Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis

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Mitochondrial heat-shock protein hsp60 functions in the folding of proteins imported into mitochondria. Folding occurs at the surface of hsp60 in an ATP-mediated reaction, followed by release of the bound polypeptides. We propose that hsp60 catalyses protein folding.

Newly synthesized cytosolic precursors of mitochondrial proteins have to maintain an unfolded conformation to be competent for membrane translocation. This is probably achieved by interaction with heat-shock proteins of relative molecular mass 70,000 (70K) in the cytosol and by the action of other factors which might include ATP-dependent 'unfolding enzymes'. Precursors traverse the mitochondrial membranes in an extended conformation at translocation contact sites between the outer and inner membranes. Amino-terminal presequences are cleaved by a matrix-localized, metal-dependent processing enzyme. Proteins remaining in the matrix compartment then have to refold, and in many cases to assemble into supramolecular complexes. The precursors of a number of proteins of the intermembrane space are re-exported across the inner membrane, and follow an evolutionarily conserved assembly pathway of procaryotic origin. These proteins probably have to remain in a more loosely folded conformation in the matrix before the second membrane-translocation event.

Little is known about how proteins fold inside cells. The mechanisms underlying the folding and assembly of proteins imported into the mitochondria are now a focus of interest. The recently identified stress protein, hsp60, is the first mitochondrial component known to be essential in these processes, which were previously assumed to occur spontaneously. Hsp60 is a nuclear-coded, constitutively expressed heat-shock protein residing in the mitochondrial matrix as an oligomer of 14 subunits. The hsp60 equivalent has been detected in mitochondria from several sources, including those of human cell lines. Together with the structurally related Escherichia coli heat-shock protein groEL and the α-component of the Rubisco subunit-binding protein, hsp60 belongs to a subclass of molecular 'chaperones' termed 'chaperonins', which are components assisting in oligomeric protein assembly by an unknown mechanism. The consequences of loss of hsp60 function have been analysed in a temperature-sensitive lethal yeast mutant defective in the gene coding for hsp60. Mutant cells are deficient in the assembly of several mitochondrial proteins of the matrix, inner membrane and intermembrane space. For example, the precursor of the β-subunit of F₁-F₄-ATPase is imported into the mutant mitochondria, but fails to assemble into the F₁-F₄-ATPase. Proteins of the intermembrane space, such as cytochrome b₅, which after import into the matrix have to be re-translocated across the inner membrane, do not reach their target compartment.

Using mitochondria as a model 'cell', we investigated whether the folding of imported proteins per se and not only the assembly of subunits into supramolecular complexes could be mediated by proteins such as hsp60. Here we describe details of the molecular folding of proteins transported into the mitochondrial matrix. At low temperatures, after depletion of mitochondrial ATP, or after treatment of mitochondria with N-ethylmaleimide, the precursor of a mitochondrial fusion protein is translocated across the mitochondrial membranes but does not assume a folded conformation. Intermediates arrested in the unfolded state are associated with hsp60 in a high-molecular weight complex. At least partial folding occurs in an ATP-dependent reaction at the surface of hsp60 before release. Essentially the same pathway is followed by authentic mitochondrial proteins. We conclude that (re)folding of proteins after translocation across the mitochondrial membranes requires a proteinaceous machinery in the matrix: hsp60 is identified as an essential component of this machinery which appears to fulfill the function of an ATP-dependent 'folding catalyst'.

Folding intermediates

To analyse the pathway of (re)folding of newly imported proteins, we used a mitochondrial precursor protein whose state of folding could be monitored by virtue of its resistance to protease. We made use of the fact that the cytosolic enzyme dihydrofolate reductase (DHFR) retains its enzymatically active conformation and folds as an independent, highly protease-resistant unit when fused to the pre-sequence of a mitochondrial precursor. The fusion protein presu9-DHFR proved to be a suitable construct: it consists of amino-acid residues 1–69 of the precursor of Neurospora crassa DHFR subunit 9, joined to the amino-terminus of the complete mouse DHFR by three linker residues. The 66-amino acid presequence is cleaved in two steps by the mitochondrial processing peptidase. Unfolding of the DHFR moiety is the rate-limiting step for the transport of similar fusion proteins across the mitochondrial membranes. To achieve rapid membrane translocation, radiolabelled presu9-DHFR was precipitated from a reticulocyte lysate with ammonium sulphate, and the precipitate dissolved in 8M urea. After dilution in the import incubation, this precursor is rapidly translocated into mitochondria from N. crassa in a membrane-potential-dependent manner. Implanted precursor was processed to the mature form, which corresponds to the complete DHFR carrying six additional residues at the amino-terminus. To determine the kinetics of refolding, import reactions were terminated after a short incubation by addition of uncoupler with immediate cooling. Precursor associated with the surface of mitochondria was removed by treatment with proteinase K. The mitochondria were then permeabilized by digitonin and the state of folding of the imported protein assayed by measuring the protease-resistance of its DHFR component. About 90% of the presu9-DHFR contained in the reaction is translocated into mitochondria within 45 s at 25°C (Fig. 1a), but only 30% of the imported protein reaches the stably folded, protease-resistant conformation. After three minutes, 70% of the imported protein has folded. Lowering the temperature considerably slows down the refolding and proteolytic processing of imported Su9-DHFR,
although the efficiency of membrane translocation is unchanged (Fig. 1b).

In these experiments, the organelles were energized for import by the addition of NADH, which in mitochondria of N. crassa and yeast is directly channelled into the respiratory chain, resulting in the production of ATP. Does the folding of imported proteins require energy in the form of ATP? We incubated isolated mitochondria in the presence of apyrase, an ATP- and ADP-hydrolysing enzyme from potato. Under these conditions, the urea-denatured precursor is readily translocated into mitochondria. The DHFR component of the imported protein, however, remains sensitive towards digestion with protease, indicating that the refolding reaction is largely inhibited (Fig. 1c). Results were similar when import was performed in the presence of the non-hydrolysable ATP analogues, AMP-PNP and AMP-PCP (AMP-PNP = adenosine-5'-β,y-imido-triphosphate AMP-PCP = adenosine-5'-β,y-methylene-triphosphate) (Fig. 1d). The non-hydrolysable ATP analogues compete with endogenous ATP present in the energized mitochondria. (In contrast to the previous view, membrane translocate of the precursor is not obligatorily coupled with its immediate refolding inside the mitochondria.) Folding of the imported protein in the matrix is mediated by an ATP-dependent reaction.

Association with hsp60

Are the folding intermediates associated with a matrix component(s) during the folding reaction? PreSu9–DHFR was imported into mitochondria and digitoxin extracts containing 70–90% of the soluble matrix marker fumarase and corresponding amounts of the imported protein were analysed on Sephacryl S-300 sizing columns. Matrix extracts of mitochondria incubated for import for 5 min at 25°C in the presence of ATP contain the mature-sized fusion protein, which fractionates with a molecular weight corresponding to the monomer (Fig. 2a; i): the DHFR part of the monomeric Su9–DHFR has reached its protease-resistant conformation. About 10% of the protein in the extract migrates as a high-molecular-weight complex of ~700K. This material represents unfolded, or at least incompletely folded, fusion protein which is sensitive to protease. In extracts of ATP-depleted mitochondria, up to 60% of the protein is recovered in these high-molecular weight fractions (Fig. 2a; ii). This material is highly sensitive to protease (Fig. 2c). No more than 1% of the radiolabel contained in Su9–DHFR is detectable on 17.5% polyacrylamide gels as distinct proteolytic fragments. An antibody directed against denatured DHFR precipitated the protease-sensitive Su9–DHFR as efficiently as the SDS-denatured fusion protein, but did not recognize the folded monomeric form (not shown). Unfolded Su9–DHFR accumulated in the presence of AMP-PNP was also recovered in the high-molecular weight column fractions (Fig. 2a; iii).

It seems that the folding intermediates are probably associated with a complex which participates in the ATP-dependent folding reaction. The digitoxin extracts analysed on Sephacryl columns contained ~ 80% of the total hsp60 present in mitochondria. This hsp60 behaves as a high-molecular weight complex which cofractionates with the incompletely folded intermediates of imported Su9–DHFR (Fig. 2). By contrast with the cofractionating Su9–DHFR, the hsp60 scaffold itself is largely resistant to digestion by protease, although a small fragment is cleaved by protease K from either the N-or C-terminus of the hsp60 subunits (Fig. 2a; iv). This does not result in the dissociation of the hsp60 oligomer, which we have demonstrated by re-chromatography of the protease-treated column fractions (not shown).

The mitochondrial proteins such as the Rieske iron sulphur (Fe/S) protein of complex III and the β-subunit of F$_i$-ATPase (F$_i$β) also form a high-molecular weight complex in the matrix. Fe/S protein imported into apyrase-treated mitochondria is readily extractable with digitoxin (Fig. 2b), unlike the protein that is assembled into complex III when imported in the presence of ATP. The Fe/S protein contained in the extracts cofractionates exactly with hsp60. Essentially the same result is obtained when F$_i$β is imported: the precursor

**FIG. 1 Import into isolated mitochondria and refolding of Su9–DHFR.** Isolated mitochondria were incubated under various conditions in the presence of preSu9–DHFR. Import (arbitrary units) at: a, 25°C; b, 10°C; c, 25°C into mitochondria treated with apyrase; d, 25°C in the presence of 5 mM AMP-PNP. Upper panels, fluorographs of SDS-polyacrylamide gels of ditigomethionylized mitochondria previously incubated in the presence (+ PK) or in the absence of protease K (− PK). Lower panels: a, intermediate; m, mature-sized form of Su9–DHFR. Lower panels: a, 5 min of total imported and protease-resistant Su9–DHFR, as determined by densitometry of the fluorographs.

**METHODS.** Pre-Su9–DHFR was synthesized in a reticulocyte lysate in the presence of [35S]methionine by transcription/translation of the complementary DNA cloned into expression vector pSP65 (refs 31, 47 and 48). Precursor was precipitated with ammonium sulphate at 66% saturation (30 min at 0°C) and the precipitate was dissolved in 8M urea/10 mM Tris, pH 7.5. Denatured precursor was diluted 20–40-fold into import reactions containing 3% BSA, 70 mM KCl, 2.5 mM MgCl$_2$, 2 mM Na$_2$HPO$_4$, 10 mM MOPS (3-N-morpholinopropanesulfonic acid), pH 7.2, and 0.8 mM isolated mitochondria of N. crassa, which were essentially free of cytochrome and endoplasmic reticulum markers.$^{15,19}$ Before addition of pre-Su9–DHFR and NADH, reaction c was incubated for 15 min with apyrase (40 U per ml final concentration; Sigma grade VIII$^{35}$) and reaction d was incubated with 5 mM AMP-PNP. After incubation at 25°C (a, c and d) or 10°C (b) for the times indicated, import reactions were cooled on ice and diluted 5-fold with ice-cold SPS (300 mM sucrose, 10 mM HEPES pH 7.2, 1 mM EDTA, 8 μM antimycin A and 20 μM oligomycin), then treated with 25 μg ml$^{-1}$ proteinase K for 10 min at 0°C. After addition of 1 mM PMSF (phenylmethylsulphonyl fluoride), mitochondria were re-isolated by centrifugation$^{19}$ and resuspended at 0.5 mg ml$^{-1}$ in 0.3% digitoxin in 10mM KCl. Half of each reaction was treated with 10 μg ml$^{-1}$ proteinase K for 10 min at 0°C. PMSF was added and trichloroacetic acid precipitates analysed by SDS-polyacrylamide gel electrophoresis SDS-PAGE; fluorography and densitometry.$^{15,19,49}$

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FIG. 2 Gel chromatography of folding intermediates. a, Analysis by Sephacryl S-300 chromatography of digigtonin extracts prepared from mitochondria which had been incubated for import of preSu9-DHFR (i) in the presence of ATP; (ii) after depletion of ATP with apyrase; (iii) in the presence of AMP-PNP (iv) Nito cellulose blot of column fractions decorated with anti-hsp60 antisem. Fractions of panel (ii) are shown representatively. Column fractions were analysed for total content in Su9-DHFR (+PK) and in protease-resistant Su9-DHFR (+PK). b, Gel chromatography of a digigtonin extract from apyrase-treated mitochondria which had imported the precursor of Fe/S protein or of F,β, respectively. Imported protein and hsp60 are shown as per cent of total protein in digigtonin extracts.

METHODS. Import of urea-denatured precursor proteins into isolated mitochondria and protease-treatment of mitochondria are described in Fig. 1. Incubation for import was for 5 min at 25 °C. Matrix was extracted by incubating reisolated mitochondria for 1 min at 0 °C in SEM containing 0.3% digitonin. The concentration of mitochondrial protein was 5 mg ml⁻¹ (ref. 18). After 5-fold dilution with SEM/100 mM KCl, reactions were centifuged for 10 min at 20,000g. Samples of pellets and supernatants were analysed for imported protein, hsp60 and the matrix marker enzyme, fumarase, 32. Supernatants were fractionated on 2.5 ml Sephacryl S-300 columns equilibrated with 100 mM NaCl, 10 mM Tris, pH 7.5. The void volume of the column was discarded and 200μl fractions collected. The peak concentration of the 700K thyroglobulin marker was in fraction 3. Half of each fraction was treated with 10 μg ml⁻¹ proteinase K for 10 min at 0 °C. Trichloroacetic acid precipitates were analysed by SDS-PAGE and fluorography, as well as by immunoblotting with anti-hsp60 antisem. Bound antibodies were detected by alkaline phosphatase-coupled to IgG directed against rabbit IgG (α-iv) or by 14C-labelled protein A (α-v). Fluorographs were quantified by densitometry.

FIG. 3 Physical association of imported proteins with hsp60. a, Co-immunoprecipitation of Fe/S protein imported into apyrase-treated mitochondria with anti-hsp60 immunoglobulins. The amount of Fe/S protein (p-Fe/S plus m-Fe/S; compare Fig. 4b) precipitated from digigtonin extracts is given as per cent of total Fe/S protein imported into mitochondria. The amounts of pre-immune and immune IgG indicated were preabsorbed to protein A-Sepharose. b, Non-denaturing polyacrylamide gel electrophoresis of Su9-DHFR-hsp60 complex partially purified by gel chromatography (corresponding to fractions 1–3 in Fig. 2a, ii). In addition, the monomeric fusion protein (fraction 7 in Fig. 2) was analysed. i–iii, Fluorograph, Coomassie blue stained and immunoblotted with anti-hsp60 antisem, respectively. The position of a purified hsp60 standard (Std) on the gel is indicated.

METHODS. a, Fe/S protein was imported into apyrase-treated mitochondria. Digigtonin extracts were prepared (see legend to Fig. 2). Aliquots of the extracts corresponding to 25 μg intact mitochondria were added to protein A-Sepharose (PAS) pellets to which 25, 50, 75 and 100 μg preimmune- and hsp60 immunoglobulin had been preabsorbed, respectively 33. Reactions were incubated for 30 min at 4 °C by rotating end-over-end in the presence of 1 μM PMSF and 30 μg ml⁻¹ protease inhibitor from N. crassa 33. PAS beads were pelleted and washed twice with SEM/KCl buffer and once with 30 mM Tris, pH 7.4. Wash solutions also contained protease inhibitors. PAS-immunoglobulin-antigen complexes were dissociated in SD5-containing buffer 15 and analysed by SDS-PAGE, fluorography and densitometry. The total amount of imported Fe/S protein contained in the extract was determined by immunoprecipitation with saturating amounts of anti-Fe/S immunoglobulin. b, Digigtonin extracts of apyrase-treated mitochondria which had imported preSu9-DHFR were fractionated by gel chromatography. 50 μl aliquots of fractions 1–3 and of fraction 7 were eluted twice on the same 4–20% non-denaturing polyacrylamide gel 35. Isolated hsp60 (1 μg) was analysed as standard. After electrophoresis, one half of the gel was blotted onto nitrocellulose and decorated with anti-hsp60 antisem; the other half was stained with Coomassie blue and analysed by fluorography.
form accumulates in the apyrase-treated mitochondria and is
detected as high-molecular weight complex (Fig. 2b).

We then demonstrated that the proteins imported at low levels
of ATP are physically associated with hsp60. Fe/S protein was
imported into apyrase-treated mitochondria, which were then
extracted with digitonin. Monospecific immunoglobulins directed
toward hsp60 could co-immunoprecipitate up to 45% of the
total Fe/S protein contained in the extracts (Fig. 3a). Compar-
able results were obtained with coprecipitation of Su9-DHFR
(not shown), demonstrating that the column cofractionation of
the imported proteins with hsp60 reflects a stable interaction
between these components. Analysis of the high-molecular
weight column fractions containing Su9-DHFR by non-
denaturing gel electrophoresis also indicated such an associ-
ation. The protease-sensitive Su9-DHFR comigrated exactly
with the hsp60 complex (Fig. 3b); between 50–70% of Su9-
DHFR and hsp60 contained in the column fractions was
recovered on the non-denaturing gel. The monomeric folded
fusion protein was not seen as a distinct band.

Together our results indicate that proteins imported into the
mitochondrial matrix transiently associate as incompletely folded
intermediates in a high-molecular weight assembly com-
plex before attaining their final, folded structure. Hsp60 is
identified as a major constituent of this complex.

**ATP-dependent folding and release**

We investigated the ATP-requiring step in the sequence of
reactions involved in folding imported proteins by adding back
ATP to the hsp60-bound folding intermediates to see if they
could be chased into the folded species. PreSu9-DHFR was
imported into ATP-depleted mitochondria and matrix extracts
were incubated with or without Mg2+ATP. At low levels of ATP,
about 60% of the imported fusion protein contained in the
matrix extract remains protease-sensitive and cofractionates
with hsp60 (Fig. 4a). In contrast, addition of ATP causes folding
into the protease-resistant conformation and the release of Su9-
DHFR from hsp60. The mature-sized protein fractionates as the
monomer (Fig. 4a). The fractionation of hsp60 is unchanged.
Apparently, the folding intermediates bound to hsp60 in the
absence of ATP are productive intermediates on the authentic
import pathway.

We also demonstrated this for the Fe/S protein associated
with hsp60 in apyrase-treated mitochondria (Fig. 4b). Readdi-
tion of ATP to the intact mitochondria causes the release of

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**FIG. 4** Folding of imported proteins and their release from hsp60. a. ATP-
dependent folding and release of imported Su9-DHFR. Before gel
chromatography, digitonin extracts of mitochondria were incubated for
15 min at 25 °C in the absence (−ATP) or presence of 5 mM Mg2+ ATP
(+ATP). Half of each column fraction was treated with protease K (+PK).
The amount of total Su9-DHFR and of protease-resistant Su9-DHFR is given
in per cent of total protein in digitonin extracts. b. ATP-dependent release
of imported Fe/S protein from hsp60. After import into apyrase-treated
mitochondria, the re-isolated organelles were incubated for 15 min at 25 °C
in the absence (left panel) or presence of 5 mM Mg2+ ATP (right panel).
Digitonin extracts were fractionated by gel chromatography. c. ATP-depen-
dence of folding of Su9-DHFR. (i) Su-DHFR-hsp60 complex partially purified
by gel chromatography (pooled fractions 2–4, see Fig. 2), was analysed
after incubation for 15 min at 25 °C in the absence or presence of 5 mM
NTPs as indicated. The amount of protease-resistant Su9-DHFR produced
is given in per cent of total Su9-DHFR analysed. (ii) Re-chromatography of
Su9-DHFR-hsp60 complex after incubation in the absence or presence of
ATP. Fractionation of hsp60 in the + ATP reaction is shown representatively.
Amounts are given as per cent of total protein analysed; (iii) Protease-
resistance of Su9-DHFR contained in the complex with hsp60 after incubation
with or without ATP, and of the monomer Su9-DHFR obtained in a. Protein
resistant to protease K at the concentrations indicated is shown as per
cent of total protein. Equal amounts of labelled Su9-DHFR were analysed.
METHODS. Import of precursor proteins into isolated mitochondria, prepa-
ration of digitonin extracts, gel chromatography and analysis of column
fractions are described in the legend to Fig. 2. c. Column fractions 2–4 (see
Fig. 2; ii) containing protease-sensitive Su9-DHFR exclusively, were pooled
and divided into five 120 μl aliquots. Before incubation for 15 min at 25 °C,
these reactions were made 5 mM in Na2ATP, Mg2+ATP, Mg2+GTP or
Mg2+AMP-PNP using 100-fold-concentrated stock solutions in water
adjusted to pH 7. Half of each reaction was treated with 10 μg ml−1 pro-
tease K for 10 min at 0 °C. Treatment with protease K for titration of
protease resistance was under the same conditions. Trichloroacetic acid
precipitates were analysed by SDS-PAGE, fluorography and densitometry.
60% of the hsp60-bound protein. Precursor is processed to m-Fe/S. Half of the released m-Fe/S is no longer extractable by digitonin and is recovered in the membrane fraction; probably this membrane-associated m-Fe/S is assembled into complex III[9]. The digitonin-extractable portion of the m-Fe/S that has been released from hsp60 migrates with a molecular weight of ~70K, which is higher than that expected for the monomeric Fe/S protein (25K). This m-Fe/S could represent protein not yet tightly assembled into complex III, or another matrix-localized intermediate on the assembly pathway. In contrast to the observations involving Su9-DHFR, ATP-dependent release of the Fe/S protein from hsp60 can only be demonstrated with intact mitochondria, and not with the matrix extracts containing the Fe/S-hsp60 complex. As the Fe/S protein has to be exported across the inner membrane, the inner membrane itself or a membrane-associated factor(s) could be required for its release from hsp60.

We investigated the nucleoside triphosphate specificity of the folding reaction using the Su9-DHFR-hsp60 complex from ATP-depleted mitochondria after its partial purification by gel chromatography. Aliquots were incubated for 15 min at 25°C with and without NTPs (Fig. 4c; i). Addition of Mg^{2+}ATP was most effective in promoting folding of the DHFR moiety, when about 60% of the total fusion protein remained resistant to proteinase K at 10 μg ml\(^{-1}\). GTP and AMP-PNP were ineffective in promoting folding. In contrast to our results using total matrix extracts (Fig. 4a), the protease-resistant protein still cofractionated with the hsp60 complex (Fig. 4c; ii) and could be co-immunoprecipitated by anti-hsp60 antibodies as efficiently as the unfolded fusion protein present in the absence of ATP (not shown). Titration with increasing concentrations of proteinase K revealed that this Su9-DHFR is less resistant to digestion than the monomeric fusion protein (Fig. 4c; iii). Several protease-resistant fragments accounting for ~10% of the radiolabel contained in Su9-DHFR were detected on SDS-polyaerylamide gels near the gel front (not shown). This indicates that ATP-mediated folding of the DHFR moiety is occurring at the surface of the hsp60 complex. It is possible that an hsp60 function necessary for release of the associated polypeptides is inactivated during gel chromatography or, more likely, that an additional component(s) of the mitochondrial matrix which does not cofractionate with hsp60 is necessary for the complete sequence of reactions leading to the folded monomeric protein.

**NEM-sensitivity of folding**

Is the folding of Su9-DHFR under physiological import conditions, that is, in the presence of intramitochondrial ATP, also dependent on protein factors? Using the membrane-permeant alkylating agent, N-ethylmaleimide (NEM), we found that modification of mitochondria with 1-5 mM NEM before import in the presence of ATP inhibited the refolding of imported Su9-DHFR into a protease-resistant conformation (Fig. 5a). Translocation of the urea-denatured precursor into the mitochondrial matrix and proteolytic processing were unaffected. NEM treatment does not cause depletion of ATP in the matrix compartment (see legend to Fig. 5). Interestingly, after short import incubation (2-5 min), Su9-DHFR can be extracted by digitonin and is associated with hsp60 (Fig. 5b), whereas in control reactions the protein is folded and exists as the monomer (Fig. 2a). But after incubation of the NEM-treated mitochondria for 15 min, most of the imported protein is no longer extractable and is recovered in the membrane fraction. This Su9-DHFR was still very sensitive to protease. Extractability of hsp60 was unchanged, indicating that Su9-DHFR had fallen off the hsp60 complex and was forming incompletely folded aggregates. After combined treatment with NEM and apyrase, readdition of ATP causes a shift of the protease-sensitive Su9-DHFR from the extractable complex with hsp60 into the membrane fraction (not shown). The aggregated fusion protein is slightly more protease-resistant than the polypeptides bound to hsp60 in the absence of ATP. Formation of insoluble aggregates of imported proteins, including a defect in the refolding of Su9-DHFR, was also observed in mitochondria of the hsp60-deficient yeast mutant, mff4 (not shown).

We conclude that folding of the imported protein must be protein 'catalysed': folding at the surface of hsp60 and ATP-dependent release are two essential but separable steps necessary to achieve the final conformation. It remains to be tested whether hsp60 itself is the target of the NEM-effect. We have detected a reaction of hsp60 with 14C-labelled NEM under conditions which inhibit folding (not shown).

**Discussion**

We have described here the surprising discovery that proteins imported from the cytosol into mitochondria do not refold spontaneously once translocation across the mitochondrial membranes is complete. The stress protein hsp60 residing in the mitochondrial matrix has been identified as a major component of an NEM-sensitive device involved in the folding of imported proteins.
proteins, which acts in conjunction with ATP. In the absence of ATP, newly imported proteins form a soluble complex with hsp60. Newly imported protein is associated in a loosely folded conformation, which is very sensitive to protease, with the surface of the hsp60 scaffold. ATP hydrolysis allows folding and release from hsp60, but our present data do not prove that this ATP effect(s) is exerted by hsp60 itself. On the other hand, ATP-dependent folding of the associated polypeptide occurs at the surface of hsp60 before release. It remains to be determined whether the release of the partially folded polypeptide from hsp60 has a direct ATP requirement, or whether it is merely a consequence of the preceeding ATP-dependent folding. One or more additional component(s) might be involved. Mitochondria could contain an equivalent to the groES protein, which in Е.coli seems to cooperate with groEL in various assembly functions by an unknown mechanism. Does hsp60 fulfill its function? A parallel study has shown that proteins traverse the mitochondrial membranes in an extended conformation (unpublished results). We propose that polypeptides entering the mitochondrial matrix have a conformation similar to nascent chains emerging from the ribosome, and that it is these unfolded polypeptides that interact with hsp60. This model predicts that the ATP-dependent folding and translocation. Our results with the DHFR-fusion protein indicate that hsp60 is not specific for the structural motifs present in only mitochondrial proteins. Notably, urea-denatured DHFR, when part of a fusion protein, can refold spontaneously in appropriate buffer solutions, but this does not happen after translocation across the mitochondrial membranes. The mitochondrial matrix is effectively a highly concentrated protein solution, which does not correspond to conditions frequently used in refolding experiments in vitro. It may be an essential function of hsp60 to capture the unfolded polypeptides entering the matrix and so prevent the formation of misfolded proteins. Cytosolic precursors of mitochondrial and secretory proteins interacting with 70K heat-shock proteins have been proposed to bind through hydrophobic regions exposed in the newly synthesized, incompletely folded polypeptides. The complex formed between hsp60 and imported polypeptides is only slightly destabilized by non-ionic detergents such as Triton X-100 (results not shown); this could indicate that here the interaction is not predominantly hydrophobic in nature.

The role of ATP in hsp60-mediated folding and release of the polypeptide is unclear. Although the chaperonins, including the mitochondrial hsp60, are structurally unrelated to the 70K heat-shock proteins, both groups of components exhibit weak ATPase activity. It has been suggested that ATP hydrolysis causes a conformational change in 70K heat-shock proteins, which is transferred to the bound polypeptide. A soluble factor seems to participate in these reactions. So far, the functions of the 70K proteins have been evident in stabilization of the translocation-competent conformation of cytosolic precursor proteins, or the disruption of protein aggregates. In contrast, the mitochondrial hsp60 is essential for protein folding. ATP hydrolysis by hsp60 or by another component could loosen the association of the unfolded polypeptides with the hsp60 scaffold, and thus allow for their ordered, domain-wise folding at the surface of hsp60. We describe this type of reaction as 'protein-catalysed protein folding'; it may be slower than the 'spontaneous folding' that occurs in the mitochondrion in the absence of the functional folding 'catalyst' resulting in misfolded protein aggregates. Hsp60 and the homologous groEL protein of Е.coli may have similar functions in folding nascent polypeptide chains synthesized in the mitochondrial matrix or in the bacterial cytosol. It is possible that protein folding in the eukaryotic or cyanobacterial membranes or some compartments such as the endoplasmic reticulum, could also be protein-mediated.

The chloroplast Rubisco-binding protein and hsp60 were originally defined as components assisting in oligomeric protein assembly. In the light of our results, these chaperoning reactions probably represent only a part of their function. The actual assembly reactions could occur spontaneously after folding of the subunits and their release from hsp60. Alternatively, complementary surfaces of subunits might be exposed only when the partially folded polypeptides are still in an assembly complex with hsp60. Likewise proteins such as the Rieskis Fe/S protein or cytochrome b6, which have to be re-exported from the matrix to the intermembrane space, could be shuttled to their respective protein export centres while associated with hsp60. A function for groEL in the stabilization of proteins for export across the bacterial plasma membrane has been suggested. Other factors in the mitochondrial matrix, such as the recently described 70K protein SSC1, could also be involved in keeping proteins in a translocation-competent conformation.

Our findings concerning the function of hsp60 should have application in the renaturation by folding catalysts of proteins obtained by overexpression in bacteria, raising interesting possibilities for biotechnologists in the future.