cis and trans Sites of the TOM Complex of Mitochondria in Unfolding and Initial Translocation of Preproteins*

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The transport of proteins across biological membranes involves the assistance of specific multi-subunit translocases (for reviews, see Refs. 1–7). Many of their subunits have been identified, but the detailed molecular events leading to the transfer of the proteins across the membranes are poorly understood. In some membrane systems, protein transport requires the unfolding of the translocating polypeptide chain. In mitochondria, targeting and translocation of a number of preproteins depends on cytosolic chaperones which keep the preproteins in a loosely folded, import-competent conformation (reviewed in Ref. 8). Some mitochondrial preproteins contain folded domains and must unfold upon their interaction with the translocation machinery. It has been suggested that the mitochondrial Hsp70 (mtHsp70)1 chaperone in conjunction with the translocation machinery of the inner membrane assists this unfolding reaction (9–13). According to one proposal, mtHsp70 participates in this process by actively “pulling” on the membrane-spanning polypeptide chain. A conformational change of mtHsp70 during the association with the preprotein is thought to be transduced into a directional force leading to the unfolding of domains still outside the mitochondrion (14, 15). In another view, mtHsp70 acts as part of a “molecular ratchet” which prevents the retrograde movement of the incoming polypeptide chain (16). Unfolding in this case is essentially due to the spontaneous breathing of the folded domain and is coupled to the reversible movement of the unfolded polypeptide chain across the membranes and trapping in the matrix space by stable binding to mtHsp70.

Using biochemical and genetic techniques, the TOM complex of the mitochondrial outer membrane has been shown to mediate specific recognition, unfolding, insertion, and translocation of preproteins (reviewed in Refs. 17 and 18). This complex contains preprotein receptors providing sites of initial interaction at the mitochondrial surface (e.g. see Refs. 19–21) and membrane-embedded components, which appear to form the translocation pore and facilitate membrane passage (22–24). Studies using intact mitochondria and isolated outer membrane vesicles (OMV) have provided a coarse picture of how preproteins containing N-terminal targeting signals (presequences) are translocated. At the mitochondrial surface, presequences are specifically recognized by the co-operative action of the receptors Tom20/Tom22 which form a presequence recognition site termed cis site (25, 26). At this site, the preprotein is bound mainly through electrostatic interactions. Translocation of the preprotein is initiated by the transfer of its presequence across the outer membrane and binding at the so-called trans site (27). The presequence of a trans site-bound preprotein is closely associated with the membrane-embedded component Tom40 (22) and is exposed to the intermembrane space (27). Preprotein binding at the trans site is accompanied by unfolding of adjacent folded domains, a prerequisite for protein import into mitochondria (9, 28).

Essential features of preprotein translocation across the

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1 The abbreviations used are: mtHsp70, mitochondrial Hsp70 chaperone; OMV, outer membrane vesicles; MPP, matrix processing peptidase; DEPF, dihydrofotolat reductase; MOPS, 4-morpholinepropanesulfonic acid; MTX, methotrexate; CCCP, carbonyl cyanide m-chlorophenylhydrazone; HS, high salt; LS, low salt; PAGE, polyacrylamide gel electrophoresis.
outer membrane, preprotein unfolding, and binding at the trans site are poorly understood. The rates of binding to the trans site and the contribution of mature domains to the interaction have not been analyzed. Further, the physiological relevance of the unfolding activity of the outer membrane is unclear. The function of the trans site as an intermediate stage in preprotein translocation has not been established in intact mitochondria.

Here, we present a biochemical characterization of the interaction of preproteins with the trans site, and we demonstrate the importance of this site as an intermediate state of translocation into intact mitochondria. Further, we present evidence that unfolding can occur at the stage of outer membrane translocation, i.e. before a contact between the preprotein and mtHsp70 chaperone is established. The unfolding event at the outer membrane was found to be rate-limiting for both the interaction of the preprotein with the trans site and for the entire translocation process.

MATERIALS AND METHODS

Biochemical Procedures—The following published procedures were used: growth of Neurospora crassa wild-type strain 74A and purification of mitochondria and mitochondrial OMV (29); treatment of OMV with trypsin, raising anti sera, and purification of immunoglobulin G (IgG; Ref. 30), with the modification that IgGs were concentrated by ultrafiltration in Centriprep tubes (Amicon); transcription and translation reactions in reticulocyte lysate using [35S]methionine (ICN Biochemicals) as radioactive label (31); preparation of both subunits of N. crassa matrix processing peptidase (MPP); and cleavage of the presequence by MPP (27, 32). Co-immunoprecipitation of the TOM complex with bound translocation intermediates was performed as described earlier using antibodies directed against various TOM complex components (33).

Preprotein Synthesis in Reticulocyte Lysates—The following fusion proteins were synthesized in reticulocyte lysate: pCyt c1-DHFR containing the presequence (amino acid residues 1–34) of N. crassa cytochrome c1, in front of mouse dihydrofolate reductase (DHFR; Ref. 34); and pSu9-DHFR containing the first 69 amino acid residues of N. crassa subunit 9 of the mitochondrial F0-ATPase (pSu9) in front of DHFR (35). Construction of a truncated version of pSu9-DHFR termed pSu9 (−7) was described earlier (22). This preprotein contains only 7 amino acid residues after the first 69 residues of pSu9 and was synthesized by run-off translation (22). The preprotein pSu9-DHFRmut has barb mutations in the DHFR domain, resulting in impaired folding (36). Ureidenedatured pSu9-DHFR (termed pSu9-DHFRdan) was prepared by precipitating reticulocyte lysate containing pSu9-DHFR with ammonium sulfate (86% saturated solution). After centrifugation for 15 min at 15,000 × g, the precipitate was dissolved in 10 mM MOPS-KOH, pH 7.2, containing 8 M urea in the same volume as the lysate input.

Binding and Import of Preproteins in Vitro—OMV were suspended in 100 μl of import buffer A (0.2 mg/ml bovine serum albumin, 2.5 mM MgCl2, 10 mM KCl, and 15 mM MOPS-KOH, pH 7.2) in the absence or presence of 1 mM NADPH and 1 mM methotrexate (MTX; Refs. 25 and 27). Import into freshly isolated mitochondria was performed in import buffer B (buffer A supplemented with 220 mM sucrose). To avoid translocation of the presequence across the inner membrane, 20 μM carbonylcyanide m-chlorophenylhydrazone (CCCP) was added to dissipate the membrane potential. OMV or mitochondria were incubated with reticulocyte lysate containing the radiolabeled preproteins for the desired times at various temperatures. Samples were diluted with 700 μl of high salt (HS) or low salt (LS) buffers (10 mM MOPS-KOH, 1 mM EDTA, pH 7.2, and 120 mM or 20 mM KCl, respectively) containing 220 mM sucrose for experiments with mitochondria. For the analysis of binding, OMV or mitochondria were resorbed by centrifugation for 20 min at 125,000 × g or 10 min at 12,000 × g, respectively. To assay for preprotein import into mitochondria, membrane protein K (50 μg/ml) was added and heated after 15 min at 60 °C by 1 ml phenylmethylsulfonyl fluoride. After addition of 1 ml of SEM buffer (220 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) and resorption of the organelles, pellets were subjected to SDS-PAGE (29). Imported proteins were visualized by fluorography and quantitated by phosphorimager analysis (FUJI X BAS 1500).

RESULTS

Conditions were established under which preproteins were bound exclusively to either the cis or trans sites. We employed fusion proteins termed pSu9-DHFR or pCyt c1-DHFR comprised of the presequences of subunit 9 of the F0-ATPase or of cytochrome c1, respectively, and mouse DHFR. The preproteins were bound at 25 °C to purified mitochondrial outer membrane vesicles (OMV) in the presence or absence of MTX and NADPH. Addition of these compounds prevents the unfolding of the DHFR moiety and thus precludes preproteins from associating with the trans site (27). In the presence of MTX/NADPH, the preproteins remained bound exclusively at the surface-exposed cis site in a salt-sensitive fashion. Bound preprotein was completely released from the OMV upon treatment with buffer containing a high concentration of KCl (Fig. 1A). In contrast, when incubated in the absence of MTX/NADPH, the preprotein remained bound to OMV even in buffers of high ionic strength (Fig. 1B). Even after treatment with 600 mM KCl, only 40% of the bound preprotein was released from the OMV (not shown), indicating that ionic interactions play a minor role for the association with the trans site. Binding to both cis and trans sites was largely dependent on the function of the surface receptors which can be degraded by treatment of the OMV with trypsin (25). We conclude from these data that we can distinguish between preprotein binding to the cis and trans sites of the mitochondrial outer membrane on the basis of their differential sensitivity to salt extraction. Binding in the presence or absence of MTX/NADPH combined with a treatment at low or high salt concentrations allows the exclusive occupation of either cis or trans sites.

Using these criteria to discriminate between cis- and trans-site-bound preproteins, we investigated the time courses of preprotein binding to these two sites. At 25 °C, the association of pSu9-DHFR with the cis site occurred within seconds, whereas only slow binding with a half time of 5 min was observed for the trans site (Fig. 2A). Binding to the trans site was significantly slowed down after removal of the surface receptors by trypsin treatment. When preprotein binding to
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Fig. 2. Kinetic analysis of preprotein binding and unfolding at the cis and trans sites. A, pSu9-DHFR was bound in import buffer A to trypsin-treated (+TP) or mock-treated (–TP) OMV in the presence or absence of MTX/NADPH as described in Fig. 1. At the indicated times at 25 °C, aliquots were withdrawn and chilled on ice, and low salt (LS) or high salt (HS) buffer (containing 20 or 120 m moles KCl, respectively) were added. OMV were reisolated and analyzed for preprotein binding as in Fig. 1. The maximal amount of bound preprotein in mock-treated untreated OMV was performed at 0 °C instead of 25 °C, association with the cis site still took place rapidly, but negligible amounts of preprotein were bound to OMV in a salt-resistant fashion (Fig. 2B), i.e. no association with the trans site was detectable under these conditions.

To analyze the time course of unfolding of the DHFR domain in these binding experiments, samples were treated with protease under conditions which leave folded DHFR intact, but completely degrade unfolded DHFR. During incubation at 25 °C in the absence of MTX/NADPH, unfolding occurred at a rate similar to that of trans site binding (cf. Fig. 2, B and C). No unfolding was observed at 0 °C, consistent with the finding that no salt-resistant state was reached under this condition. Thus, stable binding of pSu9-DHFR to the trans site of OMV is accompanied by the simultaneous unfolding of the DHFR domain. Binding to the trans site and/or unfolding of the mature part of the preprotein require incubation at higher temperature.

Can the trans site be reached after binding to the cis site? pSu9-DHFR was pre-bound at the cis site either by incubation in the presence of MTX or at 0 °C, and unbound material was removed by centrifugation. When OMV carrying cis site-bound pSu9-DHFR were incubated at 25 °C in the absence of MTX, a significant fraction of the preprotein was translocated to the trans site as indicated by the resistance to treatment at increased ionic strength (Fig. 2D). No translocation to the trans site was observed when the OMV were left on ice or were incubated in the presence of MTX. These data show that the cis site is used as an intermediate stage during preprotein translocation to the trans site of the outer membrane.

The lack of salt-resistant binding at 0 °C either could be due to the slow unfolding of the mature domain precluding translocation of the presequence across the outer membrane, or could result from a slow association of the preprotein with the trans site. In the first case, the rates of salt-resistant binding are expected to increase, if the preprotein harbors an unfolded DHFR domain. To test this experimentally, the preprotein pSu9-DHFR in the native form or after denaturation in 8 m urea (termed pSu9-DHFR\textsubscript{den}) were incubated with OMV for various times at 15 °C and 0 °C. Then, OMV were treated with high salt buffer, reisolated and analyzed for bound preprotein. pSu9-DHFR became associated with OMV in a salt-resistant samples was set to 100%. B, pSu9-DHFR was bound to OMV in import buffer A at 0 °C or 25 °C for the indicated time periods in the absence or presence of MTX/NADPH. Then, to those samples that had not received MTX/NADPH in the first place, these reagents were added to prevent further unfolding of pSu9-DHFR. After dilution with LS or HS buffers, OMV were reisolated and analyzed for bound preprotein as in Fig. 1. C, pSu9-DHFR was bound to OMV in import buffer A at 0 or 25 °C in the absence or presence of MTX/NADPH for the indicated time periods. Samples were then treated with 90 μg/ml proteinase K for 15 min at 0 °C to analyze the folding state of the DHFR domain. 1 mm phenylmethylsulfonyl fluoride was added, proteins were precipitated by trichloroacetic acid, and the unfolding of the DHFR domain was quantitated from the protease-resistant fraction of DHFR by phosphoimager analysis. D, pSu9-DHFR can be chased from the cis to the trans site. pSu9-DHFR was bound to the cis site of OMV either in the presence of methotrexate/NADPH (MTX) for 5 min at 25 °C (left panel) or in the absence of MTX for 5 min at 0 °C (right panel). The samples were adjusted to 35% sucrose and 20 m moles KCl. To remove unbound pSu9-DHFR OMV were reisolated by flotation through a 300-μl layer of 32% sucrose in import buffer A, overlaid by 1 ml of import buffer A (10 min at 259,000 × g, 2 °C). OMV were harvested and split into three aliquots. One was precipitated with trichloroacetic acid (Total bound) and served as the 100% reference. The remaining two aliquots of the left panel were incubated for 30 min at 25 °C either in the presence (− chase) or absence (+ chase) of MTX. Those of the right panel were incubated for 30 min at either 25 °C (+ chase) or 0 °C (− chase). The samples were adjusted to 100 m moles KCl, and OMV were reisolated and analyzed for bound preprotein as in Fig. 1.
fashion at 15 °C but not at 0 °C (Fig. 3). In comparison, higher amounts of pSu9-DHFRurea were bound at both temperatures, i.e. the rates of salt-resistant binding of pSu9-DHFRurea were considerably higher than those of folded pSu9-DHFR. Thus, temperature-dependent unfolding of the mature domain determines how fast the presequence can be translocated across the outer membrane and can associate with the trans site. Treatment with urea circumvents this temperature dependence, demonstrating that presequence binding at the trans site is not a rate-limiting event.

To investigate whether the mature part of the preprotein contributes to salt-resistant binding at the trans site, we used a truncated preprotein (termed pSu9(17); Ref. 22) consisting of the presequence of Su9 and only seven additional amino acid residues. Its binding under low and high salt conditions was compared with that of pSu9-DHFR. After incubation at 0 or 25 °C and treatment with low salt buffer, a large fraction of the added pSu9(17) was bound to the OMV (Fig. 4A). Following treatment with high salt buffer, only minor amounts of pSu9(17) remained associated with the OMV. This was in contrast to pSu9-DHFR, which bound in a salt-resistant fashion at 25 °C. These data suggest that the mature part of the preprotein contributes to the stable binding at the trans site. A similar conclusion can be drawn from binding studies with pSu9-DHFRmut (carrying mutations in the DHFR domain which impair stable folding; Ref. 36) and pSu9-DHFRurea. Both preproteins associated with OMV in a salt-resistant fashion, even when binding was performed at 0 °C (Fig. 4B). In conclusion, preproteins can reach the salt-resistant binding state without the need for an increased temperature provided translocation is not hindered by a folded mature domain following the presequence. Unfolded segments of the mature portion of the polypeptide chain contribute to stable interaction with the translocation machinery of the outer membrane. Receptors are unlikely to play a major role in this interaction. The low amounts of pSu9-DHFR (Figs. 1B and 2A), pSu9-DHFRmut, and pSu9-DHFRurea (not shown) bound in the absence of receptors were associated in a salt-resistant fashion as was preprotein bound in the presence of receptors.

Do the reactions of presequence translocation and of concomitant unfolding of the mature portion of the preprotein observed with isolated outer membranes reliably reflect the import process in intact mitochondria? We first followed the unfolding of the DHFR domain during the incubation of pSu9-DHFR with isolated mitochondria, and we monitored the sequestration of the presequence cleavage site into the translocation channel. To uncouple translocation across the outer membrane from that across the inner membrane, isolated mitochondria were used whose membrane potential, ΔΨ, was depleted. Upon incubation of these mitochondria with pSu9-DHFR at 25 °C, the preprotein was bound rapidly, but the...
Preprotein unfolding at the mitochondrial outer membrane.

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**A**

**B**

**FIG. 5.** Uncoupled mitochondria can unfold the mature part of preproteins and insert the presequence into the outer membrane. A, isolated mitochondria in import buffer B were treated with CCCP (24 μM), valinomycin (0.5 μM), oligomycin (8 μM), and antimycin A (20 μM) to deplete the membrane potential across the inner membrane. Radiolabeled pSu9-DHFR was added to the mitochondria, and samples were incubated for various times at 25 °C. The mitochondria were washed with SEM buffer containing 40 mM KCl, and reisolated (10 min, 10,000 × g). The pellets were resuspended in import buffer B containing the above concentrations of membrane potential-dissipating reagents. One aliquot was left untreated (Bound), another was treated with proteasome K (100 μg/ml; +PK) for 15 min at 0 °C, while the third aliquot was incubated with α- and β-MPP (20 μg/ml each) and 1.5 mM MnCl2 (+MPP). After precipitation with trichloroacetic acid, all samples were analyzed by SDS-PAGE and fluorography. B, data of part A (bound pSu9-DHFR, proteasome K-resistant DHFR and processed mature Su9-DHFR) were quantitated by phosphoimager analysis and corrected for the loss of radioactive methionines upon presequence cleavage.

Presequence was not cleaved by endogenous MPP, indicating that it had not been transferred across the inner membrane (Fig. 5, left panels). Nevertheless, the mature part of the preprotein became unfolded with a half time of less than 5 min (middle panels). The presequence cleavage site became inaccessible to externally added, purified MPP at virtually the same rate (right panels), indicating insertion of the presequence part of the preprotein into the outer membrane. Neither unfolding nor presequence sequestration was seen at 0 °C (not shown). Taken together, intact mitochondria can translocate the presequence of a preprotein across the outer membrane, and concomitantly the mature part of the preprotein unfolds. Both reactions occur independently of the further transfer of the polypeptide chain across the inner membrane.

We next asked if preprotein bound to the trans site represents a faithful intermediate of translocation into mitochondria. This has been questioned recently (37). The preprotein pSu9-DHFR<sup>trans</sup> was first bound to mitochondria which were uncoupled by the addition of CCCP. After removal of free or cis site-bound material by reisolation of the mitochondria in high salt buffer, the membrane potential, Δψ, was reestablished by the addition of dithiothreitol which quenches CCCP. A second incubation was performed to permit further transport of trans site-bound preprotein into the matrix. A significant fraction of this preprotein acquired protection against proteasome K and its presequence was processed (Fig. 6, lanes 3 and 4) similar to what was observed for the import of preprotein freshly added before the second incubation (lanes 7 and 8). In contrast, hardly any proteasome K-resistant preprotein was detected when Δψ was lacking in both incubations (lanes 1 and 2 and 5 and 6). These data indicate that preprotein transiently arrested at the trans site resumed its journey into the matrix. Thus, the trans binding stage can be considered a productive intermediate of the overall translocation process.

To prove that pSu9-DHFR bound to uncoupled mitochondria in a salt-resistant fashion was in intimate contact to the translocation machinery of the outer membrane, the TOM complex was isolated by immunoprecipitation using antibodies against Tom20, Tom22, and Tom40 and analyzed for bound preprotein. A significant fraction of added pSu9-DHFR was co-immunoprecipitated with the TOM complex after preprotein binding under conditions leading to occupation of the trans site (Fig. 7). When the preprotein was arrested at the cis site, no such stable association with the TOM complex was seen, even when co-immunoprecipitation was performed in the presence of low salt concentrations. Essentially the same was observed with isolated OMV (not shown; 22). These data demonstrate that the preprotein, after inserting its presequence into the outer membrane of uncoupled mitochondria is stably associated at the trans site of the TOM complex, whereas at the cis site, it is bound in a labile fashion. In summary, the results obtained with intact mitochondria are fully consistent with the conclusions drawn from the partial reactions detected with OMV (Ref. 27; see above). Preprotein bound at the trans site represents a functional intermediate in the translocation pathway from the cytosol into the mitochondrial matrix.

Unfolding of the mature domain at the mitochondrial outer membrane is rate-limiting for the initiation of translocation (see above, Fig. 3). To analyze whether unfolding also determines the rate of the overall translocation into the matrix, we compared the time courses of unfolding of pSu9-DHFR upon incubation with uncoupled mitochondria (by the addition of CCCP) and of translocation into the matrix space of energized mitochondria. Virtually identical rates were found for these two reactions, when they were performed at 25 °C in parallel experiments (Fig. 8A). No import was observed with uncoupled mitochondria. The similarity of the rates of unfolding and

**FIG. 6.** Preprotein arrested at the trans site of intact mitochondria can be chased into the matrix. Mitochondria were preincubated with or without radiolabeled pSu9-DHFR<sup>trans</sup> for 5 min at 0 °C in import buffer C (250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS, pH 7.2, 0.5 mM/ml bovine serum albumin) containing 10 μM CCCP to deplete the membrane potential, Δψ. After dilution of the samples with SEM buffer containing 120 mM KCl, mitochondria were reisolated and resuspended in import buffer C containing CCCP as above. One-half of each sample was left without further treatment (−Δψ), and the second half received 10 μM fresh dithiothreitol, 2 μM ATP, and 2 μM NADH (+Δψ). Then, to those samples which had not received radiolabeled pSu9-DHFR<sup>trans</sup> in the first place, this preprotein was added. All samples were incubated for 20 min at 0 °C. Half of the samples were then submitted to treatment with proteasome K while the other half were left on ice. Further analysis of bound and imported preprotein was as in Fig. 5.
translocation suggests unfolding to represent the rate-limiting step for translocation of pSu9-DHFR. This conclusion was supported by a translocation experiment with intact mitochondria and preproteins harboring unfolded DHFR domains. While there was essentially no import of folded pSu9-DHFR at 0 °C, pSu9-DHFR\textsubscript{mut} and pSu9-DHFR\textsubscript{urea} became efficiently translocated into mitochondria under these conditions (Fig. 7B). Apparently, a preprotein can translocate into mitochondria at 0 °C provided its mature domain is unfolded (see also Ref. 38).

In conclusion, unfolding of the mature domain of pSu9-DHFR at the mitochondrial outer membrane is rate-limiting for preprotein translocation. A, mitochondria were preincubated in the presence or absence of 30 μM CCCP in import buffer B containing 1 mM ATP and 3 mM NADH. pSu9-DHFR was added, and samples were transferred to 25 °C. At the indicated times, mitochondria were analyzed after import of preprotein by proteinase K treatment (circles) and for unfolding of the DHFR domain (squares) as in Fig. 5. The maximum amount of import into energized mitochondria and the amount of folded DHFR at 0 min were set to 100%. B, isolated mitochondria and the preproteins pSu9-DHFR, pSu9-DHFR\textsubscript{mut}, and pSu9-DHFR\textsubscript{urea} were incubated at 0 °C or 25 °C in import buffer B in the presence of 1 mM ATP and 3 mM NADH. At the indicated times, imported and processed protein was measured after proteinase K treatment of the mitochondria as in panel A. To ease comparison of the import rates, data were normalized to the amount of import at 0 °C (for pSu9-DHFR\textsubscript{mut} and pSu9-DHFR\textsubscript{urea}) and 25 °C (for pSu9-DHFR) after 30 min. a.u., arbitrary units.

**DISCUSSION**

The present contribution provides a biochemical characterization of the trans site of the TOM complex. This preprotein binding site can be distinguished from the cis site by several criteria. First, when a preprotein is present at the cis site, its presequence can be cleaved off by mitochondrial processing peptidase (MPP) added to the outer face but not to the inner face of OMV. When the preprotein is bound at the trans site, on the other hand, its presequence cannot be cleaved by MPP added to the cytosolic side; rather, it is exposed on the intermembrane space side of the outer membrane and can be processed by MPP enclosed in the lumen of OMV (27). Second, preproteins associated with the trans site are bound in a salt-resistant and stable fashion, whereas preproteins associated with the cis site are rapidly released, especially at higher ionic strength. Presumably, the interaction with the cis site is predominantly of electrostatic nature (25, 26). Third, binding at the cis site occurs mainly via the presequence (25), whereas both the presequence and segments of the mature part of the preprotein are required for stable association with the trans site. Apparently, different modes of interaction are operating at the two preprotein binding sites. Fourth, the presequence is bound at the cis site by Tom20 and Tom22 (22, 25). At the trans site, the presequence has left the vicinity of the former two components and is bound mainly to Tom40, a membrane-embedded component (22). The contact between the presequence and Tom40 is established early in the translocation reaction, when the preprotein is still bound to Tom20-Tom22 at the mitochondrial surface. Therefore, Tom40 appears to play an important role in guiding the presequence into and across the translocation channel. One of the small subunits of the TOM complex, Tom5, also seems to participate in the transfer of the preprotein from the mitochondrial surface into and across the outer membrane (24). In conclusion, the cis and trans sites of the mitochondrial outer membrane translocase represent distinct preprotein binding sites that become occupied in a sequential manner during preprotein transport.

The presequence is essential for the entry of the preprotein into the translocation channel and for association with the trans site (27). Our study shows that parts of the mature domain following the presequence appear to interact with the translocation machinery and contribute to the overall binding stability at the trans site. The binding of this unfolded mature domain following the presequence appears to interact with the translocation machinery and contribute to the overall binding stability at the trans site. The binding of this unfolded mature domain following the presequence appears to interact with the translocation machinery and contribute to the overall binding stability at the trans site. The binding of this unfolded preprotein from the mitochondrial surface into and across the outer membrane represents the rate-limiting step for translocation of pSu9-DHFR. This conclusion was supported by a translocation experiment with intact mitochondria and preproteins harboring unfolded DHFR domains. While there was essentially no import of folded pSu9-DHFR at 0 °C, pSu9-DHFR\textsubscript{mut} and pSu9-DHFR\textsubscript{urea} became efficiently translocated into mitochondria under these conditions (Fig. 7B). Apparently, a preprotein can translocate into mitochondria at 0 °C provided its mature domain is unfolded (see also Ref. 38).

In conclusion, unfolding of the mature domain of pSu9-DHFR at the mitochondrial outer membrane is rate-limiting for preprotein translocation. A, mitochondria were preincubated in the presence or absence of 30 μM CCCP in import buffer B containing 1 mM ATP and 3 mM NADH. pSu9-DHFR was added, and samples were transferred to 25 °C. At the indicated times, mitochondria were analyzed after import of preprotein by proteinase K treatment (circles) and for unfolding of the DHFR domain (squares) as in Fig. 5. The maximum amount of import into energized mitochondria and the amount of folded DHFR at 0 min were set to 100%. B, isolated mitochondria and the preproteins pSu9-DHFR, pSu9-DHFR\textsubscript{mut}, and pSu9-DHFR\textsubscript{urea} were incubated at 0 °C or 25 °C in import buffer B in the presence of 1 mM ATP and 3 mM NADH. At the indicated times, imported and processed protein was measured after proteinase K treatment of the mitochondria as in panel A. To ease comparison of the import rates, data were normalized to the amount of import at 0 °C (for pSu9-DHFR\textsubscript{mut} and pSu9-DHFR\textsubscript{urea}) and 25 °C (for pSu9-DHFR) after 30 min. a.u., arbitrary units.
portion to the TOM complex is reversible. The mature protein was found to be released from the import machinery in a retrograde translocation reaction, when the presence of a trans-site bound preprotein was enzymatically removed by adding MPP at the intermembrane space side of the outer membrane (27). Thus, the combination of reversible contacts of both the presequence and the mature parts are responsible for the high stability of a trans-site bound preprotein. The precise nature of interaction of the preprotein with the trans site remains to be determined. At any rate, the interactions appear to be mediated mainly or even exclusively through the TOM complex since the association of the trans site-bound preprotein was maintained when the outer membrane was lysed in detergent solutions and the TOM complex was isolated by co-immunoprecipitation.

Unfolding of a domain such as DHFR immediately following the presequence is a requirement for translocation of the presequence across the outer membrane. Only when the DHFR is unfolded does the presequence cleavage site become exposed to the lumen of OMV (27). We report here two important characteristics of the unfolding reaction. First, efficient unfolding was seen in intact uncoupled mitochondria. In the absence of a membrane potential, ΔΨ, the presequence cannot cross the inner membrane (39). Therefore, the unfolding observed in intact uncoupled mitochondria occurred at the stage of outer membrane interaction, i.e. before the preprotein made contact with components of the TIM complex, in particular Tim44, and with mtHsp70 chaperone in the matrix (40–43). Thus, at least in the experimental system used in this study, mtHsp70 does not play an essential role in the unfolding of preproteins at the mitochondrial surface (see Ref. 15).

Second, unfolding at the mitochondrial surface proceeded at rates indistinguishable from those of the entire translocation process. This argues for unfolding at the mitochondrial outer membrane being a rate-limiting step for the overall translocation process, at least in the experimental system used. This interpretation must a priori apply to preproteins with presequences so short that they cannot reach the inner membrane or matrix space components without preceding unfolding of the mature domains following the presequence part. In the case of longer presequences or presequences followed by stretches of unfolded mature portions, participation of the translocation machinery of the inner membrane, presumably of Hsp70, has been reported recently (15). Also in these cases, at least partial unfolding may occur at the stage of outer membrane interaction.

In intact energized mitochondria the N-terminal segments of longer presequences are rapidly passed on from the outer membrane to the TIM complex and subsequently to mtHsp70 in the matrix space. Binding of the preprotein to components of the TIM complex (44, 45) and to mtHsp70 prevents the reversible retrograde sliding back out of the translocation channel (16) and, thus, performs a similar function as does the reversible interaction with the trans site. Also from a kinetic point of view, our findings with pSu9-DHFR do not support an active role for mtHsp70 in the unfolding reaction, as unfolding at the stage of outer membrane interaction takes place at rates comparable with those measured for the entire translocation reaction. Any assistance in the unfolding process, however, should accelerate the rate of translocation, an observation we did not make (see Ref. 15). Thus, our data are compatible with the molecular or Brownian ratchet model. In such a minimal model, net movement of the translocating polypeptide chain across the membranes is achieved by successive binding of multiple molecules of mtHsp70, which prevent the reverse movement of the preprotein out of the translocation channel.

Taken together, our data with pSu9-DHFR suggest that mtHsp70 is not required to actively facilitate unfolding but rather may act passively by trapping those segments of the polypeptide chain that are newly imported into the matrix space.

There is no external energy requirement such as ATP hydrolysis for preprotein binding to the trans site and for the concomitant unfolding reaction (27). Since unfolding of the mature domain of pSu9-DHFR is a prerequisite for stable preprotein binding at the trans site, unfolding must occur in a spontaneous fashion. N-terminal segments of the preprotein may then slide into the translocation channel and associate with the trans site. The energy gained from interactions at the trans site seems to be sufficient to compensate for the loss of free energy of folding, thus leading to a shift in the equilibrium toward net unfolding. In this respect, the function of the trans site can be compared with that of a molecular chaperone; binding to spontaneously unfolded polypeptide segments shifts the equilibrium of folded and unfolded states to the latter. Notably, binding to the TOM complex was previously found to prevent aggregation of a preprotein in transit, reflecting a chaperone-like activity (27). This property of the TOM complex may be mechanistically useful for keeping unfolded preproteins in an import-competent conformation and may be important also for other preprotein translocases. Further movement of the preprotein from the trans site to the inner membrane is likely to be mediated by interaction with Tim23 (46). The requirement for ΔΨ at this stage may represent the energy input for relieving the tight interaction with the trans site.

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