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Autophosphorylation of the Pea Mitochondrial Heat-Shock Protein Homolog[1]

Jan A. Miernyk*, Nicholas B. Duck, Nancy R. David, and Douglas D. Randall

Department of Biochemistry, Schweitzer Hall, The University of Missouri, Columbia, Missouri 65211

ABSTRACT

Highly purified mitochondria isolated from 14-day-old pea (*Pisum sativum* L., cv Little Marvel) seedlings contain a homolog of the 70,000 molecular weight heat-shock protein. The amount of this heat-shock cognate (Hsc70) was not reduced by limited proteolysis of intact mitochondria or by preparation of mitoplasts, indicating that the protein is located within the matrix compartment. Pea mitochondrial Hsc70 binds to immobilized ATP and reacts on western blots with anti-tomato Hsc70 antiserum. When a mitochondrial matrix fraction was incubated with [γ-^{32}P]ATP, there was phosphorylation of Hsc70. The extent of phosphorylation was increased by including calcium chloride in the reactions. Phospho amino acid analysis of purified mitochondrial Hsc70, phosphorylated in the calcium-stimulated reaction, revealed only phosphothreonine. Pea mitochondrial Hsc70, purified by a combination of ATP-agarose affinity chromatography and gel permeation chromatography, was labeled when incubated with ATP plus calcium, suggesting autophosphorylation rather than phosphorylation by an associated kinase. In analogy to mammalian cells and yeast, it is likely that mitochondrial Hsc70 acts as a molecular chaperone, and it is possible that phosphorylation plays a role in chaperone function.

Short-term exposure to abruptly elevated temperature results in a markedly altered pattern of protein synthesis. Expression of typical "housekeeping" genes is curtailed after heat shock, and there is sharp increase in the synthesis of a limited number of Hsps[2] (2). It was subsequently demonstrated that the synthesis of several Hsps could additionally be induced by other types of environmental insult such as cold, pathogen attack, and exposure to heavy metals. Furthermore, the expression of some of the stress-related proteins is continuous and is not significantly stimulated by heat shock.

In eukaryotic cells, the genes encoding the major classes of Hsps are typically organized in small multigene families. This multiplicity is due, at least in part, to the localization of Hsps in various subcellular compartments. For example, members of the Hsp70 family have been found in the cytoplasm, plastids, mitochondria, and the lumen of the ER (10, 21, 28, 29). The number of Hsps and the complexity of their localization suggest multiple cellular functions or participation in events that occur in several locations. One proposed role for members of several classes of Hsps is that of molecular chaperone.

Molecular chaperones are proteins that mediate the translocation, folding, and assembly of other proteins, but are not themselves components of the final structures (7, 8). Molecular chaperones act to forestall misfolding of proteins, preventing the formation of biologically incorrect structures. In the cases of precursor proteins, molecular chaperones help maintain the polypeptide chain in an unfolded, translocation-competent form until transport through the membrane has been completed (25). The mechanisms by which molecular chaperones control protein folding are not well understood. It is thought that the chaperones recognize and bind to polypeptide surface features that will interact in the final conformation, shielding them until the appropriate time for exposure (7). In addition, it has been shown that molecular chaperones have an intrinsic ATPase activity (22), and that at least a portion of chaperone function could be in acting as ATP-dependent protein "unfoldases."

The long-term regulation of chaperone function has been addressed at the molecular level (21, 28). Conditions that alter the cellular environment to create an accumulation of damaged or misfolded proteins tend to stimulate the expression of genes encoding molecular chaperones (31). However, there has been little information on acute regulation of chaperone function. We have undertaken a study of the localization and phosphorylation of Hsc70 preliminary to studies of the roles of molecular chaperones in the assembly of multienzyme complexes in pea (*Pisum sativum* L.) seedling mitochondria.

MATERIALS AND METHODS

Reagents

Buffers were from Research Organics, Inc. Proteinase K was from Calbiochem. Unless otherwise noted, all other reagents were from the Sigma. Carrier-free ^{32}Pi was purchased from New England Nuclear and used to prepare [γ-^{32}P]ATP according to the method of Johnson and Walseth (12).

Preparation of Mitochondria

Mitochondria were isolated from 14-d-old light-grown pea (*Pisum sativum* L. cv Little Marvel) seedlings and were puri-

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[2] Abbreviations: Hsp, heat-shock protein; Hsp70, the M_r 70,000 stress-related protein; mtHsc70, the M_r 70,000 mitochondrial heat-shock cognate; PDC, pyruvate dehydrogenase complex.

fied exactly as previously described (9, 24). For limited proteolysis, purified intact mitochondria were suspended in 300 mM mannitol containing 20 mM Tes-NaOH, pH 7.4, and 2 mM DTT, plus or minus 0.05% (v/v) Triton X-100, then treated with 0.3 mg mL^{-1} proteinase K for 45 min at 4°C. Mitoplasts were prepared as described in ref. 17.

Immunochemical Methods

A portion of the 3′ coding region of a tomato Hsc70 cDNA (15) was cloned into the bacterial expression vector pGEX-2T (Pharmacia LKB Biotechnology) to produce a GSH *S*-transferase-Hsc70 fusion protein (18). The fusion protein was purified using a column of GSH-agarose. The affinity-purified fusion protein was then used to produce rabbit polyclonal antibodies. Samples were analyzed by SDS-PAGE plus western blotting as described in ref. 20. A 1:1000 dilution of antiserum was used for the immunoblots.

Isolation of the mtHsp70 Homolog

Purified mitochondria were suspended in 100 mM Tes, pH 7.4, containing 50 mM KCl, 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol, then disrupted by two 30-s bursts with a Polytron homogenizer at a setting of 6. The homogenates were clarified by centrifugation for 30 min at 10,000 rpm using an SS-34 rotor in a Sorvall preparative centrifuge. The supernatant was loaded directly onto a 2-mL ATP-agarose column (30) previously equilibrated with the mitochondrial homogenization buffer. The column was washed with 14 mL of equilibration buffer, 2 mL of equilibration buffer containing 2 mM GTP, and the mtHsc70 eluted with 2 mL of equilibration buffer containing 3 mM ATP. Samples of each fraction were assayed for protein content, and the protein-containing peaks analyzed by SDS-PAGE and western blotting. The mtHsc70-containing fractions were combined and loaded onto a 95 × 2.6 cm column of Sephacryl S200 previously equilibrated with 50 mM BisTris-HCl, pH 6.0, containing 20 mM $CaCl_2$. mtHsc70-containing fractions were again determined by SDS-PAGE plus western blotting. Peak fractions were combined and the mtHsc70 concentrated by the addition of ammonium sulfate to 80% of saturation. A typical preparation of mitochondria, starting with 500 g of 14-d seedlings, yielded 53 mg of total soluble protein and, after purification, 420 μg of mtHsc70. This suggests a relatively low abundance (0.8%) of Hsc70 in the mitochondria, although the amount could vary with age or developmental state (23).

Phosphorylation of the Pea mtHsc70 Homolog

Phosphorylation of pea mtHsc70 was assayed under the conditions described in ref. 14, using endogenous Hsc70 as the substrate. Incorporation of ^{32}P from [γ-^{32}P]MgATP into mtHsc70 was quantitated using an AMBIS Radioanalytic Imaging System. To test for autophosphorylation, highly purified pea mtHsc70 was incubated with [γ-^{32}P]MgATP for 30 min at room temperature. After incubation, the sample was mixed with an equal volume of 20% (w/v) TCA and the acid-insoluble material was collected by centrifugation. The acid-insoluble pellet was dissolved, subjected to SDS-PAGE, and radioactivity detected using an AMBIS Radioanalytic Imaging System.

Phosphoamino Acid Analysis

For phosphoamino acid analysis, samples were precipitated with 10 volumes of ice-cold acetone, washed three times, airdried, acid-hydrolyzed, and the hydrolysate was examined by two-dimensional thin layer electrophoresis (11). The positions of the phosphoamino acid standards were detected by spraying the plates with 0.25% (w/v) ninhydrin in acetone. Radioactive spots were detected by radioanalytic imaging.

Other Analytical Methods

The methods for sample preparation, SDS-PAGE, and western blotting have been described (20). For quantitation of protein phosphorylation, gels were dried, then scanned using the AMBIS Imaging System. Quantitation was accomplished using the AMBIS software and is based upon total cpm detected per protein band minus the sample background chosen for each lane. The methods for both catalytic and immunochemical assay of the PDC have been described (24).

RESULTS AND DISCUSSION

Hsp70 homologs have been found in the cytoplasm, mitochondria, and ER of mammalian and yeast cells, and in the cytoplasm, chloroplasts, and ER of higher plant cells (21, 28). In addition, an Hsp70 homolog has been identified in mitochondria from *Euglena* (1, 14) and higher plants (21). Furthermore, Watts et al. (29) recently reported the isolation of

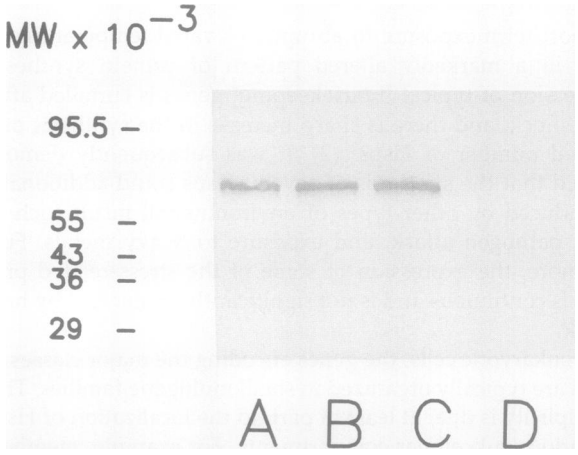

Figure 1. Immunoblot analysis of pea mtHsc70. Lane A, Purified mitochondria; lane B, a mitoplast fraction; lane C, purified mitochondria subjected to limited proteolysis; lane D, mitochondria treated with Triton X-100 at a final concentration of 0.05% prior to proteolysis. In each case, an equivalent amount of sample was analyzed. Samples were precipitated with TCA, washed, dissolved, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was then incubated with anti-tomato Hsc70 antiserum followed by alkaline phosphatase-conjugated secondary antibody.

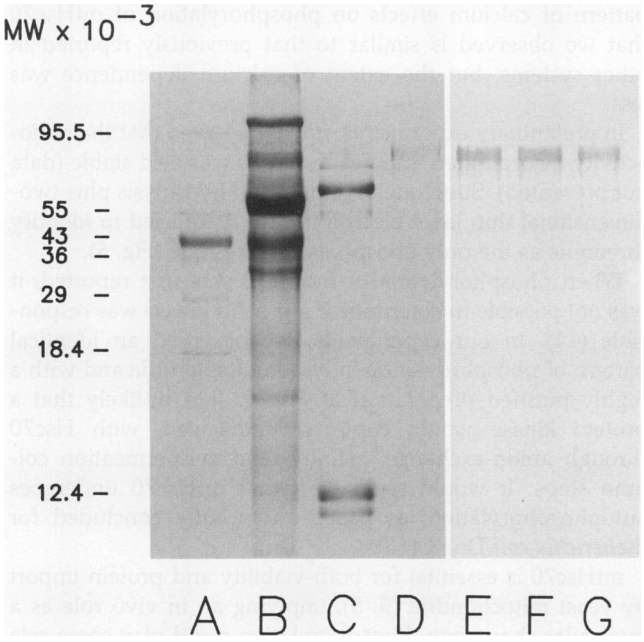

Figure 2. Purification of the pea mtHsc70 homolog. Lane A, Marker proteins; lane B, the matrix fraction isolated from purified mitochondria; lane C, proteins eluted from the ATP-agarose column; lane D, the Hsc70 peak from gel-permeation chromatography. Lanes E through G are a repeat of samples B through D, transferred to a nitrocellulose membrane and detected with anti-tomato Hsc70 antiserum.

a cDNA that encodes a mtHsc70 from pea. Using anti-tomato Hsc70 (Fig. 1) or monoclonal anti-rat Hsp70 (data not shown) antibodies, we have verified the presence of an Hsp70 homolog in mitochondria isolated from light-grown pea seedlings. Because the mitochondrial Hsp70 homolog does not require heat shock for expression, it is more appropriate to refer to it

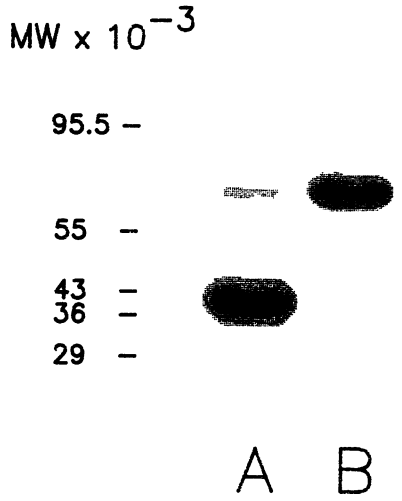

Figure 3. Phosphorylation of pea seedling mitochondrial proteins in vitro. Lane A, Mitochondrial matrix fraction incubated with 200 μM [γ-^{32}P]ATP at pH 7.5; lane B, a matrix fraction incubated with ATP at pH 6.0 in the presence of 20 mM $CaCl_2$.

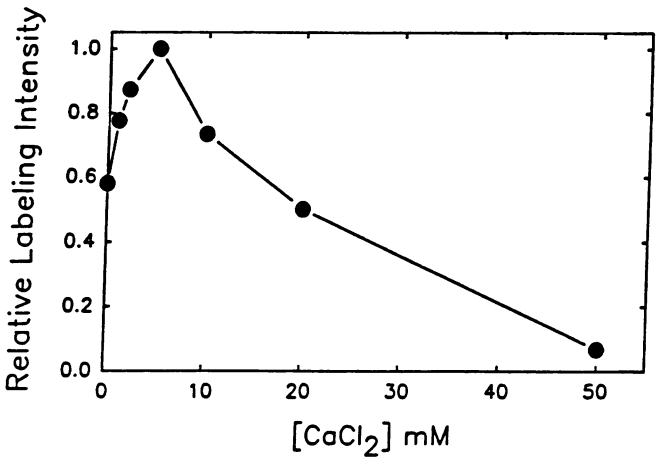

Figure 4. The effects of calcium concentration upon phosphorylation of the pea seedling mtHsc70. The zero calcium samples contain 2 mM EGTA. Samples were incubated for 30 min at room temperature in 50 mM BisTris-HCl, pH 6.0, with 200 μM MgATP. After incubation, proteins were separated by SDS-PAGE and the extent of phosphorylation quantitated by radioanalytic imaging.

as a mtHsc70. Neither physical removal of the outer membrane nor limited proteolysis of intact pea mitochondria significantly reduced the amount of mtHsc70 (Fig. 1). Mild alkaline treatment (25 mM 3-[cyclohexylamino)]-2-hydroxy-1-propanesulfonic acid-KOH, pH 9.1) of mitoplasts, however, released nearly all the mtHsc70 (data not shown). Distribution of mtHsc70 during isolation and subfractionation of pea mitochondria was identical to that of the PDC, suggesting that it is a soluble protein localized within the matrix compartment.

We further characterized the pea mtHsc70 by ATP-agarose

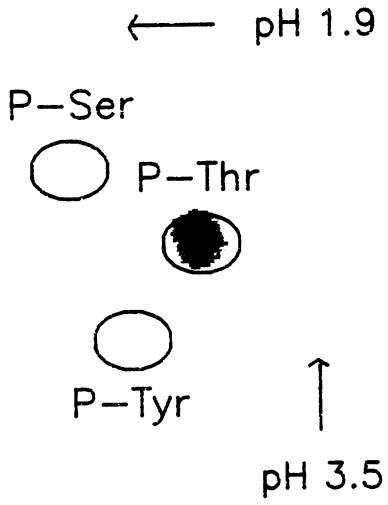

Figure 5. Phosphoamino acid analysis of the pea seedling mtHsp70 homolog. After phosphorylation in vitro, samples were acid-hydrolyzed, then analyzed by two-dimensional thin layer electrophoresis. The positions of authentic standards were determined by spraying the plates with ninhydrin. The positions of radioactive samples were determined by radioanalytic imaging.

affinity chromatography (30). Several mitochondrial matrix proteins bound to and were eluted from the column by ATP (Fig. 2). Based upon electrophoretic mobility, we believe that the major ATP-binding protein is mitochondrial Hsp60, an abundant plant mitochondrial protein (23). From the mixture of proteins that bound to ATP-agarose, a fraction containing apparently homogeneous mtHsc70 was selected by gel permeation chromatography (Fig. 2).

The purified protein gave a strong signal on western blots probed with anti-tomato Hsc70 antibodies (Fig. 2). Verifying the specificity, no signal was observed on western blots when an excess of Hsc70, purified from maize endosperm cells, was included during incubation with the antibodies (data not shown). Thus, our identification of the pea protein as an authentic mtHsc70 is based upon electrophoretic mobility, cross-reaction with anti-Hsc70 and Hsp70 antiserum, subcellular localization, and the ability to bind to immobilized ATP.

When mitochondria were disrupted at pH 7.5 and the matrix fraction incubated with [γ-^{32}P]ATP, a single major radioactive band with an M_r of 43,000 was observed after SDS-PAGE and radioanalytic imaging (Fig. 3). We have previously identified this band as the $E_{1\alpha}$-subunit of the PDC (24). However, when mitochondria were both disrupted and incubated at pH 6.0 plus 20 mM $CaCl_2$, labeling of the M_r 43,000 band was greatly decreased and there was labeling of an M_r 70,000 band (Fig. 3). A similar $CaCl_2$-stimulated phosphorylation of an M_r 70,000 protein has been seen with mitochondrial preparations from mammalian cells, yeast, and *Euglena gracilis* (1, 2, 14).

Protein phosphorylation is often regulated by calcium in both animal and plant systems, and calcium plays an important role in regulating mammalian mitochondrial metabolism (6). Increasing intramitochondrial calcium decreases the proportion of PDC that is in the inactive, phosphorylated state. However, physiological micromolar concentrations of calcium were without effect upon the phosphorylation state of the PDC in intact mitochondria isolated from pea seedlings (4). The labeling of mammalian mtHsc70 was essentially dependent upon high concentrations of added calcium (14). In contrast, we observed considerable labeling of pea mtHsc70 when incubations were conducted without added calcium in the presence of 2 mM EGTA (Fig. 4). Labeling of pea mtHsc70 was increased by the addition of calcium, with a 2-fold stimulation at 5 mM. Higher concentrations inhibited labeling of pea mtHsc70, as was seen with mammalian and *Euglena* mtHsc70s (1, 14). Hsc70 sequences contain a potential calmodulin-binding domain (27), and it is possible that this regulator plays some role in calcium-stimulated autophosphorylation. This was addressed by preincubation of purified mtHsc70 with the potent sulfonamide calmodulin antagonist W-7 (19), at a final concentration of 200 μM, prior to addition of MgATP. Under these conditions, W-7 had no effect upon the autophosphorylation of pea mtHsc70 (data not presented), providing no support for an involvement of calmodulin. The relatively high concentrations of calcium that stimulate the basal phosphorylation of mtHsc70 could be an artifact of the *in vitro* experiments. It is possible that essential factors are lost or diluted to excess during isolation and disruption of mitochondria, and that more physiological concentrations of calcium are active in vivo. Overall, the pattern of calcium effects on phosphorylation of mtHsc70 that we observed is similar to that previously reported in other systems, but the extent of calcium dependence was less.

In preliminary experiments, it was observed that the radioactivity incorporated into pea mtHsc70 was acid stable (data not presented). Subsequently, mild acid hydrolysis plus two-dimensional thin layer electrophoresis were used to identify threonine as the only phosphorylated residue (Fig. 5).

When phosphorylation of mtHsc70 was first reported, it was not possible to determine if a protein kinase was responsible (14). In our experiments, we observed an identical pattern of phosphorylation in lysed mitochondria and with a highly purified preparation of Hsc70. It is unlikely that a protein kinase would copurify, undetected, with Hsc70 through anion-exchange, affinity, and gel-permeation column steps. It would seem then that mtHsc70 undergoes autophosphorylation, as has been recently concluded for *Escherichia coli* DnaK (16).

mtHsc70 is essential for both viability and protein import by yeast mitochondria (3, 5), implying an in vivo role as a molecular chaperone. Phosphorylation could play some role in chaperone function. We are currently undertaking studies of the synthesis and assembly of the mitochondrial PDC to directly evaluate the potential of mtHsc70 to function as a molecular chaperone in these processes.

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