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The Occurrence of hsp70 in the Outer Membrane of Plant Mitochondria

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Hsp70 was localized to the mitochondrial outer membranes of bean and cauliflower mitochondria. Western blotting showed that the outer membrane hsp70 was antigenically distinct from the mitochondrial-matrix hsp70, but was similar to the cytosolic form. The protein was resistant to solubilization with 200 mM sodium carbonate which showed the hsp70 was tightly bound to the outer membrane. Proteinase K studies suggested that the hsp70 was partially exposed to the cytosol with approximately 17% of the protein protease-accessible. It is suggested that the position of the outer membrane hsp70 could relate to a precursor unfolding function during protein import into mitochondria.

Hsp70 is a heat shock protein with a molecular mass of approximately 70kDa. Hsp70 functions during heat shock to prevent the formation of insoluble aggregates of denatured proteins, and to promote the refolding of denatured proteins following a heat shock (1). At normal growing temperatures cytosolic hsp70 associates with proteins during their translation and aids their subsequent folding or import into organelles (2). Cytosolic and organellar hsp70 (e.g. of mitochondria, chloroplasts) play a role in the import of proteins and also in the folding of these proteins into functional structures within the organelle (3,4).

During import of precursor proteins into mitochondria hsp70 maintains the precursors in an extended (unfolded) import-competent conformation (5). The precursor binds to receptors on the outer surface of the mitochondria via its signal sequence. Protein import occurs, with ATP-mediated release of bound hsp70, through the import machinery (made up of a number of proteins) of the outer and inner membranes (6). Mitochondrial import of artificially unfolded proteins was shown to occur in the absence of soluble hsp73, the mammalian form of cytosolic hsp70 (7). Lithgow *et al* (8) have localized a hsp70 to the outer membranes of rat liver mitochondria at a level similar to that of precursor receptors. A similar hsp70 has not been shown in yeast or *Neurospora.* Mitochondrial hsp70 participates in the translocation of the precursor across the mitochondrial membranes and aids its subsequent folding (9). Hsp70 in the outer membrane may be involved in the translocation of precursors through the outer membrane (7).

This communication discusses the occurrence of hsp70 in the outer membrane of bean and cauliflower mitochondria. The hsp70 is tightly associated with the outer membrane, distinct from mitochondrial matrix hsp70 and is partly accessible to externally added protease.

MATERIALS AND METHODS

Mitochondria were isolated according to Douce *et al* (10) and outer membrane fractions were prepared as outlined below.

Purification of isolated mitochondria. Intact mitochondria were purified through a sucrose cushion (600mM sucrose, 10mM Tris-HCl pH 7.4, 5mM EDTA, 2mM PMSF) by centrifugation at RCF 47,000 for 30 minutes. The mitochondrial pellet was resuspended in 3 volumes of 5mM phosphate buffer, pH 7.4 and incubated on ice for 45 minutes. The mitochondrial membranes were then ruptured by subjecting the swollen mitochondria to 20 strokes in a Potter homogeniser. Outer membranes were then purified according to Söllner *et al* (11).

Discontinuous sucrose gradient. The ruptured mitochondria were placed on a discontinuous SEM gradient. The gradient consisted of 12ml 32% SEM buffer (32% sucrose, 1mM EDTA, 10mM MOPS pH 7.4, 2mM PMSF), 8ml 15% SEM and

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5ml ruptured mitochondria. The gradient was centrifuged at RCF 114,000 for 1 hour at 4°C. The outer membrane band was recovered from the 15%/32% SEM interface, with a syringe and a wide bore needle with a flattened end.

Flotation gradient. The outer membrane fraction was made 50% (with respect to sucrose) by addition of 1.2 volumes of 70% SEM. A flotation gradient was prepared with 10ml outer membrane in SEM, 10ml 32% SEM and 5ml 0% SEM (buffer without sucrose). The gradient was centrifuged at RCF 240,000 for 6 hours. The purified outer membrane was recovered from the 0%/32% interface.

Sodium carbonate extraction of outer mitochondrial membrane. Purified outer membrane was extracted with 200mM Na_2CO_3, pH 11.5 by incubation on ice for 30 minutes. This was then centrifuged at RCF 234,000 for 1 hour. Supernatants and pellets were then boiled in an equal volume of 2X sample buffer (12) for 10 minutes.

Protease treatment of mitochondria. Whole mitochondria were treated with proteinase K (at 60 μg/ml) for 15 minutes on ice, PMSF (to 2mM) was added for a further 15 minutes on ice. A washed double membrane fraction was then prepared. An equal volume of buffer (1% Triton X-100, 200mM NaCl, 30mM Tris-HCl pH 8.5) was added to the mitochondria, with mixing. PMSF was added to 2mM, and incubated at 4°C for 1 hour with constant shaking. This was then centrifuged at RCF 12,100 for 20 minutes. The pellet represented a membrane fraction of the mitochondria. The pellet was washed twice with buffer, to produce a pure membrane fraction.

SDS-PAGE (12) and western blotting (13) were then used to localize hsp70 to the samples prepared above.

RESULTS

Western blotting of outer and inner mitochondrial membranes with antibody to hsp70. The purity of the outer membrane was verified by immunoblotting with antibody to, the inner membrane protein, adenine nucleotide translocator, and the outer membrane protein, porin (not shown). The outer membrane was free of ANT but positive for porin and MOM42. The hsp70 antibody used was raised to isolated cytosolic hsp70 from *Vicia faba* epicotyls, but it also recognised hsp70 homologues from cauliflower and cabbage. Samples of purified outer and inner membrane were separated on SDS-PAGE, transferred to nitrocellulose and immunodecorated with the antibody. The antibody bound to a single band of 70kDa in the outer and inner membrane samples (Fig. 1). The outer membrane preparation was free from inner membrane-bound and matrix hsp70, and also free from cytosolic hsp70 contamination. Therefore, the hsp70 present must be an integral part of the outer membrane.

Outer membrane hsp70 is antigenically distinct from inner membrane/matrix hsp70. Samples of outer and inner membrane were immunodecorated with monoclonal antibodies (a gift from T. Elthon) to matrix hsp70 (Fig. 2). The monoclonal antibodies bound to a hsp70 band in the inner membrane sample but not in the outer membrane sample. This band was the same molecular mass as the inner membrane hsp70 immunolabelled by the bean hsp70 antibody. The outer membrane

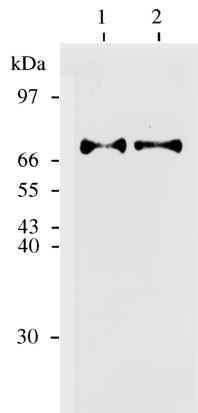

FIG. 1. Localization of hsp70 to purified outer and inner membrane fractions of cauliflower mitochondria using Western blotting. Outer and inner membranes were purified on a sucrose gradient. 10μg of outer membrane (lane 1) and inner membrane (lane 2) were separated on SDS-PAGE, electroblotted to nitrocellulose and immunodecorated with bean hsp70 antibody at a dilution of 1:1000. The blot was developed using enhanced chemiluminescence (ECL) system (AMERSHAM). The positions of protein molecular mass (in kilodaltons) markers are shown on the left.

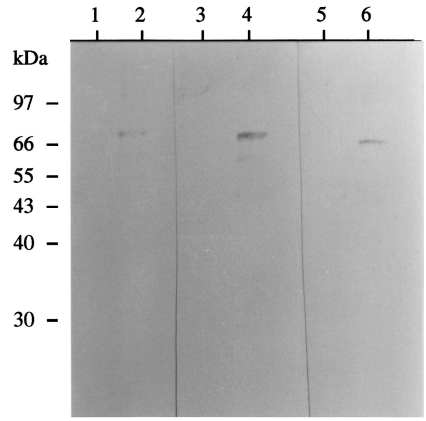

FIG. 2. Localization of hsp70 to cauliflower mitochondrial membranes using monoclonal antibodies to mitochondrial matrix/inner membrane hsp70. 10μg of purified outer membrane (lanes 1, 3, and 5) and inner membrane (lanes 2, 4, and 6) were separated on SDS-PAGE and transferred to nitrocellulose. Monoclonal antibodies raised to mitochondrial matrix hsp70 were used at 1:100 dilution. Monoclonals used HSP70A (lanes 1 and 2), HSP70B (lanes 3 and 4), and HSP70C (lanes 5 and 6). Positions of the molecular mass markers is shown.

hsp70 was shown to be antigenically distinct from the inner membrane/matrix hsp70, because the monoclonal antibodies to matrix hsp70 did not bind to the outer membrane sample. The outer membrane hsp70 may be more homologous to the cytosolic form of hsp70, than the mitochondrial matrix hsp70.

Outer membrane hsp70 is tightly associated with the outer membrane. Outer membrane samples were extracted with 200mM sodium carbonate to release loosely-bound membrane proteins. When pellet and supernatant fractions were prepared from this sodium carbonate extraction, the pellet represented membrane associated protein, and the supernatant represented released loosely-bound protein. Hsp70 antibody bound to a single band, with the same molecular mass, in each sample (Fig. 3). There was a much greater intensity of binding to hsp70 in the pellet fraction. This showed that the majority of the outer membrane hsp70 was tightly bound.

Hsp70 in the outer membrane is surface-exposed. Whole mitochondria were treated with Proteinase K (at 60 μg/ml final concentration) and a washed membrane fraction containing outer and

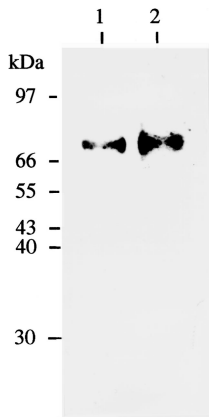

FIG. 3. Western blot showing hsp70 in pellet and supernatant fractions of a sodium carbonate extraction of outer mitochondrial membranes. 10μg of sodium carbonate supernatant (lane 1) and pellet (lane 2) fractions of outer membrane were separated on SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was immunodecorated with hsp70 antibody and developed with the ECL system.

inner membranes was prepared. Western blots revealed two bands in the protease treated sample relative to one in the control when immunodecorated with the hsp70 antibody (Fig. 4, compare lanes 1 and 2). The higher molecular mass band represented the inner membrane hsp70, while the lower molecular mass band represented a protease fragment of the outer membrane hsp70 still associated with the outer membrane. The lower molecular mass band is approximately 12kDa smaller than the hsp70 band. This suggests that about 17% of the outer membrane hsp70 is exposed on the outer surface. As a control samples treated with Proteinase K were also immunodecorated with antibody to MOM42, an integral protein of the outer membrane GIP complex. MOM42 was little affected by the addition of proteinase K which agrees with its integral position in the membrane and verifies the integrity of the membrane.

DISCUSSION

Hsp70 localized to the outer membrane is similar in molecular mass to the cytosolic form, and the mitochondrial inner membrane/matrix hsp70. Mitochondrial hsp70 in yeast (Ssc1p), has a molecular mass of 70.2kDa which is similar to the molecular mass of the cytosolic forms of hsp70 in yeast. Ssc1p is synthesized as a precursor in the cytoplasm and is imported into mitochondria where it is processed to its mature form (14). Hsp70 in the outer membrane detected in these experiments is similar to the cytosolic form but it is antigenically distinct from the mitochondrial matrix form. The majority of the outer membrane hsp70 was resistant to extraction with sodium carbonate. This shows that the hsp70 is not transiently associated with the outer membrane, but is tightly-bound. The surface-exposure of the outer membrane hsp70 suggests that it could be involved in the import of precursors during their translocation through the outer membrane. It is possible that transfer of the precursor from cytosolic to membrane hsp70 occurs during insertion of the precursor into the outer membrane. Precursor released from the cytosolic hsp70 may then bind to the membrane hsp70, with subsequent binding and release occurring until the entire precursor has inserted into the outer membrane. This binding and release would maintain the precursor in an import competent (unfolded) form throughout import. However, the orientation of the hsp70, i.e. whether the ATPase domain or the protein-binding domain is surface-exposed, has yet to be determined. This orientation information would help to elucidate the true function of the outer membrane hsp70.

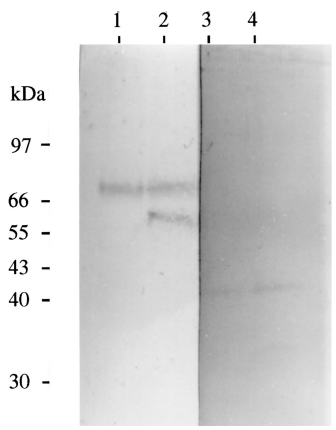

FIG. 4. Western blot of outer membrane hsp70 susceptibility to Proteinase K digestion. Whole mitochondria were treated with Proteinase K at 60μg/ml for 15 minutes. A mitochondrial membrane fraction was prepared which contained outer and inner membranes. The samples were immunodecorated with antibody to bean hsp70 (lanes 1 and 2) and antibody to MOM42 (lanes 3 and 4). Lanes 1 and 4 untreated membranes. Lanes 2 and 3 Proteinase K treated membranes. Positions of molecular mass markers is shown on left, values are in kilodaltons.

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