The C-terminus of yeast plasma membrane H^+-ATPase is essential for the regulation of this enzyme by heat shock protein Hsp30, but not for stress activation

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Abstract Several stresses cause additional activation to the glucose-stimulated plasma membrane H^+-ATPase activity of yeast, but it is not clear how this is achieved. We recently reported that cells lacking the integral plasma membrane heat shock protein Hsp30 display enhanced increases in plasma membrane H^+-ATPase activity with either heat shock or weak organic acid stress (Piper, P.W., Ortiz-Calderon, C., Holyoak, C., Coote, P. and Cole, M. (1997) Cell Stress and Chaperones 2, 12–24), indicating that the stress induction of Hsp30 acts to reduce stress stimulation of the H^+-ATPase. In this study it is shown that Hsp30 acts to reduce the V_{max} of H^+-ATPase in heat shocked cells. Its action is lost with deletion of the C-terminal 11 amino acids of the H^+-ATPase, a deletion that does not abolish the stress stimulation of this enzyme. Mutation of the Thr-912 residue within the C-terminal domain of H^+-ATPase, a potential site of phosphorylation by the Ca^{2+}-calmodulin-dependent protein kinase, also abolishes any effect of Hsp30. Hsp30 may therefore be acting on a Thr-912 phosphorylated form of the H^+-ATPase.

Key words: *Saccharomyces cerevisiae*; Membrane heat shock protein; Plasma membrane H^+-ATPase; Heat shock

1. Introduction

The plasma membrane H^+-ATPase of yeast uses the energy of ATP hydrolysis to transport protons from the cytosol to the extracellular medium. This is a vital process, needed not just for the maintenance of intracellular pH but also for providing the electrochemical potential that acts as the driving force for nutrient uptake (for review see [1]). The important physiological role of the H^+-ATPase, also its consumption of much of the ATP generated by the cell, means that it is an enzyme that has to be tightly regulated in response to growth conditions. Previous research has demonstrated that the H^+-ATPase in yeast is rapidly activated in response to either glucose metabolism [2] or medium acidification [3]. Glucose increases both expression of the gene for H^+-ATPase [4] and catalytic activity of the enzyme itself [5–10]. The mechanism of the glucose activation is still controversial, but it is known to rely upon elimination of an inhibitory interaction of the C-terminus of the H^+-ATPase with its ATP-binding region [6–10]. Phosphorylation of this C-domain is probably important in this regulation, through reactions thought to be catalysed by either casein kinase I or Ca^{2+}/calmodulin-dependent protein kinase [8–10].

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Several stresses further stimulate the high plasma membrane H^+-ATPase activity in glucose-deregulated cells, including growth at supraoptimal temperatures [11], heat shock [11,12], the presence of ethanol [13–15] and weak organic acids [12,16–18]. The mechanism of this stress stimulation is unknown. Although the cytoplasmic acidification that occurs with several of these stresses may be a factor, the stress-stimulated state of the H^+-ATPase is maintained during plasma membrane purification [11–17], indicating that stress induces posttranslational modifications of the enzyme which influence its activity. We recently reported that cells lacking heat shock protein Hsp30 display enhanced stimulation of plasma membrane H^+-ATPase activity with either heat shock or weak organic acid stress [12], indicating that the Hsp30 induction caused by these stresses acts directly or indirectly to reduce stress stimulation of the H^+-ATPase. Hsp30 is a highly hydrophobic integral plasma membrane protein that is induced in *S. cerevisiae* not just in response to heat shock, but also with exposure to ethanol, weak organic acids and glucose limitation [12,19–21]. This study sought to further characterise the Hsp30 influence over plasma membrane H^+-ATPase activity.

2. Materials and methods

2.1. Yeast strains

The *S. cerevisiae* strains used are listed in Table 1. In RS72 the wild-type promoter of the *PMA1* gene for plasma membrane H^+-ATPase has been replaced by the galactose-inducible *GAL1* gene promoter [22]. Strain matings, sporulation and tetrad dissections were according to standard procedures [23].

2.2. Yeast gene replacement and transformation

A cassette for replacement of the *HSP30* gene by *TRP1* sequences was generated. The −613 to −782 region of the *HSP30* gene was PCR amplified using the primers CGTAAGAAAGTTGTGT and GCGGATCCGACTGTATG; also the +1025 to +1233 region was amplified using primers GCGGATCCATTCTTTGCTTAACAC and ATTGATTAGACTATTAG. Following *Bam*HI digestion, these products were ligated to the *TRP1* gene fragment released by *Bam*HI digestion of YDp-W [24], the ligation mix being used for transformation of strain RB1 (Table 1) by a modified lithium acetate procedure [25]. Replacement of the entire *HSP30* gene with *TRP1* sequences was verified by PCR on transformant colonies [26], using primers GCAGTATTACTATTAC and CCGCGAATGTATCCAGTGAT (sequences −865 to −850 and +1380 to +1361 respectively relative to the ATG of *HSP30*). RB1 and its Δ*hsp30::TRP1* derivative (RB1h) were then transformed with the pSB32 derivative plasmids in Table 1. pSB32 is a centromeric episomal vector that bears the *LEU2* gene for transformant selection and either the wild-type *PMA1* gene or the stated *pma1* mutant allele [8].

2.3. Yeast culture

For analysis of the effects of Hsp30 and heat shock on the kinetic properties of glucose-deregulated H^+-ATPase activity shake-flask cultures of BJ2168 and KT3 (Table 1) were grown with aeration to mid-

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Table 1
Yeast strains and plasmids employed

Yeast strain	Genotype	Source/ref.
BJ2168	a *leu2-3,112,trp1,ura3-52,prb1-1122,pep4-3,prc407,gal2*	[12]
KT3	BJ2168 *hsp30::URA3*	[12]
YGXC18	a *leu2-3,112,trp1,pep4-3*	E. Jones
RS72	a *leu2-3,112,ade1-100,ura3-52,his4-519,pma1::URA3,GAL1-PMA1*	[22]
RB1	a *leu2-3,112,trp1,ura3-52,pep4-3,pma1::URA3-GAL1-PMA1*	This study
RB1h	RB1 *Δhsp30::TRP1*	This study
pSB32-derived plasmids		
pSB32.*PMA1*		[6]
pSB32.*Δpma1-244*		[6]
pSB32.*pma1(S911A)*		[8]
pSB32.*pma1(T912A)*		[8]

exponential phase at 30°C (5×10^6–1×10^7 cells ml^{-1}) on YPD medium (2% (w/v) bactopeptone, 1% yeast extract, 2% glucose). To heat shock cells, cultures were rapidly shifted to 39°C for the stated times prior to harvesting. RB1 and RB1h cultures bearing pSB32-derived plasmids were inoculated from freshly grown galactose minimal plates into YPGal (2% bactopeptone, 1% yeast extract, 2% galactose), grown to 2.5–5×10^6 cells ml^{-1} then transferred to YPD for 6 h so as to allow expression of the plasmid-borne *PMA1* allele.

2.4. Plasma membrane purification and plasma membrane H^+-ATPase assay

All measurements of H^+-ATPase activity employed strains deficient in vacuolar protease activities, so as to minimise the problems of proteolysis that can arise when purifying plasma membranes with intact proteins from heat-stressed yeast cells [20] and any possibility of activation of yeast ATPase through limited proteolysis of its C-terminal domain. Analysis of the H^+-ATPase activity of glucose-metabolising (GM) and glucose-starved (GS) stationary cultures (Table 2) used partially purified membrane preparations prepared according to Serrano [2]. All other H^+-ATPase assays were on plasma membranes purified by banding on isopycnic sucrose gradients, intactness of the H^+-ATPase protein being verified by 1D gel analysis as described earlier [20]. Protein determinations were performed using the Bio-Rad Protein Assay Kit and bovine serum albumin as standard. H^+-ATPase activities were determined according to the procedure of Serrano [27], with the inclusion of molybdate, nitrate and azide in the assay mixture (to inhibit any residual contamination by acid phosphatase, mitochondrial ATPase and vacuolar ATPase respectively). Plasma membrane H^+-ATPase activities are expressed as μmol Pi released per mg of total plasma membrane protein per min under these assay conditions.

3. Results

3.1. Influences of Hsp30 on the kinetic properties of plasma membrane H^+-ATPase

Unravelling the influence of Hsp30 on the plasma membrane H^+-ATPase of heat shocked cells involves distinguishing this influence from the direct effects of heat stress on the enzyme. The latter effects should be those seen in Hsp30 null cells. The data of Ahlers [28] indicate that the apparent K_m of yeast H^+-ATPase for ATP varies very little with temperature. Our data, obtained using plasma membranes from wild-type and Hsp30 null cells, confirmed that this K_m is essentially unaffected by heat stress. In addition it showed that K_m is not influenced by the presence or absence of Hsp30, within the experimental error of our measurements (Table 2). Although loss of Hsp30 increases activation of H^+-ATPase by heat shock [12], the pH optimum was also found to be essentially unaltered by heat stress or the absence of Hsp30 (Fig. 1). Vanadate inhibition was also unaffected (data not shown). Hsp30 induction therefore appears to lower the V_{max} of H^+-ATPase in glucose-deregulated cells, without affecting other properties of this enzyme (Table 2).

Previous studies have shown that glucose rapidly activates yeast plasma membrane H^+-ATPase at least 7- to 8-fold, increasing V_{max}, reducing K_m and displacing the pH optimum to more alkaline values. It also increases sensitivity to vanadate inhibition [2]. The lack of a functional *HSP30* gene does not influence this glucose stimulation of H^+-ATPase (Table 3). Hsp30 loss is therefore not acting like certain H^+-ATPase mutations which cause a constitutively activated H^+-ATPase enzyme [5,8,9]. Indeed the influence of Hsp30 would appear to be restricted to suppressing stress-induced increases in enzyme activity (Fig. 1 and Table 2). Under the relatively severe heat shock conditions employed, a stress-induced increase in activity relative to the total protein of purified plasma membranes was only apparent in Hsp30 null cells.

3.2. Hsp30 action on glucose-deregulated H^+-ATPase requires the C-terminal regulatory domain of this enzyme

Yeast H^+-ATPase is thought to be regulated partly by phosphorylation of its C-terminus, a kinasing that modulates inhibitory interaction of this domain with the enzyme active site [6–10]. We investigated whether an Hsp30 influence was still apparent in cells expressing H^+-ATPase mutated in this C-domain. For this purpose a *trp1,pep4-3* strain (RB1; Table 1) was constructed, this being a single spore segregant of the diploid produced by mating strains RS72 and YGXC18 (Table 1). In RB1 the single (RS72-derived) chromosomal gene for plasma membrane H^+-ATPase is under *GAL1* promoter control (*GAL1-PMA1*). A Hsp30 null version of this strain

Table 2
Effects of heat shock stress and Hsp30 loss on the kinetic properties of H^+-ATPase in sucrose gradient-banded plasma membranes

Strain	K_m for ATP[a] (mM)	V_{max}* (μmol Pi/min per mg protein)
BJ2168, unstressed	0.72 ± 0.03	1.01 ± 0.21
KT3, unstressed	0.58 ± 0.06	0.83 ± 0.11
BJ2168, 80 min 39°C	0.60 ± 0.04	1.11 ± 0.21
KT3, 80 min 39°C	0.76 ± 0.11	2.03 ± 0.32

[a]Mean and S.D. from assays on three identically prepared sucrose gradient-banded membrane preparations. ATPase activity was assayed at pH 5.7 and 0.1–2 mM ATP, total ATP consumption during the assay being < 15%. Apparent K_m and V_{max} were extrapolated from double-reciprocal plots fitted using a standard linear regression programme ($P = 0.96$–0.99).

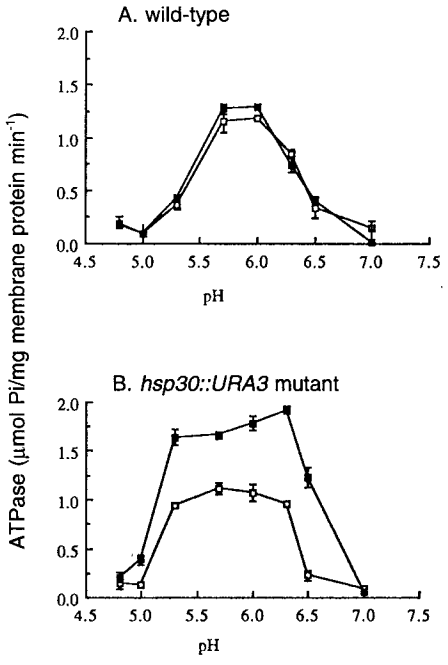


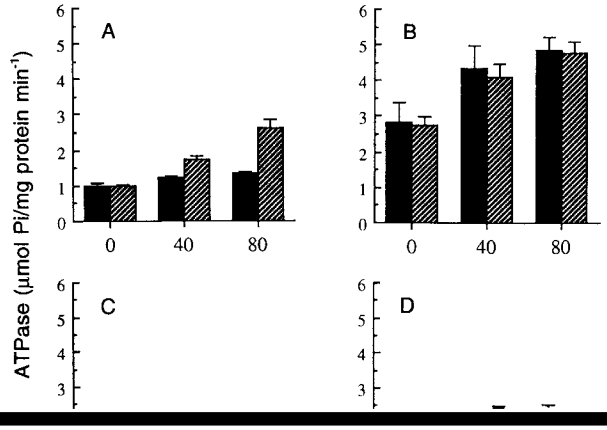
Fig. 1. Effects of pH on H^+-ATPase activity in plasma membranes purified from A: wild-type cells (strain BJ2168) and B: Hsp30 null cells (strain KT3). Membranes were prepared from cultures either in 30°C growth (□), or grown at 30°C then heat shocked at 39°C for 80 min (■). Error bars indicate S.D. of data from three independent assays.

(RB1h) was also generated by transformation with a PCR-generated DNA cassette (see Section 2). This *Δhsp30::TRP1* strain and its RB1 parent were both maintained on galactose. On glucose media they displayed the characteristic growth arrest of strains possessing only a *GAL1* promoter-regulated *PMA1* gene [22].

The isogenic strains RB1 and RB1h were transformed to leucine prototrophy with the pSB32-derived plasmids listed in Table 1. These carry either the wild-type *PMA1* gene (pSB32-*PMA1*) or mutant *pma1* genes (pSB32.*Δpma1-244*, pSB32.*pma1(S911A)* and pSB32.*pma1(T912A)*), and are described in [6,8,29]. These transformants were cultured on galactose, then transferred to liquid glucose medium for 6 h so as to suppress expression of the chromosomal *GAL1-PMA1* fusion and enable expression of the plasmid-borne mutant H^+-ATPase (see Section 2). One third of each culture was then harvested, the other cells being heat shocked at 39°C for either 40 or 80 min so as to determine the effects of the presence or absence of Hsp30 on activity of these mutant H^+-ATPases.

Plasma membranes were purified from the unstressed and heat shocked RB1 and RB1h transformants by sucrose gradient banding and their H^+-ATPase activities determined relative to total membrane protein content (Fig. 2). The relative activities in unstressed cells expressing either the pSB32-*PMA1* or pSB32.*Δpma1-244* plasmids confirmed that deletion of the C-terminal 11 amino acids of the H^+-ATPase leads to activation of this enzyme (compare zero time point samples, Fig. 2A, B). It is noteworthy however that further activation of the mutant enzyme by heat shock is not abolished by this deletion (Fig. 2B). Activation of yeast H^+-ATPase by stress has been noted in a number of studies [11–18]. The determinants of this stress activation are unknown, but Fig. 2B shows that it is at least in part independent of the enzyme C-terminal domain. However deletion of this domain totally eliminates any effect of Hsp30 (Fig. 2B), whereas an Hsp30 influence is seen in the equivalent heat shocked cells expressing wild-type H^+-ATPase (Fig. 2A). The C-terminal regulatory domain of H^+-ATPase is therefore essential for the stress stimulation of this enzyme to be suppressed through Hsp30 action.

The effects of two point mutations in the H^+-ATPase C-terminal domain were also investigated. The influence of Hsp30 during heat shock was unaffected by the *pma1-S911A* mutation, which replaces Ser-911 with Ala (Fig. 2C). However Hsp30 influence was lost with the expression of another H^+-ATPase mutation, *pma1-T912A* causing the change of Thr-912 to alanine (Fig. 2D). The latter mutation is known to reduce the glucose activation of H^+-ATPase [5–8]. Since Thr-912 is a potential site of phosphorylation by Ca^{2+}/calmodulin-dependent kinase [8–10] and needed for Hsp30 action, Hsp30 may be exerting its influence on H^+-ATPase that is phosphorylated at Thr-912 (see Section 4).



4. Discussion

Prior to this work there were few details of how stress activates plasma membrane H^+-ATPase. Recently we found that this activation was enhanced in cells lacking the heat shock protein Hsp30, indicating that Hsp30 is somehow involved in the stress regulation of H^+-ATPase [12]. This study shows that its action leads to a lowering of the apparent V_{max} of the H^+-ATPase (Tables 2 and 3). In addition the C-terminal regulatory domain of the H^+-ATPase is shown to be nonessential for the heat shock activation of this enzyme, but essential for Hsp30 to suppress this activation (Fig. 2B). This suggests: (i) that Hsp30 is influencing, possibly enhancing, autoinhibitory interactions of this C-terminal domain with the enzyme active site; and (ii) that the mechanisms of H^+-ATPase stimulation by stress and Hsp30 action differ, even though the overall effect of Hsp30 on enzyme activity levels is to suppress stress-induced increases (Fig. 2A).

In cells wild-type for the *HSP30* gene the T912A mutant H^+-ATPase shows a degree of activation with stress normally only seen in *Hsp30* mutant cells (Fig. 2D). Thr-912, a residue within the H^+-ATPase C-terminal domain, is therefore needed for Hsp30 action. Even though it is a potential site of phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase [8–10], evidence has yet to be presented that this residue is indeed phosphorylated in vivo [9]. Fig. 2D nevertheless indicates that the influence of Hsp30 in glucose-grown heat shocked cells may be on a Thr-912 phosphorylated form of H^+-ATPase.

The data in Fig. 2 would be consistent with a model in which Hsp30 increases the autoinhibitory action of the H^+-ATPase C-terminal domain. It is conceivable that Hsp30 might partly occlude this domain from activating kinase or phosphatase activities. Our results seem inconsistent with Hsp30 protein containing a domain that can functionally replace the autoinhibitory C-terminal domain of H^+-ATPase, since Hsp30 loss is without effect when this enzyme lacks its C-terminal domain (Fig. 2B). However much more work will be needed to distinguish between these models.

The effects of Hsp30 on the stress-stimulated, glucose-deregulated H^+-ATPase seem to be, at most, about a 2-fold lowering of activity levels (Fig. 2A). This compares with the rapid 7- to 8-fold activation of this same enzyme with glucose addition to starved cells [2]. Hsp30 is nonessential for such glucose stimulation (Table 3), even though glucose-starved cells are known to express the *HSP30* gene [19]. Hsp30 should therefore possibly be viewed as a component involved in the fine-tuning of H^+-ATPase activity and, as a stress protein, primarily of importance in stressed cells. This is consistent with it being a nonessential protein in yeast. *Hsp30* mutant cells are unaffected in their tolerances of extreme stress but take longer than wild-type cells to adapt to growth under conditions of stress which are less severe, but nevertheless still energy-demanding as regards homeostasis maintenance [12]. Plasma membrane H^+-ATPase action consumes a significant fraction of all the ATP generated by the cell [1], a usage will be greatly increased during stress stimulation. We have therefore proposed that Hsp30 induction may serve an energy conservation role, limiting excessive ATP consumption by the H^+-ATPase during extended stress or glucose limitation [12]. Consistent with this are: (i) the lower final cell densities of *hsp30* mutant cultures; and (ii) lowered ATP levels of *hsp30* cultures at the diauxic lag stage of batch fermentation when Hsp30 is normally induced, the latter possibly reflecting higher H^+-ATPase activity in this mutant [12]. It will be interesting to know if homologues of Hsp30 exist in other fungi and in plants, where a tightly regulated H^+-ATPase is also essential for homeostasis maintenance, also if other P-type cation pumps require regulation during stress.

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