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The mitochondrial small heat-shock protein protects NADH:ubiquinone oxidoreductase of the electron transport chain during heat stress in plants

Craig A. Downs*, Scott A. Heckathorn

Department of Biology, University of Charleston, 58 Coming Street, Charleston, SC 29424, USA

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Abstract Functional inactivation of the mitochondrial small heat-shock protein (lmw Hsp) in submitochondrial vesicles using protein-specific antibodies indicated that this protein protects NADH:ubiquinone oxidoreductase (complex I), and consequently electron transport from complex I to cytochrome *c*:O_2 oxidoreductase (complex IV). Lmw Hsp function completely accounted for heat acclimation of complex I electron transport in pre-heat-stressed plants. Addition of purified lmw Hsp to submitochondrial vesicles lacking this Hsp increased complex I electron transport rates 100% in submitochondrial vesicles assayed at high temperatures. These results indicate that production of the mitochondrial lmw Hsp is an important adaptation to heat stress in plants.

Key words: Small heat-shock protein; Mitochondrion; Oxidative phosphorylation; Heat stress

1. Introduction

NADH:ubiquinone oxidoreductase (complex I) is usually the most thermolabile protein complex of oxidative phosphorylation [1–4]. Under most physiological conditions, complex I has a molecular mass of 700–900 kDa and is composed of 26–32 subunits [5–8]. During heat stress conditions, complex I breaks down to a lower molecular mass form of about 350 kDa and exhibits lower NADH:ubiquinone oxidoreductase activity rates [1,5]. At heat stress temperatures, components of oxidative phosphorylation other than complex I often exhibit increased rates of activity [1,9–11], but oxidative phosphorylation as a whole shows deceased rates of activity, indicating that complex I may be the limiting element of oxidative phosphorylation during heat stress [11]. Adaptations that protect complex I from heat stress are largely unknown. However, evidence suggests that the heat-shock protein response, and specifically a low-molecular-weight heat-shock protein (lmw Hsp), is involved in the thermotolerance of oxidative phosphorylation. For example, several studies have demonstrated that thermotolerance of oxidative phosphorylation is correlated with the induction of Hsps [12–16]. Potent inhibitors of electron transport or ATP production, specifically inhibitors of complex I, act as inducers of Hsps [12]. Induction of Hsps by heat stress before the addition of complex I inhibitors confers tolerance to complex I against these inhibitors, while suppression of Hsp synthesis prevents tolerance to these inhibitors [13]. However, a definite role for a specific Hsp in the thermotolerance of complex I or oxidative phosphorylation has not been demonstrated.

The mitochondrial lmw Hsp is usually produced only in response to environmental stress [14–19]. It contains a conserved C-terminal 'heat-shock domain' as well as a second conserved domain that exhibits roughly 70% amino acid homology among all known members of mitochondrial lmw Hsp [19] and strongly associates in a temperature-dependent manner with the matrix side of the mitochondrial inner membrane [20,21]. Correlative evidence suggests that the mitochondrial lmw Hsp is associated with the thermotolerance of oxidative phosphorylation, but no correlation was demonstrated between the presence of the lmw Hsp and protection of electron transport from the succinate:ubiquinone oxidoreductase complex (complex II) to the cytochrome *c*:O_2 oxidoreductase complex (complex IV) from heat stress [11].

Purified lmw Hsps from both plants and animals have been shown to prevent aggregation of other proteins in vitro [22,23]. Recently, it was demonstrated the lmw Hsp in the chloroplast protects photosynthetic electron transport during heat stress [24]. These studies indicate that lmw Hsps are an important adaptation to heat stress, perhaps by functioning as protective molecular chaperones. In this study, we directly test whether the mitochondrial lmw Hsp confers thermotolerance to electron transport in mitochondria and which component of electron transport it protects.

2. Materials and methods

Pyrus pumila (P. Mill.) K. Koch var. 'McIntosh' fruit (apple) submitochondrial vesicle samples were prepared from both unstressed and heat-stressed fruits using a method modified from Douce [25,26]. Apples were homogenized in a buffer (MIB) consisting of 0.30 M sorbitol, 50 mM HEPES/KOH (pH 7.4), 2 mM EGTA, 5 mM DTT, 4 mM L-cysteine, 3% (w/v) PVP, and 30 units of trypsin per 1 ml of buffer. Trypsin treatment of the mitochondria during preparation ensures that mitochondria are intact and inhibits contamination of the mitochondria from non-mitochondrial lmw Hsps. The homogenate were filtered through muslin cloth and subjected to differential centrifugation (as in [25,26]). Mitochondria were then isolated on a discontinuous Percoll step gradient [27,28]. The mitochondrial fraction was collected and washed with MIB lacking trypsin, but containing trypsin inhibitor, and then centrifuged at $10\,000\times g$ for 10 min. The resulting pellet was resuspended in either a potassium phosphate buffer or triethanolamine buffer, depending on the assay (see below). The samples were frozen at −80°C, thawed, sonicated, and then centrifuged at $100\,000\times g$ for 20 min. The pellet was resuspended in either the oxidase assay buffer or the NADH dehydrogenase buffer.

Electron transport from complex I through complex III and complex IV (NADH oxidase) or complex II to complex IV (succinate oxidase) was measured polarographically using a Clark-type electrode. Submitochondrial vesicles were suspended in a 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM EGTA. NADH concentration to produce maximal NADH oxidase activity was determined and NADH oxidase activity was measured using 0.25 mM NADH,

*Corresponding author.
E-mail: cadowns@technologist.com

Abbreviations: lmw Hsp, low-molecular-weight heat-shock protein

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0.5 mM thenoyltrifluoroacetone (an inhibitor of complex II), and 10 mM SHAM (an inhibitor of alternative oxidase activity) [1]. Succinate concentration to produce maximal succinate oxidase activity was determined and succinate oxidase activity was measured using 20 mM succinate, 50 μM rotenone, and 10 mM SHAM [1,29,30]. NADH was added 30 s before the succinate during the succinate oxidase assay to convert succinate dehydrogenase from an inactive to an active state [29]. Succinate oxidase activity was measured at 28°C and 48°C, while NADH oxidase activity was measured at 28°C, 40°C, and 48°C. For each oxidase assay, submitochondrial vesicles were incubated with either: no protein added; BSA (0.4 mg/ml); Ab_α pre-immune serum (1:350 v/v); or Ab_α (1:350 v/v). In preliminary experiments, dilutions (v/v) of Ab_α of 1:50, 1:200, 1:350, 1:1400, and 1:5600 were used to determine the optimal concentration for lmw Hsp inactivation. The rate of electron transport was determined from the rate of liquid-phase O_2 exchange. Protein concentration was determined as described [31]. A concentration of 25 μg/ml of submitochondrial vesicle protein was used for all oxidase assays. Electron transport rates were measured for 7–15 min to ensure linearity.

Polyclonal rabbit antiserum with broad specificity (among species and lmw Hsp classes) to the α-crystallin region of plant lmw Hsps (Ab_α) was generated and characterized as previously described [24]. Mitochondrial lmw Hsp was purified by immuno-affinity column chromatography, using the method previously described [24]. Mitochondria were isolated from fruit that had been incubated at 40°C for 36 h in a growth chamber. Hsp purity was determined by SDS-PAGE (12.5% gel; 5 μg total protein), followed by silver staining for protein detection (Fig. 2A). Identity of the lmw Hsp was confirmed by immuno-blotting as previously described [24] (Fig. 2B). Replicate Western blots of the samples were assayed with mitochondrial Hsp 60 antibody and chloroplast chaperonin 70 antibody to ensure purity of the mitochondrial sample (data not shown).

NADH dehydrogenase activity was assayed spectrophotometrically by the rate of NADH-dependent ferricyanide reduction at 420 nm [1]. Ferricyanide concentrations of 0.15, 0.35, 0.60, 0.85, 1.0 and 1.25 mM were used to determine V_{max} or K_m for each assay temperature treatment. Submitochondrial vesicles were incubated with either no added protein, or the addition of either Ab_α (1:350 v/v), BSA (0.4 mg/ml), or purified mitochondrial lmw Hsp (4.85 μg/ml) for 7.5 minutes at either 28°C, 40°C, or 48°C before being assayed at each indicated temperature. Additions of 4 mM EGTA and/or 5 mM SHAM did not affect activity rates at any of the three assay temperatures.

Results were analyzed by three-way (control/heat stress × incubation temperature × protein addition) analysis of variance (ANOVA). Differences among proteins within each treatment combination were analyzed by one-way ANOVA within each non-stressed and heat-stressed sample × incubation temperature combination followed by Fisher's least significant difference test.

3. Results

To examine the importance of the lmw Hsp to mitochondrial electron transport, we used polyclonal antibodies specific to the conserved 'heat-shock domain' common to all plant lmw Hsps (Ab_α) to disrupt the function of the lmw Hsp in mitochondria isolated from *Pyrus pumila* fruits (apples). We then monitored electron transport either (1) from complex II through ubiquinone:cytochrome *c* oxido:reductase (complex III) to complex IV (here referred to as succinate oxidase electron transport), or (2) electron transport from complex I through complex III to complex IV (referred to as NADH oxidase electron transport). We monitored electron transport by measuring oxygen exchange in submitochondrial vesicles isolated from unstressed control fruits and fruits that had been heat-stressed at 43°C (pre-heat-stressed fruit). Succinate oxidase electron transport in control and pre-heat-stressed fruits was assayed at both 28°C and 48°C. NADH oxidase electron transport in control and pre-heat-stressed fruits was assayed at 28°C, 40°C, and 48°C.

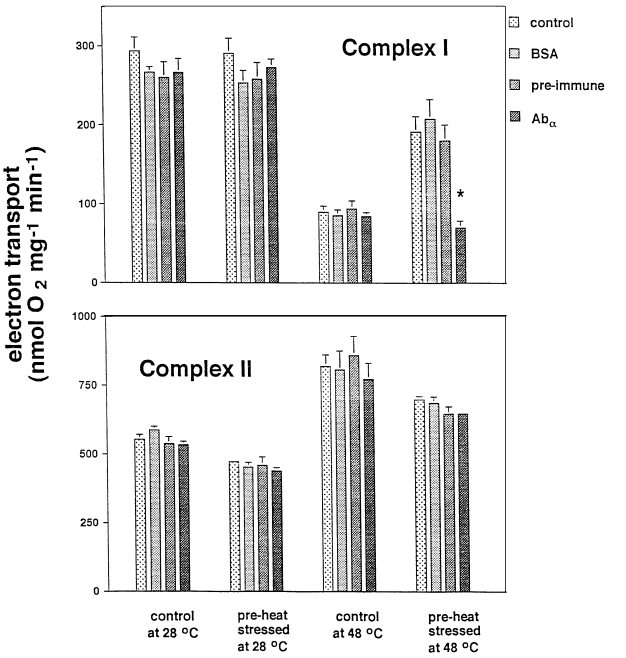

Fig. 1. The effect of antibodies to the lmw Hsp on mitochondrial electron transport. *Pyrus pumila* apples were incubated at either 25°C or 40°C for 36 h. Submitochondrial vesicles purified from either non-stressed (control) or heat-stressed fruit were incubated at 28°C or 48°C for either (A) complex I (NADH oxidase) or (B) complex II (succinate oxidase) electron transport. Either no added protein, BSA (0.4 mg/ml), pre-immune antiserum of Ab_α (1:350 v/v), or antiserum specific to the 'heat-shock domain' of plant low-molecular-weight heat-shock proteins (Ab_α) (1:350 v/v) was added to non-stressed or pre-heat-stressed submitochondrial vesicles. Results are means ± 1 S.E.; $n = 3$–4 (each from separate sets of submitochondrial vesicle preparations). Differences ($P < 0.05$) among protein additions within each control/heat stress × incubation-temperature combination are indicated by an asterisk.

The rate of complex I (NADH oxidase) electron transport was lower when assayed at 48°C than at 28°C (ANOVA; $P < 0.0001$), but electron transport was greater in pre-heat-stressed samples at 48°C relative to non-stressed samples at 48°C ($P < 0.05$), indicating that acclimation to high temperature occurred in pre-heat-stressed samples (Fig. 1, upper panel). This acclimation appeared to be due entirely to the production of the mitochondrial lmw Hsp, because addition of Ab_α, which we predicted would disrupt the function of the mitochondrial lmw Hsp, decreased electron transport in pre-heat-stressed plants incubated at 48°C by 63% in comparison to pre-heat-stressed control samples at 48°C. Addition of pre-immune serum or BSA had no effect on electron transport of mitochondria from pre-heat-stressed samples assayed at 28°C or 48°C, or in non-stressed samples assayed at 28°C or 48°C. No effect of Ab_α was observed in non-stressed samples at either 28°C of 48°C, as expected, since we observed no significant accumulation of the mitochondrial lmw Hsp in plants in the absence of heat stress (data not shown). Also, no effect of Ab_α was observed at 28°C in pre-heat-stressed samples. Nearly identical results were obtained when samples were incubated at 40°C, rather than at 48°C (data not shown).

In contrast to complex I/NADH oxidase function, the rate of electron transport of complex II (succinate oxidase) was greater when assayed at 48°C than at 28°C (ANOVA; $P < 0.0001$) (Fig. 1, lower panel), as is often the case [11].

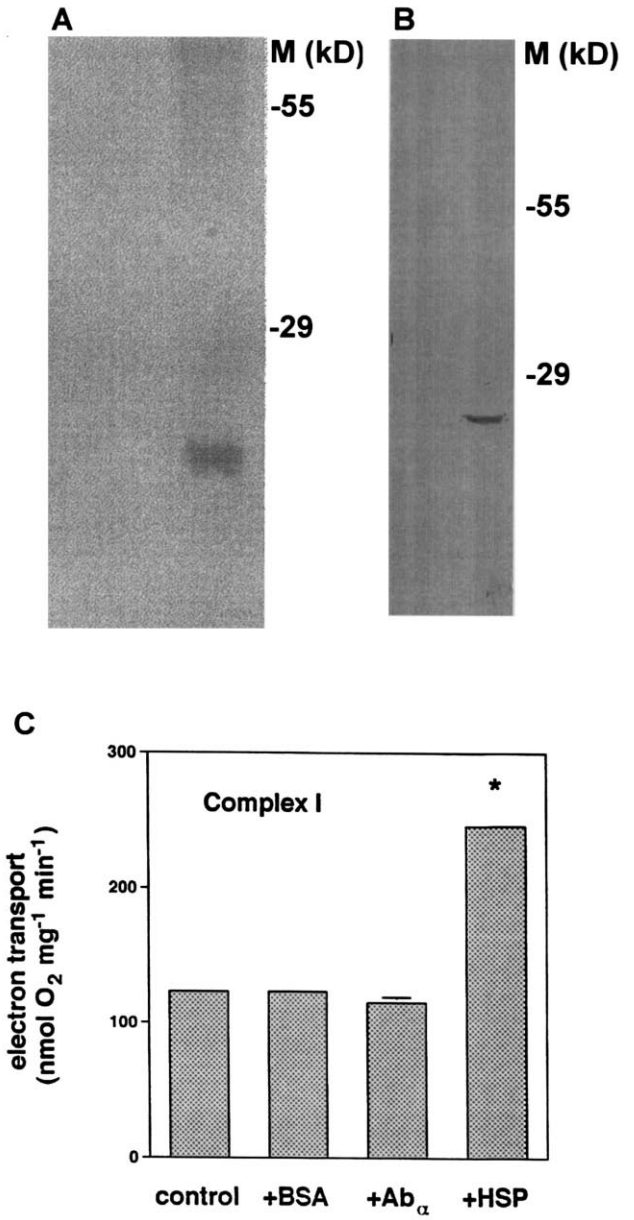

Fig. 2. A, B: Homogeneity of the mitochondrial sHsp purified from intact mitochondria isolated from heat-stressed *Pyrus pumila* apples. Silver stain (A) and immunoblot (Western) (B) of protein samples eluted from Ab column. Eluted proteins were fractionated by gel electrophoresis. C: The effect of purified mitochondrial lmw Hsp on complex I (NADH oxidase) electron transport. *Pyrus pumila* apples were incubated at 25°C for 36 h before submitochondrial vesicles were prepared. Submitochondrial vesicles were incubated with either no added protein, BSA (0.4 mg/ml), antiserum specific to the 'heat-shock domain' of plant low-molecular-weight heat-shock proteins (Ab_α) (1:350 v/v), or purified mitochondrial lmw Hsp (9.7 μg/ml) and assayed at 40°C (for rates at 28°C, see Fig. 1, top panel). Differences ($P < 0.05$) among protein additions are indicated by an asterisk.

In addition, pre-heat-stressed samples had slightly lower rates of electron transport than did controls ($P < 0.0001$). Succinate oxidase electron transport was unaffected by antibody/protein additions in both non-stressed and pre-heat-stressed samples ($P = 0.58$). Collectively, results from these two experiments indicate that (1) there was no detrimental effect of heat stress on complex II, complex III, or complex IV, since electron transport from complex II to III to IV did not decrease at high temperatures; (2) complex I was negatively affected by heat stress, however; and (3) the mitochondrial lmw Hsp protects complex I during heat stress.

To determine if the mitochondrial lmw Hsp could protect complex I/NADH oxidase electron transport during heat stress when added to submitochondrial vesicles lacking this protein, we purified the mitochondrial lmw Hsp from intact mitochondria isolated from heat-stressed apple fruits to apparent homogeneity by antibody affinity-column chromatography using Ab_α, as in [24], and then monitored the effects of purified lmw Hsp on NADH oxidase electron transport (Fig. 2C). Silver staining of total protein eluted from the columns and subjected to SDS-PAGE indicated that only one protein (ca. 24 kDa) was purified (Fig. 2A). There were no differences in electron transport rates of submitochondrial vesicles from unstressed plants among samples incubated at 40°C with either no added protein (control), or with BSA or Ab_α added (Fig. 2C). When purified mitochondrial lmw Hsp was added to submitochondrial vesicles from unstressed control plants (i.e. plants that did not contain the lmw Hsp) incubated at 40°C, the rate of electron chain transport was 100% greater of that exhibited by control samples incubated at 40°C and was approximately equal to the rates of pre-heat-stressed samples (see Fig. 1). These results confirm that the mitochondrial lmw Hsp confers thermotolerance to NADH oxidase electron chain transport.

To confirm if the mitochondrial lmw Hsp specifically protected complex I, we monitored the activity of NADH dehydrogenase of complex I spectrophotometrically by measuring the reduction of ferricyanide (coupled to oxidation of NADH

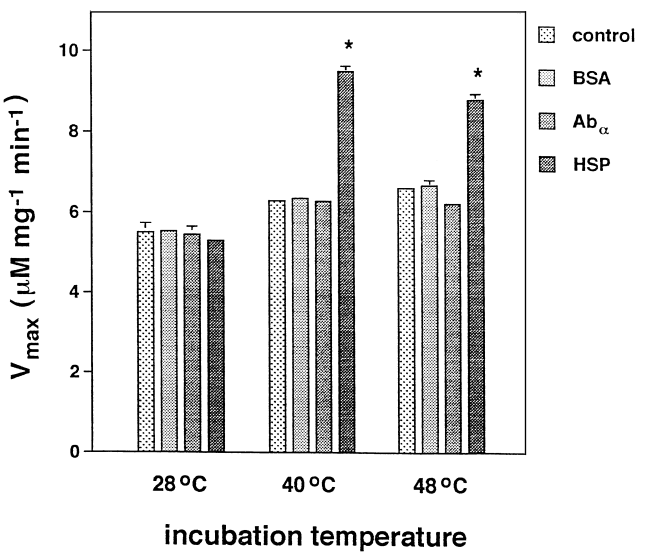

Fig. 3. The effect of purified mitochondrial lmw Hsp on NADH dehydrogenase activity. NADH dehydrogenase activity was assayed spectrophotometrically by the rate of NADH-dependent ferricyanide reduction at 420 nm. *Pyrus pumila* apples were exposed to 25°C temperatures for 36 h before submitochondrial vesicles were prepared. Submitochondrial vesicles were incubated with either no protein added, BSA (0.4 mg/ml), antiserum specific to the 'heat-shock domain' of plant low-molecular-weight heat-shock proteins (Ab_α) (1:350 v/v), or purified mitochondrial lmw Hsp (9.7 μg/ml), and assayed at either 28°C, 40°C or 48°C. Results are means ± 1 S.E.; n = 3–5. Differences ($P < 0.05$) among protein additions within each incubation temperature are indicated by an asterisk.

by complex I) (Fig. 3). At 28°C, submitochondrial vesicles from unstressed fruits incubated with either no added proteins, BSA, Ab_{α}, or purified mitochondrial lmw Hsp showed no differences in activity (Fig. 3). When submitochondrial vesicles were assayed at high temperatures, no significant differences in activity were observed among samples incubated with either no protein, BSA, or Ab_{α}, but activity increased 51% and 35% (at 40°C and 48°C, respectively) when samples were incubated with purified mitochondrial lmw Hsp (Fig. 3). These results confirm that the mitochondrial lmw Hsp specifically protects complex I activity during heat stress.

4. Discussion

Taken together, our results demonstrate that the mitochondrial lmw Hsp is an important determinant of the thermotolerance of oxidative phosphorylation. The mitochondrial lmw Hsp (a) increases electron transport from complex I through complex III and complex IV during heat stress, but has no effect on electron transport from complex II to complex IV; (b) completely accounts for all of the observed heat acclimation of electron transport from complex I through complex III and complex IV; and (c) specifically protects complex I during heat stress. To our knowledge, results from this study are the first direct evidence that mitochondrial Hsps play a role in the thermotolerance of oxidative phosphorylation. Since it is well established that complex I is a highly thermolabile component of mitochondrial oxidative phosphorylation, production of the mitochondrial lmw Hsp represents an important adaptation to acute heat stress in plants.

Similar to the function of the chloroplast lmw Hsp in protecting photosynthetic electron transport [24], disruption of this lmw Hsp with a specific antibody had an almost immediate effect (e.g. <1 min) on NADH oxidase function (data not shown). But unlike the near immediate protection from the addition of chloroplast lmw Hsp to chloroplasts lacking this Hsp, submitochondrial vesicles lacking the mitochondrial lmw Hsp had to be incubated for at least 5 min at a sub-heat-stress temperature (28–30°C) before significant differences in assay rates (NADH oxidase and NADH dehydrogenase) could be observed at 40°C and 48°C from controls. These results suggest that either the prior existence of an association between the mitochondrial lmw Hsp and complex I is necessary for thermoprotection or the rate of protection by the mitochondrial lmw Hsp is much slower than its chloroplast counterpart.

The mechanism of thermal protection or the specific site of interaction between lmw Hsp and complex I is unknown, but the results from the NADH dehydrogenase assay suggest that the lmw Hsp might be interacting with the hydrophilic arm of complex I since electron transfer in the NADH dehydrogenase assay is believed to occur between FMN to the iron-sulfur N-1 site [1,6]. It is well documented that acid-ethanol-heat treatments induce a decomposition of complex I to smaller fragments giving rise to conformational changes, transiently altered catalytic properties, decreased proton transfer ability, and changes in substrate specificity [1–4]. One hypothesis is that during heat stress, the lmw Hsp may stabilize the structural interactions between the hydrophilic and hydrophobic arms of complex I, thereby maintaining complex I's function of reducing ubiquinone and proton translocation. An alternative hypothesis is that the lmw Hsp is specifically protecting the FMN site (NADH oxidation site) of complex I. Clearly, further work is needed in order to elucidate the mechanism of interaction between the mitochondrial lmw Hsp and complex I.

The onset of heat stress is concomitant with increasing stress imposed by radical oxygen species (oxidative stress). Mitochondria are one of the earliest targets for these compounded insults [32], while injury to complex I has been reported to occur before other complexes of mitochondrial electron transport [33]. Polla and co-workers have established that the induction of heat-shock proteins protects mitochondrial function from both heat stress and oxidative injury [34]. Though conflicting observations exist concerning the ability of human Hsp 27 and other animalian lmw Hsps to bestow a measure of protection against oxidative stress [35–37], we hypothesize that the mitochondrial lmw Hsp in plants could specifically protect complex I from oxidative stress and are currently examining whether such a protective role for the mitochondrial lmw Hsp exists.

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