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Heat shock proteins of barley mitochondria and chloroplasts

Identification of organellar hsp 10 and 12: putative chaperonin 10 homologues

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Tissue slices from barley seedlings were subjected to heat shock and metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine. Mitochondria and chloroplasts were isolated and shown to contain two novel heat shock proteins of 10 and 12 kDa, respectively. The possibility that these proteins, like a mitochondrial 10 kDa stress protein recently isolated from rat hepatoma cells [(1992) Proc. Natl. Acad. Sci. 89, in press] represent eukaryotic chaperonin 10 homologues is discussed.

Heat shock protein; Chaperonin; Chloroplast; Mitochondrion; Barley; Rat; Metabolic labelling; Electrophoresis

1. INTRODUCTION

A universal cellular response to heat shock and a number of stressing agents is the synthesis of a select set of proteins collectively termed heat shock proteins (hsps) (reviewed in [1,2]). HSPs are ubiquitous and highly conserved from bacteria to man indicating that they play central roles in cellular metabolism. Although the precise function of many hsps remain an enigma, recent evidence has made it clear that the constitutive expression of hsp 60 in *E. coli* and hsp 70 homologues in *S. cerevisiae* is essential for cell viability due to their involvement in protein targetting and folding [1,2]. Hsp 70 homologues have been identified in chloroplast of higher plants [3] and *Euglena gracilis* [4] and in mitochondria from *Euglena gracilis* [4], pea [5], fungi [6], trypanosomes [7] and mammalian cells [8]. Members of the hsp 60 family, collectively termed chaperonin 60 (cpn 60) [9] have been found in both chloroplasts [10] and mitochondria of many species [8,11–13] where they assist the folding and assembly of both imported and organellar synthesized proteins [9,13,14]. Direct evidence for cpn60 mediated protein folding and assembly in vivo [15] and in vitro [16,17] has come from studies on *E. coli* cpn60 (GroEL), the action of which is critically dependent on a physical interaction with *E. coli* chaperonin 10 (cpn10), also known as GroES [17,18].

Abbreviations: cpn60, chaperonin 60; cpn10, chaperonin 10; hsp, heat shock protein, DMEM, Dulbecco's modified Eagle's medium, MW, molecular weight.

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Bacterial cpn10 is also a requirement for both mitochondrial and chloroplastic cpn60 function in vitro [17]. These observations argue for the existence of an as yet unidentified cpn10 homologue in plant mitochondria and chloroplast. We recently identified and purified a novel rat mitochondrial 10 kDa hsp and showed that this protein represents a functional mammalian cpn10 homologue [19]. The identification of this protein was achieved by in vitro metabolic labelling of stressed cells, followed by cell fractionation, SDS-PAGE and fluorography. Here we use the same approach to identify two novel hsps of 10 and 12 kDa apparent molecular weight (MW) in barley mitochondria and chloroplasts, respectively, and discuss the possibility that these two novel hsps may represent plant cpn10 homologues involved in protein folding and assembly of, organellar proteins in higher plants.

2. MATERIALS AND METHODS

2.1. *Heat shock and metabolic labelling*

Seeds of barley (*Hordeum vulgare* cv. Clipper) were germinated and grown at 24°C for six days on moist vermiculite either in the dark (for mitochondrial isolations) or in continuous light (for chloroplasts isolations). For mitochondrial heat shock experiments 8.0 g of etiolated primary leaf tissue was chopped into 1–2 mm^2 segments with a razor blade and incubated at 25°C for 20 h in 50 ml of sterile water. Following this preincubation, which was required for subsequent efficient metabollic labelling, the tissue slices were incubated for 30 min at 25°C (control) or 37°C (heat shock) and then metabolically labelled for three hours at the same temperatures in the presence of 500 μCi [^{35}S]methionine and [^{35}S]cysteine (New England Nuclear, 'Translabel', specific activity >1000 Ci/mmol) followed by an additional two hours at 25°C for both control and heat shocked leaf segments. The labelled segments were recovered and chlorophyll-free mitochondria purified as described by Day and Hanson [20]. For chloroplast heat shock

experiments 4.9 g of green primary leaf tissue was cut into 1–2 mm^2 segments and incubated at 25°C under constant illumination (30 $\mu E/m^2/s$) for 21 h at 25°C in 50 ml of sterile water prior to addition of 530 μCi [^{35}S]methionine and [^{35}S]cysteine (specific activity >1000 Ci/mmol). Following the addition of labelled amino acids, the leaf segments were incubated for an additional 3 h at 25°C (control) or 37°C (heat shock) and finally for 3 h at 25°C prior to chloroplast isolation as described by Shioi et al. [21]. The heat shock treatment and metabolic labelling of clonal rat hepatoma cells was as described in [19]. Specifically the cells were heat shocked at 42°C for 15 min. Rat cpn 10 was purified as described by Hartman et al. [19] and *E. coli* chaperonin 10 was a kind gift from Drs. N. Dixon and B. Surin of the Australian National University.

2.2. *Additional analytical techniques*

SDS-PAGE in 12% (w/v) Tris-glycine gels [22] and in (16%T, 3%C) Tris-tricine gels [23] was carried out as in [19]. Prior to electrophoretic analysis isolated chloroplasts were precipitated with 90% (v/v) methanol at −20°C overnight, recovered by centrifugation and solubilized in SDS-PAGE sample buffer. Mitochondria were lysed directly in sample buffer without prior precipitation. For fluorography equal amounts of trichloroacetic acid-insoluble radioactive material were loaded in each sample well and the gel treated with Amplify (Amersham) before drying. Western blotting was carried out essentially as in [24] using rabbit anti-yeast cpn 60 (a generous gift from Dr. R.L. Hallberg) and a chemiluminescent detection kit supplied by Amersham. Bovine ubiquitin was obtained from Sigma and ^{14}C-marker proteins were supplied by Amersham. The low molecular weight markers for Coomassie staining were myoglobin CNBr-fragment obtained from Sigma.

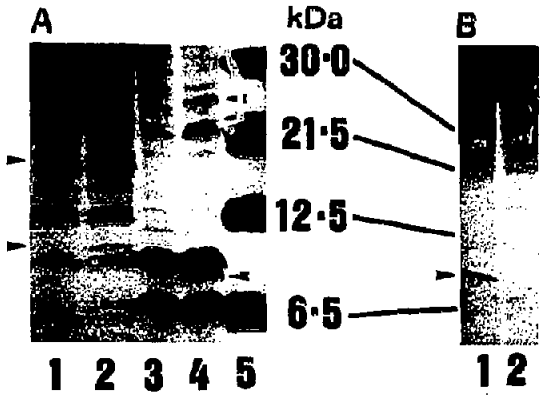

Fig. 1. Analysis of low molecular weight proteins synthesized in response to heat shock in mitochondria and chloroplasts. (A) Chloroplasts were recovered from control (lane 1) or heat shocked (lane 2) leaf segments and mitochondria were recovered from control (lane 3) or heat shocked (lane 4) leaf segments and the content of ^{35}S-labelled polypeptides revealed by electrophoresis in Tris-Tricine gels followed by fluorography. The [^{14}C]methylated molecular weight markers (lane 5) were obtained from Amersham. (B) Mitochondria were derived from heat shocked (lane 1) or control (lane 2) rat hepatoma cells and the ^{35}S-labelled polypeptide content analysed as in (A). Arrows indicate proteins that are synthesized at an elevated rate in response to the heat shock. *Note:* the apparent molecular weights assigned to the stress inducible proteins are not based on the [^{14}C]molecular weight markers employed in here but on the use of stained protein markers shown in legend to Fig. 2 and on the co-electrophoresis with rat mitochondrial chaperonin 10 of MW 10.8 kDa.

3. RESULTS

Tissue slices of 6 day old etiolated barley seedlings were pre-incubated in water at 25°C for 20 hours and then further incubated for 3 hours in the presence of [^{35}S]Met and [^{35}S]Cys at either 25°C (control) or 37°C (heat shock) followed by an additional 2 hours labelling at 25°C. Following metabolic labelling chlorophyl-free mitochondria were isolated and analysed by SDS-PAGE in Tris-tricine gels to identify low molecular weight mitochondrial heat shock proteins. Two low molecular weight proteins of apparent MW 24 kDa and 10 kDa were clearly induced (Fig. 1A, lanes 3 and 4). The electrophoretic mobility of the novel 10 kDa protein is identical to that of rat hsp10, a eukaryotic cpn10 homologue recently identified by its distinctive migration during the SDS-PAGE system employed herein (Fig. 1B) [19].

When labelled low molecular weight chloroplast proteins derived from heat shocked tissue slices and control tissue slices were analysed two distinct hsps of 18 kDa and 12 kDa were seen (Fig. 1A, lanes 1 and 2). The difference in electrophoretic mobility between the mitochondrial and chloroplast components of apparent molecular weights 10 and 12 kDa, respectively, does not necessarily reflect true differences in molecular mass. Similar differences in electrophoretic mobilities are observed when rat mitochondrial chaperonin 10 (true molecular weight 10,813.4 Da [19], apparent molecular weight 10 kDa) and *E.coli* chaperonin 10 (true molecular weight 10,370 Da [10], apparent molecular weight 12 kDa are analysed (Fig. 2). The electrophoretic mobility of the 10 and 12 kDa stress proteins was clearly different from that of bovine ubiquitin, a known stress protein [25] which migrates with an apparent molecular weight of 7 kDa in the Tris-tricine gel system (data not shown).

Analysis of high molecular weight proteins induced in response to the heat shock treatment was also performed and revealed a clear induction of a 72 kDa and a 60 kDa component in mitochondria (Fig. 3B). In chloroplasts, high molecular weight compounds of 75, 72 and 60 kDa were clearly induced (Fig. 3A). Western blot analysis of barley mitochondrial and chloroplast extracts using rabbit anti-yeast chaperonin 60 antibodies revealed crossreactive components which co-electrophoresed exactly with the inducible 60 kDa polypeptides (data not shown).

4. DISCUSSION

Identification of heat shock proteins in higher plants has primarily relied on 2D-gel electrophoretic analysis of ^{35}S-labelled proteins extracted from whole tissues (e.g. [26,27]) and in some cases subcellular fractionation has been employed prior to electrophoretic analysis [28–31]. These studies have revealed that plants in addition to hsps of other eukaryotes synthesize a large number of low molecular weight hsps of apparent MW 15–24 kDa. Some of these low molecular weight heat shock proteins are located in chloroplast [29] and some may

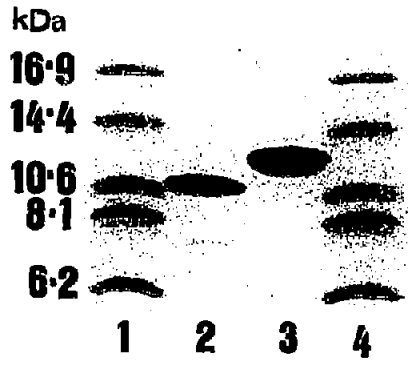

Fig. 2. Electrophoretic analysis of two purified cpn10 homologues. Purified rat cpn10 (lane 2) and *E. coli* (lane 3) cpn10 were electrophoresed in a Tris-tricine gel and visualized by staining with Coomassie brilliant blue R-250. The CNBr fragments of myoglobin (Sigma) were used as molecular weight markers (lanes 1 and 4).

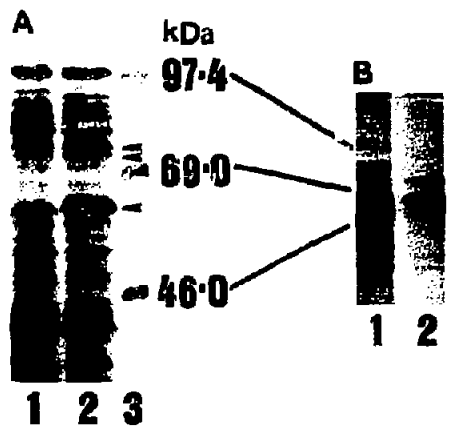

Fig. 3. Analysis of high molecular weight polypeptides synthesized in barley chloroplasts and mitochondria in response to heat shock. (A) Chloroplasts were recovered from control (lane 1) or heat shocked leaf segments and the ^{35}S-labelled polypeptide content revealed by electrophoresis in a Tris-glycine gel followed by fluorography. (B) Mitochondria were recovered from control (lane 1) or heat shocked leaf segments (lane 2) and the [^{35}S]polypeptide content revealed by electrophoresis in a Tris-tricine gel followed by fluorography. When analysed on the same gel the inducible chloroplast and mitochondrial hsp 60 comigrate (data not shown).

be found in mitochondria [28,31]. Most, if not all, electrophoretic procedures employed in earlier studies, however, have an apparent inability to resolve proteins of subunit molecular weights below 12–14 kDa and as a consequence the presence of very low molecular weight plant hsps may have escaped detection. In this study we have employed the Tris-tricine system of Schäger and von Jägow capable of resolving proteins in the 2–14 kDa range [23] to analyse the polypeptide content in mitochondria and chloroplasts derived from heat shocked barley leaf segments. The induction pattern of mitochondria was remarkably simple revealing the heat shock enhanced synthesis of only four polypeptides of apparent MWs 72 kDa, 60 kDa, 24 kDa and 10 kDa. The identity of the 72 kDa polypeptide is unknown, but it could be a mitochondrial hsp70 homologue. Heat inducible hsp 70 homologues have now been positively identified in plant [5], fungal [6], mammalian [8], trypanosomic [7] and algal [4] mitochondria. The 60 kDa polypeptide is likely to represent mitochondrial cpn60 which previously has been shown to exist as a heat-inducible component of maize mitochondria [12] or alternatively it represents the mitochondrially encoded hsp60 of maize and Brassica described by Sinibaldi and Turpen [32]. In any case barley mitochondria contain a cpn 60 molecule [12] and it is in this context that the identification of the novel 10 kDa stress protein is significant. Chaperonin 60 in the matrix of *S. cerevisiae* mitochondria is essential for folding of imported protein in vivo [13]. Bacterial, plant and fungal chaperonin 60 homologues have been shown to catalyse protein-folding in vitro but in most cases this folding could only be shown in the presence of the heat inducible *E. coli* cpn 10 [16]. These observations argue for the presence of an organellar version of cpn 10. Plant cpn 10 versions have not yet been reported but we have recently used heat shock treatment to identify a novel mitochondrial 10 kDa heat shock protein in cultured rat hepatoma cells [19] (see Fig. 1C) and shown this to be a cpn10 homologue. It was therefore expected, that a plant cpn10 homologue would be identifiable by its subcellular location and heat inducibility. The novel 10 kDa hsp identified herein, fits these criteria and may therefore represent plant mitochondrial cpn10. The identity of the 24 kDa stress protein heat induced in these studies is not known but, mitochondrial 22 kDa and 24 kDa hsps have been observed in soybean [33].

Analysis of chloroplast proteins revealed the presence of heat shock inducible proteins with apparent MWs of 75, 72, 60, 18 and 12 kDa, respectively. Three hsp70 homologues of apparent MW 75 kDa, 75 kDa and 78 kDa, respectively, have been detected in pea chloroplasts by immuno-blotting [3]. The heat shock inducible 75 kDa and 72 kDa polypeptides revealed in this study therefore probably reflect the presence of these hsp70 members in barley chloroplasts and the apparent lack of heat shock induction of these proteins in pea [3] may be related to the differing methods of detection and/or stress application used in this and the previous study [3].

Likewise, although the chloroplast RUBISCO binding protein is a member of the heat shock inducible cpn 60 family, no information concerning the expression of this protein during heat stress has been published [2]. We observed a very considerable induction of a 60 kDa polypeptide in chloroplasts. The nuclear encoded RUBISCO-binding protein may therefore be synthesized at elevated rates in response to heat shock. On the other hand, Krishnasamy et al. [34] reported the presence of a plastid encoded 60 kDa hsp in 7-day-old *Vigna sinensis*. The identity of the 60 kDa component induced by heat shock in this study therefore is uncertain as is the identity of the 18 kDa heat shock protein. The identifi-

cation of the novel 12 kDa heat shock protein is intriguing for the same reasons stated for the mitochondrial 10 kDa component. The role of these two low molecular weight proteins in the higher plant stress response is not clear but the fact that mammalian mitochondria synthesize a similar sized cpn10 homologue in response to stress indicates that they, like cpn60 homologues, are of ubiquitous presence and therefore may represent plant cpn 10 homologues. Based upon their heat shock inducibility these proteins can now be purified and characterized to test this possibility.

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