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[1,2]. 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'[3-9]. Precursors traverse the mitochondrial membranes in an extended conformation at translocation contact sites between the outer and inner membranes[10-14]. Amino-terminal presequences are cleaved by a matrix-localized, metal-dependent processing enzyme[15-17]. 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[18,19]. 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[20,21], which were previously assumed to occur spontaneously[22]. Hsp60 is a nuclear-coded, constitutively expressed heat-shock protein residing in the mitochondrial matrix as an oligomer of 14 subunits[23,24]. The hsp60 equivalent has been detected in mitochondria from several sources, including those of human cell lines[25-27]. Together with the structurally related *Eschericia 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[25,28,29]. The consequences of loss of hsp60 function have been analysed in a temperature-sensitive lethal yeast mutant defective in the gene coding for hsp60[20]. 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 F_1-ATPase is imported into the mutant mitochondria, but fails to assemble into the F_oF_1-ATPase. Proteins of the intermembrane space, such as cytochrome b_2, 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[13,30]. The fusion protein preSu9-DHFR proved to be a suitable construct: it consists of amino-acid residues 1-69 of the precursor of *Neurospora* F_o-ATPase subunit 9, joined to the amino-terminus of the complete mouse DHFR by three linker residues[31]. The 66-amino acid presequence is cleaved in two steps by the mitochondrial processing peptidase[32,33]. Unfolding of the DHFR moiety is the rate-limiting step for the transport of similar fusion proteins across the mitochondrial membranes[13,34]. 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. Imported 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. 1*a*), 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. 1*b*).

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. 1*c*). 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,γ-imido)-triphosphate AMP-PCP = adenosine-5′-(β,γ-methylene)-triphosphate) (Fig. 1*d*). The non-hydrolysable ATP analogues compete with endogenous ATP present in the energized mitochondria. (In contrast to the previous view, membrane translocation 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 digitonin 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. 2*a*; 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. 2*a*; ii). This material is highly sensitive to protease (Fig. 4*c*). 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. 2*a*; iii).

It seems that the folding intermediates are probably associated with a complex which participates in the ATP-dependent folding reaction. The digitonin 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 of 1–2K is cleaved by proteinase K from either the N-or C-terminus of the hsp60 subunits (Fig. 2*a*; 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).

Authentic mitochondrial proteins such as the Rieske iron sulphur (Fe/S) protein of complex III and the β-subunit of F_1-ATPase ($F_1\beta$) also form a high-molecular weight complex in the matrix. Fe/S protein imported into apyrase-treated mitochondria is readily extractable with digitonin (Fig. 2*b*), unlike the protein that is assembled into complex III when imported in the presence of ATP[18]. The Fe/S protein contained in the extracts cofractionates exactly with hsp60. Essentially the same result is obtained when $F_1\beta$ is imported: the precursor

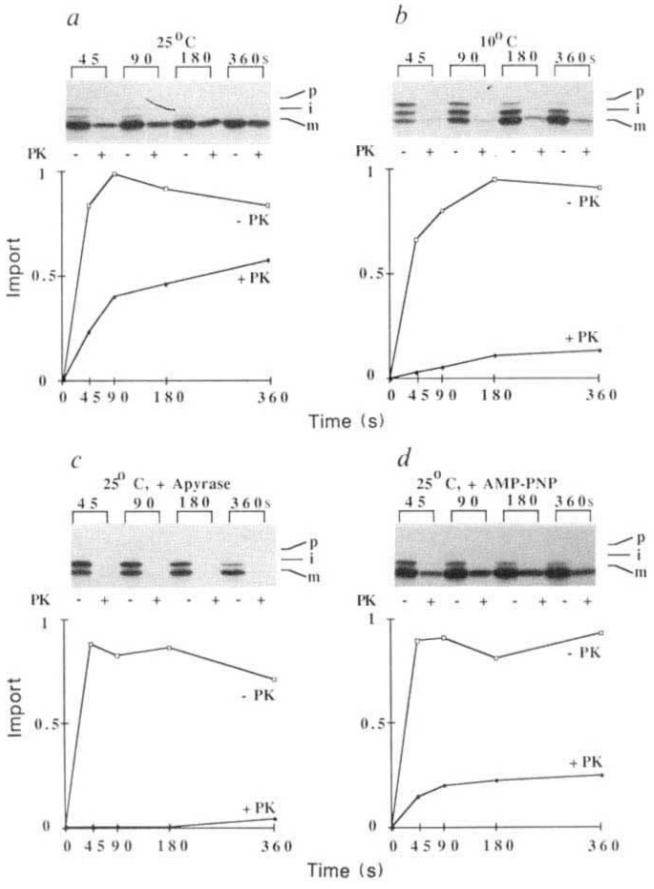

FIG. 1 Import into isolated mitochondria and refolding of Su9-DHFR. Isolated mitochondria were incubated under various conditions in the presence of preSu9-DHFR. Import (arbitrary units) at *a*, 25 °C; *b*, 10 °C; *c*, 25 °C into mitochondria treated with apyrase; *d*, 25 °C in the presence of 5 mM AMP-PNP. Upper panels, fluorographs of SDS-polyacrylamide gels of digitonin-permeabilized mitochondria previously incubated in the presence (+PK) or in the absence of proteinase K (−PK). *p*, Precursor; *i*, intermediate; *m*, mature-sized form of Su9-DHFR. Lower panels: amount 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 [^{35}S]methionine by transcription/translation of the complementary DNA cloned into expression vector pSP6 (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 8 M 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 NADH, 10 mM MOPS (3-[N-morpholino] propanesulphonic acid), pH 7.2, and 0.8 mg ml^{-1} isolated mitochondria of *N. crassa*, which were essentially free of cytosolic and endoplasmic reticulum markers[18,19]. Before addition of preSu9-DHFR and NADH, reaction *c* was incubated for 15 min with apyrase (40 U per ml final concentration; Sigma grade VIII)[31] and reaction *d* was incubated with 5 mM AMP-PNP. After incubation at 25° (*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 SEM-buffer (0.25 M sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) containing 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[18] and resuspended at 0.5 mg ml^{-1} in 0.3% digitonin in SEM/100 mM 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].

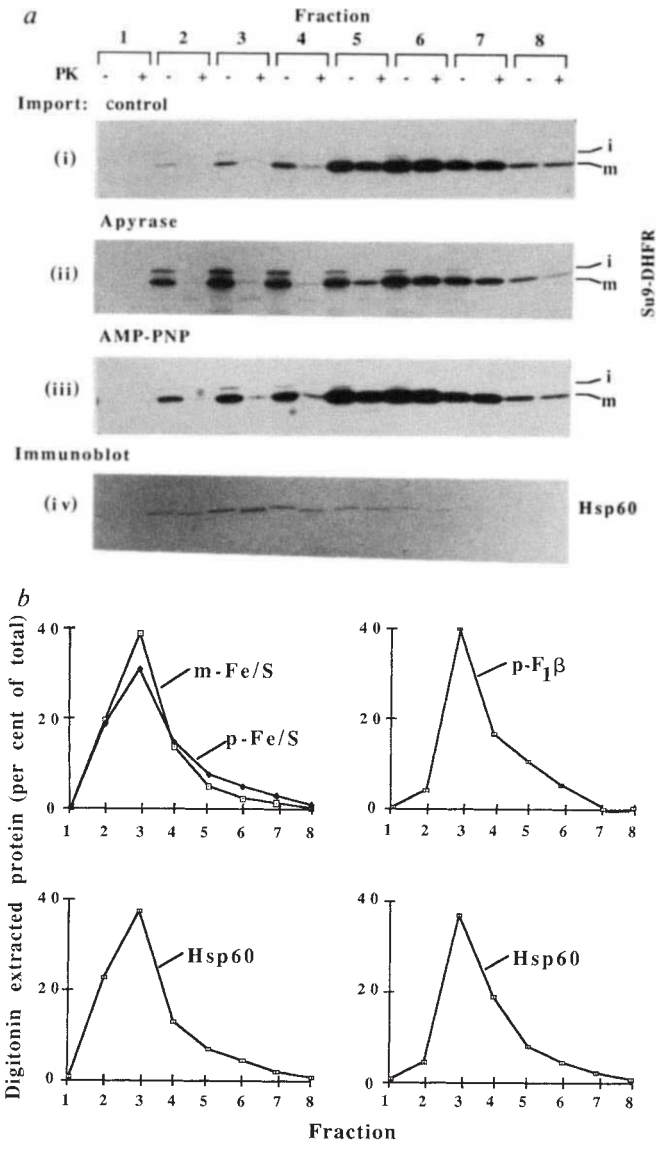

FIG. 2 Gel chromatography of folding intermediates. *a*, Analysis by Sephacryl S-300 chromatography of digitonin extracts prepared from mitochondria which had been incubated for import of preSu9-DHFR: (i) in the presence of ATP; (ii) after depletion of ATP with apyrase; (iii) in the presence of AMP-PNP (iv) Nitrocellulose blot of column fractions decorated with anti-hsp60 antiserum. 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 digitonin extract from apyrase-treated mitochondria which had imported the precursor of Fe/S protein or of $F_1\beta$, respectively. Imported protein and hsp60 are shown as per cent of total protein in digitonin 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 20,000*g*. Aliquots of pellets and supernatants were analysed for imported protein, hsp60 and the matrix marker enzyme, fumarase[33]. 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 200 μl 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[50] with anti-hsp60 antiserum. Bound antibodies were detected by alkaline phosphatase[51] coupled to IgG directed against rabbit IgG (*a*; iv) or by ^{14}C-labelled protein A[15] (*b*). Fluorographs were quantified by densitometry.

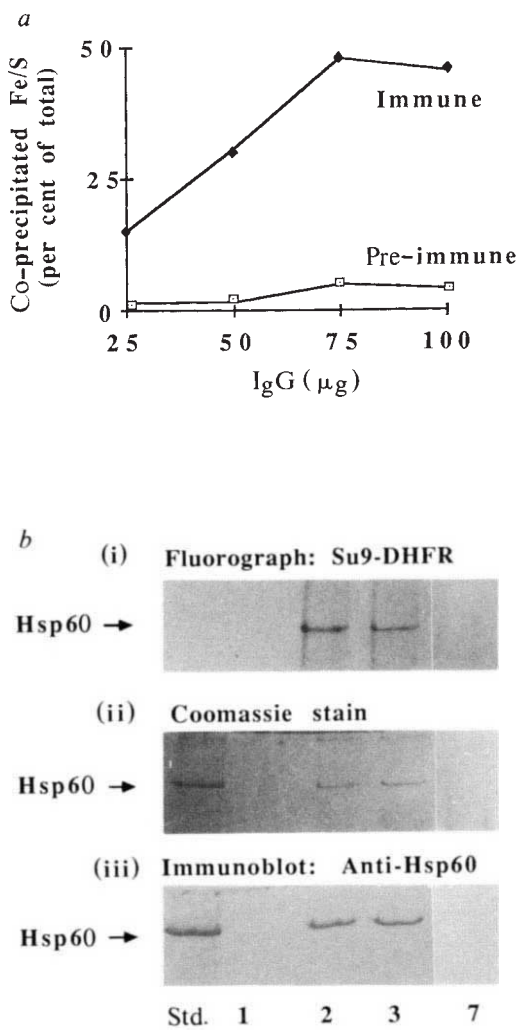

FIG. 3 Physical association of imported proteins with hsp60. *a*, Co-immunoprecipitation of Fe/S protein imported into apyrase-treated mitochondria with anti-hsp60 immunoglobulins. The amount of Fe/S protein (p-Fe/S plus m-Fe/S; compare Fig. 4*b*) precipitated from digitonin extracts are given as per 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. 2*a*, 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 antiserum, 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. Digitonin 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[15]. Reactions were incubated for 30 min at 4 °C by rotating end-over-end in the presence of 1 mM 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 SDS-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*, Digitonin 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 (see legend to Fig. 2) were loaded 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 antiserum; 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. 2*b*).

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 against hsp60 could co-immunoprecipitate up to 45% of the total Fe/S protein contained in the extracts (Fig. 3*a*). Comparable 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[35] also indicated such an association. The protease-sensitive Su9-DHFR comigrated exactly with the hsp60 complex (Fig. 3*b*); 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 complex 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 Mg^{2+}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. 4*a*). 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. 4*a*). 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. 4*b*). Readdition of ATP to the intact mitochondria causes the release of

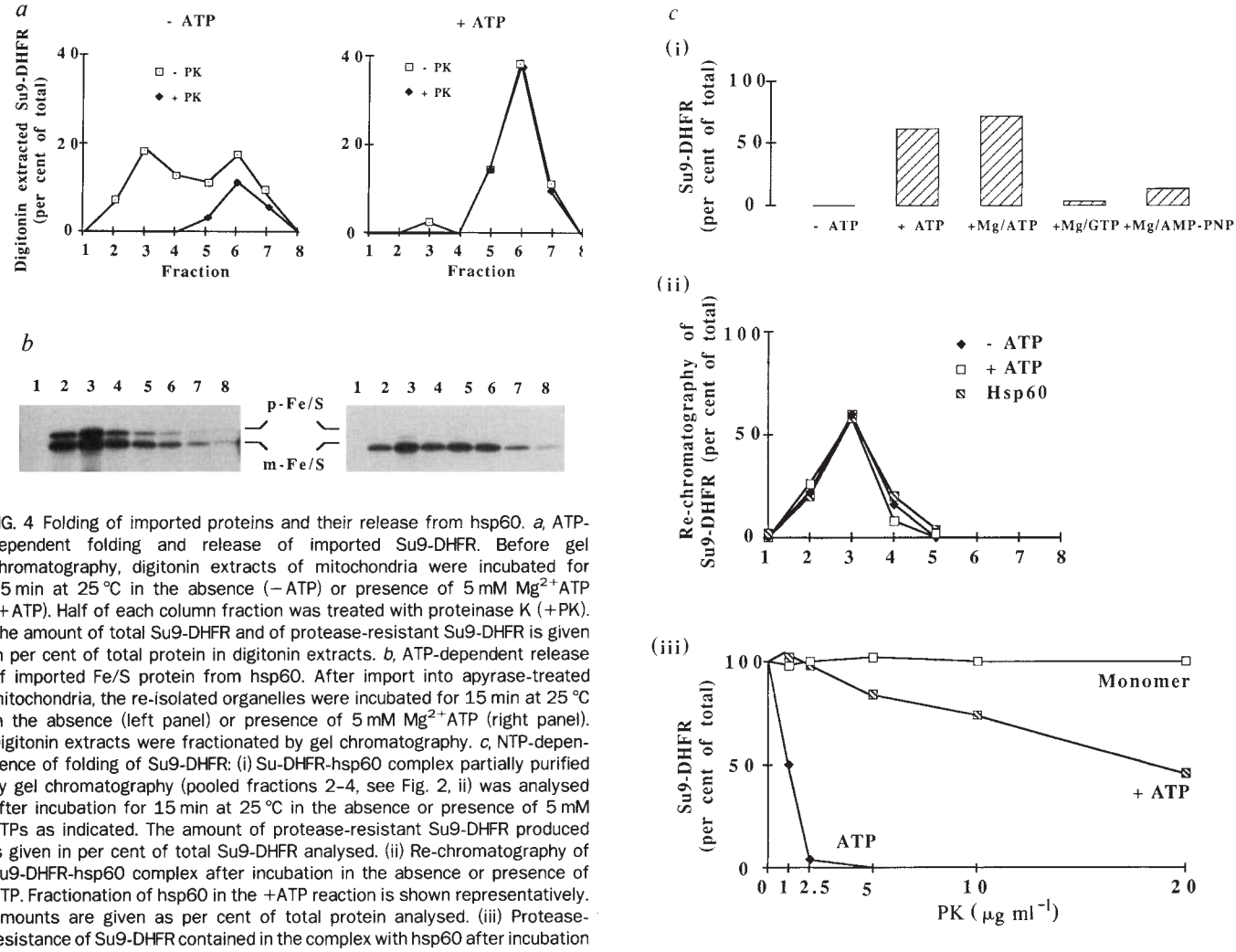

FIG. 4 Folding of imported proteins and their release from hsp60. *a*, ATP-dependent folding and release of imported Su9-DHFR. Before gel chromatography, digitonin extracts of mitochondria were incubated for 15 min at 25 °C in the absence (−ATP) or presence of 5 mM Mg^{2+}ATP (+ATP). Half of each column fraction was treated with proteinase 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 Mg^{2+}ATP (right panel). Digitonin extracts were fractionated by gel chromatography. *c*, NTP-dependence of folding of Su9-DHFR: (i) Su-DHFR-hsp60 complex partially purified by gel chromatography (pooled fractions 2–4, see Fig. 2, ii) 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 proteinase 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, preparation 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 Na_2ATP, Mg^{2+}ATP, Mg^{2+}GTP or Mg^{2+}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} proteinase K for 10 min at 0 °C. Treatment with proteinase 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[18]. 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 re-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. 4*c*; 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. 4*a*), the protease-resistant fusion protein still cofractionated with the hsp60 complex (Fig. 4*c*; 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. 4*c*; 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. 5*a*). Translocation of the urea-denatured precursor into the mitochondria 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. 5*b*), whereas in control reactions the protein is folded and exists as the monomer (Fig. 2*a*). 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, mif4 (not shown).

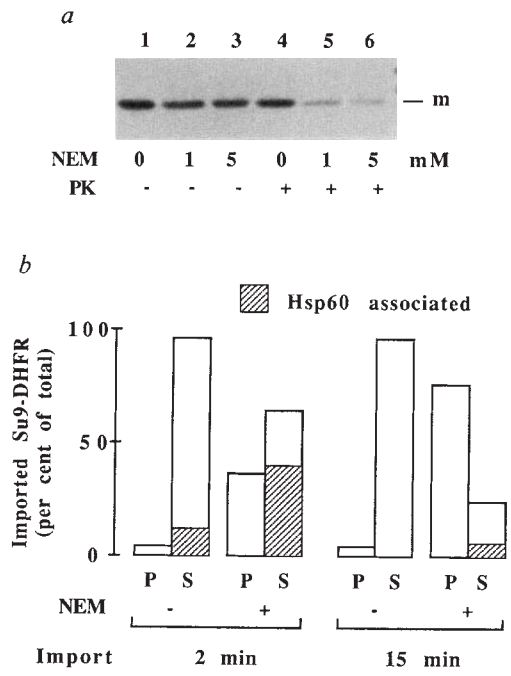

FIG. 5 Inhibition of folding in NEM-treated mitochondria. *a*, Import of preSu9-DHFR for 15 min at 25 °C into isolated mitochondria pretreated with NEM at the concentrations indicated. Mitochondria were permeabilized with digitonin and were incubated in the absence (lanes 1-3) or in the presence (lanes 4-6) of proteinase K. *b*, Extractability by digitonin of Su9-DHFR imported for 2 min or 15 min into control mitochondria and into mitochondria pretreated with 5 mM NEM. Extracts were separated into pellets (*P*) and supernatants (*S*). Supernatants were analysed by gel chromatography. Column fractions 3 and 4 containing the highest concentrations of hsp60-complex were pooled. Imported Su9-DHFR is given as per cent of total imported protein. The shaded area of the columns marked supernatant represents Su9-DHFR recovered in hsp60-containing column fractions.
METHODS. Mitochondria were incubated for 10 min at 25 °C at a protein concentration of 2 mg ml^{-1} in SEM-buffer containing 0, 1 or 5 mM NEM. Dithiothreitol was added to a concentration 5-fold higher than NEM and incubation continued for 5 min at 25 °C. Mitochondria reisolated by centrifugation were resuspended in SEM and used for import of Su9-DHFR as described in Fig. 1. Import reactions contained 2 mM Mg^{2+}ATP. Digitonin treatment, preparation of digitonin extracts and analysis on Sephacryl S-300 columns are described in the legends to Figs 1 and 2. Oligomycin-sensitive ATP hydrolysis of NEM-treated mitochondria was assayed: mitochondria were incubated as for import in the presence of 1 mM Mg^{2+}ATP for various times at 25 °C either with or without 20 μM oligomycin and ATP in the mitochondrial supernatant was determined by coupled enzyme assay[52]. Compared with controls, NEM-treated mitochondria internalized and hydrolysed identical amounts of ATP.

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 ^{14}C-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, 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 *E. coli* seems to cooperate with groEL in various assembly functions by an unknown mechanism[36–38].

How does hsp60 fulfil 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 may even occur during 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[34], 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[3,4] have been proposed to bind through hydrophobic regions exposed in the newly synthesized, incompletely folded polypeptides[6,7,39,40]. 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[39–43]. It has been suggested that ATP hydrolysis causes a conformational change in 70K heat-shock proteins, which is transferred to the bound polypeptide[6,7,39,40,44]. An additional soluble factor seems to participate in these reactions[4,5,9]. So far, the function of the 70K proteins has been evident in stabilization of a loose, 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 *E. 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 cell cytosol or in other membrane 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[20,21,25,28,29]. 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 while the partially folded polypeptides are still in an assembly complex with hsp60. Likewise proteins such as the Riesks Fe/S protein or cytochrome b_2, which have to be re-exported from the matrix to the intermembrane space[18,19], 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[45]. Other factors in the mitochondrial matrix, such as the recently described 70K protein SSC1[46], 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. □

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