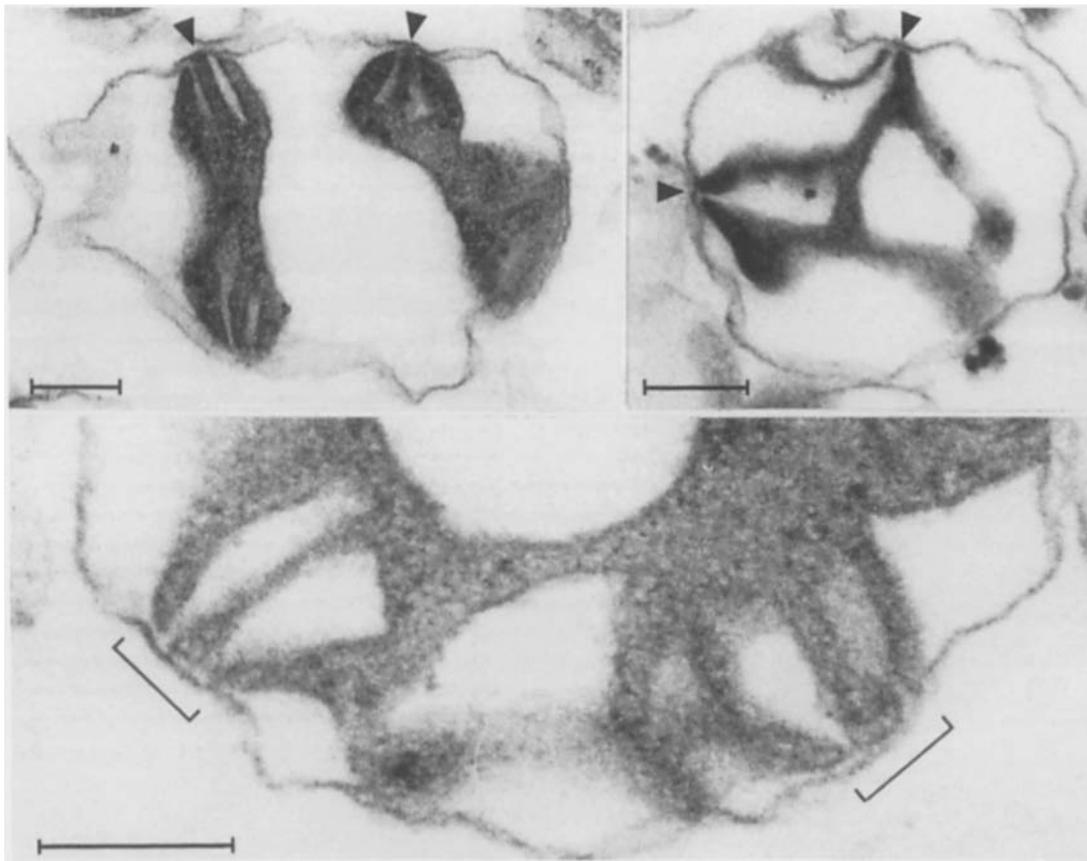
#### **Contact Site Regions in Neurospora Mitochondria**

By protein A-gold labeling of membrane spanning intermediates we found in a previous study (Schwaiger et al., 1987) that the biochemically defined translocation contact sites corresponded to the morphologically observed sites of close contact between outer and inner membranes. In the context of our present results we were interested in analyzing the total area of contact site regions per mitochondrion.

Contact sites were clearly distinguished in electron micrographs of isolated mitochondria which had been exposed to conditions resulting in contraction of the matrix compartment. Analysis of 100 ultra-thin sections of mitochondria with an average diameter of 1.2  $\mu$ m revealed rather short contour lengths of contact sites between outer and inner membranes (Fig. 6). On average, 10 such contacts were detected per mitochondrial section, they were often clustered in



**Figure 5.** Inhibition of import of F<sub>1</sub> $\beta$  and Fe/S-protein by b<sub>2</sub>DHFR translocation intermediate. Mitochondria were incubated for 40 min at 25°C with increasing amounts of radiolabeled pb<sub>2</sub>DHFR in the presence of methotrexate (see legend to Fig. 3). Then 20  $\mu$ l of reticulocyte lysate containing either radiolabeled precursor to F<sub>1</sub> $\beta$  or Fe/S-protein of complex III were added and incubation continued for 20 min. The reactions were cooled to 0°C and treated with 20  $\mu$ g/ml proteinase K. Mitochondria were reisolated and analyzed by SDS gel electrophoresis and fluorography. Protease protected processed F<sub>1</sub> $\beta$  and Fe/S protein was quantified by densitometry. In parallel reactions the corresponding degree of saturation of mitochondria by translocation intermediate was determined after incubation for 40 min at 25°C (see legend to Fig. 4). Control import of F<sub>1</sub> $\beta$  and Fe/S protein measured in the absence of pb<sub>2</sub>DHFR was set to 100%.



**Figure 6.** Morphology of translocation contact sites. Mitochondria were isolated as described in Materials and Methods and resuspended in 0.5 M sucrose, 10 mM MOPS, pH 7.5. Mitochondrial pellets were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in Epon. 50-nm-thin sections were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 102. Contact site regions are indicated with arrowheads (*top*) or parentheses (*below*). For morphometric evaluation of contact sites the micrographs of 100 mitochondrial thin sections were analyzed. The circumference of mitochondrial sections and the contour length of contact sites (where the diameter of both membranes was <20 nm) were determined. For the calculation of the surface area of contact sites mitochondria were assumed to be of spherical form. Bars, 0.2  $\mu\text{m}$ .

groups of three to five. At these sites outer and inner membranes were in close contact over  $\sim 30$  nm. The distance across outer and inner membranes at the contact sites was up to 20 nm. Assuming that the extension of contacts in the third dimension is <50 nm (the thickness of the sections), the number of contact sites per single mitochondrion would be  $\sim 270$ . Occasionally however, contact site regions were observed which extended over 200–600 nm. It has been claimed from freeze-fracture data that contacts between outer and inner membranes can be viewed as extended linear structures (van Venetie and Verkleij, 1982; Knoll and Brdiczka, 1983; Cline et al., 1985; Cremers et al., 1988). However, functional coincidence of contacts observed in classical cross-sections and in freeze-fracture micrographs has never been shown. Considering contact sites as being narrow stripes extending along the origin of cristae, their appearance in cross sections as predominantly spotlike areas could be easily explained. To get a more precise idea of the spatial arrangement of contact sites a detailed morphometric analysis involving three-dimensional reconstructions will be undertaken. Within the limitations of the present study it therefore seemed more reasonable to quantitatively express contact sites as relative surface area. In these terms contact sites occupy  $7.1 \pm 2.4\%$  of the total surface area of the outer membrane, or  $0.34 \mu\text{m}^2$  per single average mitochondrion.

We tested whether the surface area of contact sites was dependent on the amount of  $\text{b}_2\text{DHFR}$  accumulated as membrane spanning intermediates. In these experiments larger amounts of the fusion protein had to be used which were obtained by in vivo expression in *Escherichia coli* of the original construct shown in Fig. 1 cloned into the expression vector pJLA502 (Schauder et al., 1987). The fusion protein was purified as inclusion bodies and dissolved in 8 M urea. Mitochondria were incubated in the presence of methotrexate bound  $\text{pb}_2\text{DHFR}$  sufficient to reach half maximal or complete saturation of translocation contact sites. Compared to controls, no significant difference with respect to the number of membrane contacts and the total area of close contact between the two membranes were observed (data not shown). Neither was uncoupling of mitochondria by antimycin A and oligomycin of any obvious effect on these parameters. We conclude that, at least in the time range of our experiments and with mitochondria isolated from cultures in the logarithmic growth phase, the number of morphologically observed contact sites was independent of the metabolic state of the mitochondria or the amount of membrane spanning intermediates accumulated.

Based on its average protein content of 0.1 pg (Bahr and Zeitler, 1962) a single average mitochondrion was able to accumulate 4,200 molecules of  $\text{pb}_2\text{DHFR}$  spanning contact

sites. Since the three-dimensional structure of dihydrofolate reductase is known (Volz et al., 1982; Matthews et al., 1985) one may compare the area occupied by these intermediates with the total area of close contact between the two membranes. It can be estimated that the DHFR parts of the membrane spanning intermediates occupied roughly 10–20% of the area of contact site regions corresponding to 0.7–1.4% of the total outer membrane surface.

## Discussion

Our results allow several conclusions: (a) There is a limited number of translocation sites for proteins across the mitochondrial membranes indicating a defined number of assemblies of the translocation machinery; (b) These sites can be reversibly blocked by occupying them with a spanning intermediate which is unable to completely traverse the site because its major carboxy-terminal domain is prevented from unfolding; and (c) Common translocation sites are used for the transport of at least three different precursor proteins.

Import of various precursor proteins of matrix, inner membrane, and intermembrane space has been shown to occur via contact sites of the mitochondrial membranes (Schleyer and Neupert, 1985; Schwaiger et al., 1987). In the light of our present findings it seems very likely that all these precursor proteins use a common translocation apparatus. Based on our morphological observations it has to be assumed that at least for *Neurospora* mitochondria contact sites are not dot-like structures but rather form bands probably extending over several hundred nanometers. The determination of the number and the exact three-dimensional arrangement of these contact regions is presently the subject of a more detailed morphometric analysis. The total area of morphologically observed contact sites accounts for ~7% of the total surface of the outer membrane. This was independent of the metabolic state of mitochondria or the amount of translocation intermediate accumulated in contact sites. Both, the morphometric data and the titration analysis with the pb<sub>2</sub>DHFR fusion protein indicate that there were no additional translocation sites formed in response to the precursor. The biochemically defined area of translocation contact sites (corresponding to the area occupied by the translocation intermediates) would be only ~1% of the outer membrane surface, thus potentially leaving enough adjacent room for protein components of the translocation machinery.

Accumulation of a chemically modified fusion protein as translocation intermediate has recently been shown to reduce the import of authentic precursor proteins into yeast mitochondria (Vestweber and Schatz, 1988). However, it was not clear how much of the accumulated intermediate was indeed in contact sites since the conjugated used was neither digested by externally added protease nor could it be chased into a fully imported form. Nevertheless, a number of contact sites was indirectly calculated which is similar to the number of sites directly determined in our titration experiments.

The molecular organization of the apparatus for protein translocation is unknown. We have previously presented evidence that translocation contact site intermediates are present in a hydrophilic membrane environment (Pfanner et al., 1987a). This and the findings described here support the idea that proteinaceous pores or channels exist at contact sites which accomplish the translocation of precursor proteins. The process of protein translocation requires nucleoside

triphosphates (Pfanner and Neupert, 1986; Pfanner et al., 1987b; Hartl et al., 1987b; Eilers et al., 1987) and the electrical component  $\Delta\Psi$  of the membrane potential across the inner membrane (Pfanner and Neupert, 1985). While NTP hydrolysis has been shown to be necessary for “unfolding” of precursor proteins in the cytosol or at the surface of the outer membrane (Chen and Douglas, 1987; Pfanner et al., 1987), the role of  $\Delta\Psi$  is unclear. With respect to the possible mechanism of translocation it is important that ~135 amino acid residues were found sufficient to span contact sites. The distance from outer face of outer membrane to inner face of inner membrane at contact sites was measured on our micrographs to be 18–20 nm. This corresponds roughly to two times the diameter of a typical protein-rich membrane. About 50 or 130 amino acid residues, respectively, would be required to bridge this distance as an extended  $\beta$  sheet or an  $\alpha$  helix. Fusion proteins between amino-terminal parts of the precursor of subunit 9 of F<sub>0</sub>ATPase and DHFR having up to 70 residues between the cleavage site of the processing peptidase and the DHFR moiety were not processed by isolated mitochondria in the presence of methotrexate (Müller, H., and W. Neupert, unpublished data). This would indicate that the critical length of a polypeptide chain to span contact sites must be somewhere between 70 and 135 residues. Although never observed by us morphologically, it has to be noted that a fusion between the two bilayers at very distinct locations at translocation contact sites, which would reduce the distance to be bridged by the polypeptide chain, cannot be ruled out completely. Our data indicate that the region of a stable translocation intermediate spanning contact sites is essentially devoid of tertiary structure. The necessity for cytosolic precursor proteins to assume an “unfolded” conformation may directly reflect mechanistic requirements at the molecular level of the translocation process itself.

So far no mitochondrial component directly involved in protein translocation has been identified. However, based on functional studies of binding and membrane insertion of precursor proteins, we have proposed the existence of at least three classes of proteinaceous surface receptors each of which recognizes subclasses of cytosolic precursors. Insertion of bound precursors into the outer membrane has been suggested to be facilitated by a “general insertion protein” in the outer membrane (Pfaller et al., 1988). It seems possible that these components involved in the initial steps of the import pathway are in close topological arrangement with the translocation apparatus at contact sites. Interestingly, however, the number of translocation sites determined here is ~10–20 times higher than the number of general insertion protein sites measured for the outer membrane protein porin and the ADP/ATP translocator of the inner membrane.

Protein translocation via contact sites and surface proteins involved as “receptors” is also found in chloroplasts (Pain et al., 1988). Based on the endosymbiont hypothesis for the origin of mitochondria and chloroplasts we have proposed that preexisting contact sites might have been adapted or new sites have been established after the endosymbiotic event and upon gene transfer from the endosymbiont to the nucleus of the host cell had taken place (Hartl et al., 1986; Hartl et al., 1987a). Further analysis of translocation contact sites using the pb<sub>2</sub>DHFR translocation intermediate as a “molecular handle” may reveal interesting principles of protein translocation specific to mitochondria and chloroplasts.

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