Volume 277, number 1,2, 281-284 FEBS 09299 December 1990

Precursor proteins in transit through mitochondrial contact sites interact with hsp70 in the matrix

Joachim Ostermann[1,*], Wolfgang Voos[1], Pil Jung Kang[2], Elizabeth A. Craig[2], Walter Neupert[1] and Nikolaus Pfanner[1]

[1]*Institut für Physiologische Chemie, Universität München, Goethestr. 33, W-8000 München 2, Germany* and [2]*Department of Physiological Chemistry, University of Wisconsin at Madison, Madison, WI 53706, USA*

Received 16 November 1990

We previously reported that hsp70 in the mitochondrial matrix (mt-hsp70 = Ssc1p) is required for import of precursor proteins destined for the matrix or intermembrane space. Here we show that mt-hsp70 is also needed for the import of mitochondrial inner membrane proteins. In particular, the precursor of ADP/ATP carrier that is known not to interact with hsp60 on its assembly pathway requires functional mt-hsp70 for import, suggesting a general role of mt-hsp70 in membrane translocation of precursors. Moreover, a precursor arrested in contact sites was specifically co-precipitated with antibodies directed against mt-hsp70. We conclude that mt-hsp70 is directly involved in the translocation of many, if not all, precursor proteins that are transported across the inner membrane.

Mitochondria; hsp70; Contact site; Protein transport; ADP/ATP carrier

1. INTRODUCTION

A member of the 70 kDa heat shock protein family (hsp70s) is located in the mitochondrial matrix [1,2]. Mitochondrial hsp70 (mt-hsp70), also termed Ssc1p in yeast, is an essential protein [3]. By analysing a yeast mutant that is defective in mt-hsp70 in a temperature-sensitive manner, we recently observed that mt-hsp70 was required for the import of nuclear-encoded precursor proteins destined for the mitochondrial matrix or intermembrane space [2]. Precursor proteins that were denatured by a pre-incubation in urea were able to circumvent the translocation defect of mitochondria impaired in mt-hsp70 function. These precursor proteins then accumulated in a complex with the mutant mt-hsp70 in the matrix in a loosely folded conformation [2]. This indicated that mt-hsp70 was also required in the process of refolding of precursor proteins, probably by donating the precursor to the 'folding machinery' hsp60 [4,5].

Here we report a more detailed analysis of the role of mt-hsp70 in membrane translocation of precursor proteins. We found that the precursors of two mitochondrial inner membrane proteins, cytochrome c_1 and ADP/ATP carrier, which follow different intramitochondrial assembly pathways, both required functional mt-hsp70 for complete import into mitochondria. Furthermore, a precursor protein arrested in contact sites between mitochondrial outer and inner membranes [6,7] was found in a complex with mt-hsp70. We conclude that mt-hsp70 is directly involved in the translocation of precursor proteins through mitochondrial contact sites and plays an essential role in the general mitochondrial import pathway.

Correspondence address: N. Pfanner, Institut für Physiologische Chemie, Universität München, Goethestr. 33, W-8000 München 2, Germany

**Present address:* Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014, USA

2. MATERIALS AND METHODS

The following procedures were performed as published previously: growth of wild-type and mutant cells of the yeast *Saccharomyces cerevisiae* at 23°C and isolation of mitochondria [2,8,9]; synthesis of mitochondrial precursor proteins in rabbit reticulocyte lysates and labelling with [^{35}S]methionine [10,11]; import of the radiolabelled precursor proteins into isolated energized mitochondria (pre-incubated for 10 min at 37°C) in the presence of 3% (w/v) bovine serum albumine at 25°C [2,12,13]; treatment with proteinase K and reisolation of mitochondria [13]; analysis by SDS-polyacrylamide gel electrophoresis [14], fluorography [15], and laser densitometry [16].

3. RESULTS

We used mitochondria from the temperature-sensitive yeast strain BC100(*ssc1-2*) that carries a disruption of the chromosomal mt-hsp70 gene (*SSC1*) and a mutant mt-hsp70 gene (*ssc1-2*) on a centromeric plasmid (mutant) [2]. The mutant cells were able to grow at 23-25°C, but not at 37°C. When mitochondria were isolated from the mutant cells after growth at permissive temperature and pre-incubated for 10 min at 37°C, the mitochondria were defective in functional mt-hsp70 even at 25°C [2]. As a control we used

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

mitochondria from the yeast strain BC100(SSC1) that carries the wild-type mt-hsp70 gene (*SSC1*) on a centromeric plasmid (wild-type) [2].

We analysed the import of cytochrome c_1 into mutant and wild-type mitochondria at 25°C. The cytochrome c_1 precursor was efficiently processed to the intermediate-sized form by both mitochondria (Fig. 1A) (in the absence of NADH, virtually no processing to the mature-sized form occurs [9,17]). The processing of cytochrome c_1 to the intermediate-sized form is performed by the enzyme processing peptidase in the mitochondrial matrix [18,19], demonstrating that the amino-terminal presequence of the precursor reached into the matrix even in the mutant mitochondria. In parallel, mitochondria were treated with proteinase K after the import reaction to analyse for complete import of the precursor molecules. While the processed cytochrome c_1 was protected against added proteinase K in wild-type mitochondria, it was accessible to proteinase K added to the mutant mitochondria (Fig. 1B). By analysis of several marker proteins, we previously demonstrated that the mutant mitochondria possessed intact membranes that were not permeable to added protease, excluding an increased protease susceptibility of mitochondria defective in mt-hsp70 [2]. The processed cytochrome c_1 accumulated in the mutant mitochondria thus was accessible to proteases both on the cytosolic side (proteinase K) and on the matrix side of the membranes (processing peptidase). These precursor molecules reached from the cytosolic side across outer and inner membranes into the matrix, thereby spanning mitochondrial contact sites [6,7,20,21]. We conclude that the presequence of cytochrome c_1 can be imported into mitochondria defective in mt-hsp70, while the translocation of the major portion of the precursor requires functional mt-hsp70.

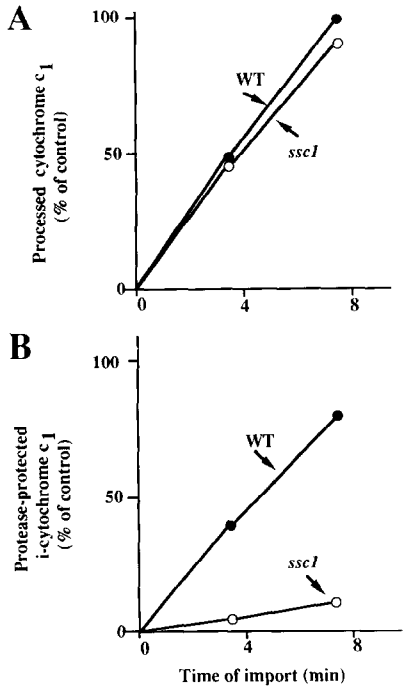

Fig. 1. Mt-hsp70 (Sscl p) is required for translocation of the mature part of cytochrome c_1 through contact sites. Mitochondria were isolated from the yeast strain BC100(*ssc1-2*) that carried a mutant gene for mt-hsp70 (*ssc1-2*) and from the control strain BC100(*SSC1*) that carried the wild-type gene for mt-hsp70. The mitochondria were preincubated for 10 min at 37°C and then incubated with reticulocyte lysate containing radiolabeled precursor of cytochrome c_1 at 25°C for the indicated times as described in Materials and Methods. For energization of the mitochondria, 8 mM potassium ascorbate and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine (TMPD) were included in the import reaction [13]. The samples of panel B were treated with proteinase K (120 μg/ml) after the import reaction. The mitochondria were reisolated and analysed by SDS-polyacrylamide gel electrophoresis, fluorography and laser densitometry of intermediate-sized i-cytochrome c_1. The amount of i-cytochrome c_1 present in wild-type mitochondria after 7.5 min import was set to 100%. Panel A, without proteinase K; panel B, with proteinase K; WT, mitochondria with wild-type mt-hsp70; *ssc1*, mitochondria with defective mt-hsp70.

The precursor of ADP/ATP carrier is synthesized without a cleavable presequence [22] and, in contrast to most other precursor proteins that are translocated to mitochondrial matrix, inner membrane or intermembrane space, does not require the interaction with the heat shock protein hsp60 in the matrix for import and assembly [23–25]. To analyse how general the role of mt-hsp70 in protein translocation is, we studied the import of ADP/ATP carrier into mitochondria defective

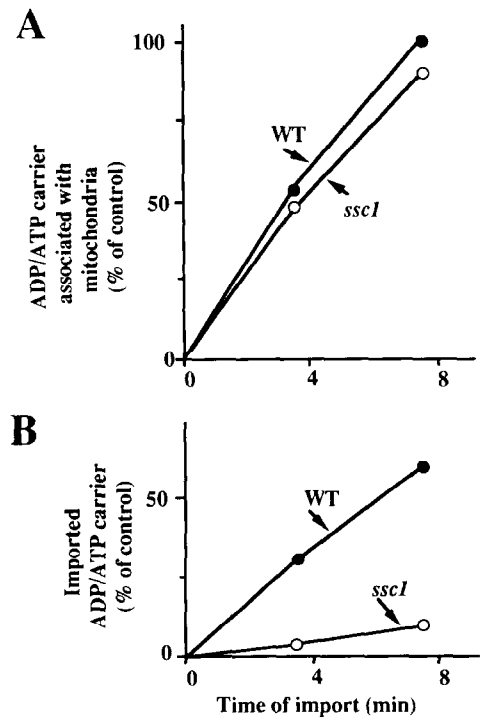

Fig. 2. Mt-hsp70 (Sscl p) is required for complete import of ADP/ATP carrier into mitochondria. The experiment was performed as described in the legend of Fig. 1 except that reticulocyte lysate containing the precursor of ADP/ATP carrier was used. The amount of ADP/ATP carrier associated with wild-type mitochondria after 7.5 min import was set to 100%.

in mt-hsp70. The total amount of precursor associated with mitochondria was the same with wild-type and mutant mitochondria (Fig. 2A), indicating that a defect in mt-hsp70 did not impair the binding of ADP/ATP carrier to the receptor sites on the mitochondrial outer membrane [11,13,26,27]. When the mitochondria were treated with proteinase K after the import, however, it became apparent that the membrane translocation of ADP/ATP carrier was strongly inhibited by the defect in mt-hsp70 (Fig. 2B). It can be excluded that the import defect in the mutant mitochondria is caused by a dissipation of the membrane potential $\Delta\Psi$ across the inner membrane since the studies with cytochrome c_1 (Fig. 1A) and other precursor proteins [2] demonstrate that the $\Delta\Psi$-dependent translocation of the amino-terminal presequence [6] is virtually not affected whereas the $\Delta\Psi$-independent completion of translocation requires functional mt-hsp70. We conclude that the translocation of ADP/ATP carrier into the inner membrane requires mt-hsp70 in the matrix.

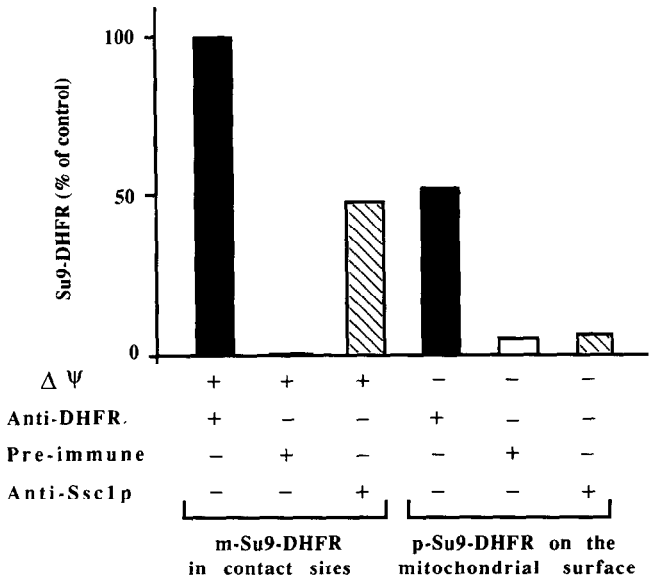

Fig. 3. Mt-hsp70 (Ssc1p) forms a complex with Su9-DHFR accumulated in contact sites. Reticulocyte lysate containing radiolabelled precursor of Su9-DHFR and isolated mitochondria from the strain BC100(*ssc1-2*) that is defective in mt-hsp70 were incubated for 10 min at 25°C as described in Materials and Methods. For energization of mitochondria ($+\Delta\Psi$), 2 mM NADH were included; thereby Su9-DHFR accumulated in mitochondrial contact sites [2]. For dissipation of the membrane potential ($-\Delta\Psi$), 1 μM valinomycin was included. The mitochondria were reisolated, lysed in a buffer containing 0.1% Triton X-100, and immuno-precipitations with antibodies directed against DHFR (anti-DHFR) or mt-hsp70 (anti-Ssc1p) or antibodies from pre-immune sera were performed [2]. The immuno-precipitates were analysed by SDS-polyacrylamide gel electrophoresis, fluorography and laser densitometry. The amount of m-Su9-DHFR precipitated with anti-DHFR antibody was set to 100%. It has to be noted that the interaction of Su9-DHFR with mt-hsp70 is not limited to the processed mature-sized form (m-Su9-DHFR), since the unprocessed precursor form (p-Su9-DHFR) imported into mutant mitochondria was also found in a complex with mt-hsp70 (data not shown).

Do precursor proteins in transit through the mitochondrial membranes directly interact with mt-hsp70? To test this idea, a radio-labelled hybrid protein between the presequence of F_o-ATPase subunit 9 and the entire dihydrofolate reductase (Su9-DHFR) [16] was accumulated in contact sites of the mutant mitochondria by importing it from reticulocyte lysate [2]. The reisolated mitochondria were lysed with non-ionic detergent and immuno-precipitations with antibodies directed against DHFR or a carboxyl-terminal sequence of mt-hsp70 (Ssc1p) not conserved in other hsp70s [2] were performed. A major fraction of Su9-DHFR that could be immuno-precipitated with anti-DHFR antibodies was co-precipitated with the anti-mt-hsp70 (= anti-Ssc1p) antibodies (Fig. 3). As a control, the precursor of Su9-DHFR was accumulated on the mitochondrial surface in the absence of a membrane potential and found not to be co-precipitated with anti-mt-hsp70 antibodies (Fig. 3). These results show that mt-hsp70 forms a complex with precursor proteins in transit across the mitochondrial membranes.

4. DISCUSSION

We report here that two integral proteins of the mitochondrial inner membrane, cytochrome c_1 and ADP/ATP carrier, require functional mt-hsp70 for complete import into mitochondria. The presequence of cytochrome c_1, but not the mature portion of the precursor, could be translocated into the matrix of mitochondria defective in mt-hsp70. Mt-hsp70 is needed for transport of the mature portion of precursor proteins through mitochondrial contact sites. This extends our previous studies that showed a requirement for mt-hsp70 for import of soluble proteins of the matrix and intermembrane space (Su9-DHFR, cytochrome b_2) and of proteins peripherally associated with the inner membrane (F_1-ATPase subunit β, Fe/S protein of the bc_1-complex) [2]. Mt-hsp70 thus possesses a general function in mitochondrial protein import.

Most mitochondrial proteins that are imported via contact sites are fully translocated into the mitochondrial matrix and interact with hsp60 [4,23,28]. Several of these proteins are retranslocated to the inner membrane or intermembrane space, thereby following the conserved folding and assembly pathways established in the putative prokaryotic ancestors of mitochondria ('conservative sorting') [9,23,29]. It is assumed that the prokaryotic ancestors did not contain an equivalent of the ADP/ATP carrier, but that this protein was contributed by the nucleated 'host' in light of the endosymbiont theory of mitochondrial origin [30]. This would agree with the observation that the assembly pathway of ADP/ATP carrier does not require functional hsp60 ('non-conservative sorting') [23]. However, its import requires functional mt-hsp70 as do the precursor proteins that follow the conservative sorting pathway,

strongly suggesting that mt-hsp70 is a component of the general translocation machinery of mitochondrial contact sites. We propose that during its import a part of the ADP/ATP carrier reaches into the matrix and interacts with mt-hsp70. The release of proteins from hsp70s seems to require the hydrolysis of ATP [31]. Since the translocation of radiochemical amounts of ADP/ATP carrier from the outer membrane to the inner membrane can occur at very low levels of ATP [32], it is suggested that mt-hsp70 carries bound ATP which is sufficient to drive one cycle of import. It has to be expected that the function of mt-hsp70s in several cycles of import by using chemical amounts of ADP/ATP carrier should require the addition of ATP as found with other precursor proteins [5,33,34].

The isolation of a complex between a precursor protein spanning contact sites and mt-hsp70 provides the first demonstration that mt-hsp70 directly partakes in membrane translocation of precursor proteins. We conclude that mt-hsp70 and possibly further proteins which were not identified so far bind to the precursor proteins in transit through contact sites and thereby promote the completion of translocation. It is suggested that BiP, the hsp70 in the endoplasmic reticulum (ER) [31,35], also directly interacts with precursor proteins in transit across the ER membrane and thus is important for protein translocation.

Acknowledgements: We thank Alexandra Weinzierl for expert technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 184), the Fonds der Chemischen Industrie, the National Institute of Health, and a fellowship to P.J.K. by the Korean Ministry of Education and the Lucille P. Markey Charitable Trust.

REFERENCES

[1] Craig, E.A., Kramer, J., Shilling, J., Werner-Washburne, M., Holmes, S., Kosic-Smithers, J. and Nicolet, C. M. (1989) Mol. Cell. Biol. 9, 3000-3008.
[2] Kang, P.J., Ostermann, J., Shilling, J., Neupert, W., Craig, E.A. and Pfanner, N. (1990) Nature 348, 137-143.
[3] Craig, E.A., Kramer, J. and Kosic-Smithers, J. (1987) Proc. Natl. Acad. Sci. USA 84, 4156-4160.
[4] Ostermann, J., Horwich, A. L., Neupert, W. and Hartl, F.-U. (1989) Nature 341, 125-130.
[5] Neupert, W., Hartl, F.-U., Craig, E.A. and Pfanner, N. (1990) Cell (in press).
[6] Schleyer, M. and Neupert, W. (1985) Cell 43, 339-350.
[7] Schwaiger, M., Herzog, V. and Neupert, W. (1987) J. Cell Biol. 105, 235-246.
[8] Daum, G., Gasser, S.M. and Schatz, G. (1982) J. Biol. Chem. 257, 13075-13080.
[9] Hartl, F.-U., Ostermann, J., Guiard, B. and Neupert, W. (1987) Cell 51, 1027-1037.
[10] Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.
[11] Pfaller, R., Steger, H.F., Rassow, J., Pfanner, N. and Neupert, W. (1988) J. Cell Biol. 107, 2483-2490.
[12] Pfanner, N. and Neupert, W. (1985) EMBO J. 4, 2819-2825.
[13] Pfanner, N. and Neupert, W. (1987) J. Biol. Chem. 262, 7528-7536.
[14] Laemmli, U.K. (1970) Nature 227, 680-685.
[15] Chamberlain, J.P. (1979) Anal. Biochem. 98, 132-135.
[16] Pfanner, N., Müller, H. K., Harmey, M. A. and Neupert, W. (1987) EMBO J. 6, 3449-3454.
[17] Nicholson, D.W., Stuart, R.A. and Neupert, W. (1989) J. Biol. Chem. 264, 10156-10168.
[18] Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U. and Neupert, W. (1988) Cell 53, 795-806.
[19] Yang, M., Jensen, R.E., Yaffe, M.P., Oppliger, W. and Schatz, G. (1988) EMBO J. 7, 3857-3862.
[20] Vestweber, D. and Schatz, G. (1988) J. Cell Biol. 107, 2037-2043.
[21] Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.-U. and Neupert, W. (1989) J. Cell Biol. 109, 1421-1428.
[22] Zimmermann, R., Paluch, U., Sprinzl, M. and Neupert, W. (1979) Eur. J. Biochem. 99, 247-252.
[23] Mahlke, K., Pfanner, N., Martin, J., Horwich, A. L., Hartl, F.-U. and Neupert, W. (1990) Eur. J. Biochem. 192, 551-555.
[24] Hartl, F.-U. and Neupert, W. (1990) Science 247, 930-938.
[25] Pfanner, N. and Neupert, W. (1990) Annu. Rev. Biochem. 59, 331-353.
[26] Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W. (1990) Cell 62, 107-115.
[27] Steger, H.F., Söllner, T., Kiebler, M., Dietmeier, K.A., Pfaller, R., Trülzsch, K.S., Tropschug, M., Neupert, W., and Pfanner, N. (1990) J. Cell Biol. (in press).
[28] Cheng, M.Y., Hartl, F.-U., Martin, J., Pollock, R.A., Kalousek, F., Neupert, W., Hallberg, E.M., Hallberg, R.L. and Horwich, A.L. (1989) Nature 337, 620-625.
[29] Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H. and Neupert, W. (1986) Cell 47, 939-951.
[30] Klingenberg, M. (1985) Ann. NY Acad. Sci. 456, 279-288.
[31] Rothman, J.E. (1989) Cell 59, 591-601.
[32] Pfanner, N., Tropschug, M. and Neupert, W. (1987) Cell 49, 815-823.
[33] Hwang, S.T. and Schatz, G. (1989) Proc. Natl. Acad. Sci. USA 86, 8432-8436.
[34] Pfanner, N., Rassow, J., Guiard, B., Söllner, T., Hartl, F.-U. and Neupert, W. (1990) J. Biol. Chem. 265, 16324-16329.
[35] Vogel, J.P., Misra, L.M. and Rose, M.D. (1990) J. Cell Biol. 110, 1885-1895.