Glucose-induced activation of the plasma membrane $H^+$-ATPase in *Fusarium oxysporum*

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Addition of glucose and other sugars to derepressed cells of the fungus *Fusarium oxysporum* var. *lili* triggered activation of the plasma membrane $H^+$-ATPase within 5 min. Glucose was the best activator while galactose and lactose had a lesser effect. The activation was not prevented by previous addition of cycloheximide and it was fully reversible when the glucose was removed. The activation process *in vivo* also caused changes in the kinetic properties of the enzyme. The non-activated enzyme had an apparent $K_m$ of about 3-2 mM for ATP whereas the activated enzyme showed an apparent $K_m$ of 0-26 mM. In addition, the pH optimum of the $H^+$-ATPase changed from 6-0 to 7-5 upon activation. The activated enzyme was more sensitive to inhibition by vanadate. When *F. oxysporum* was cultivated in media containing glucose as the major carbon source, enhanced $M^+$-ATPase activity was largely confined to the period corresponding to the lag phase, i.e. just before the start of acidification of the medium. This suggests that the activation process might play a role in the onset of extracellular acidification. Addition of glucose to *F. oxysporum* var. *lili* cells also caused an increase in the cAMP level. No reliable increase could be demonstrated for the other sugars. Addition of proton ionophores such as DNP and CCCP at pH 5-0 caused both a large increase in the intracellular level of cAMP and in the activity of the plasma membrane $H^+$-ATPase. Inhibition of the DNP-induced increase in the cAMP level by acridine orange also resulted in inhibition of the activation of plasma membrane $H^+$-ATPase. These results suggest a possible causal relationship between the activity of *F. oxysporum* var. *lili* plasma membrane $H^+$-ATPase and the intracellular level of cAMP.

Introduction

Plasma membrane $H^+$-ATPases of fungi and plants participate in the creation and maintenance of electrochemical proton gradients necessary for the transport of nutrients. Internal alkalization and external acidification apparently resulting from $H^+$-ATPase activity are associated with growth induction of yeast cells in glucose-containing media. Acidification of the external medium is due to metabolism of the sugar. When the extracellular pH is lower than the cytoplasmic pH, passive proton movement tends to acidify the cytoplasm. However, due to plasma membrane $H^+$-ATPase activity the internal pH of yeast cells is maintained between 6-0 and 7-5 even when great variations of extracellular pH occur (Serrano, 1985, 1988). Genetic evidence has confirmed the importance of the $H^+$-ATPase in both proton pumping and active nutrient transport (Serrano *et al.*, 1986a, b).

Recently, much interest has been paid to the post-translational mechanisms which regulate $H^+$-ATPase activity. In *Saccharomyces cerevisiae*, it has been demonstrated that addition of glucose or related sugars to derepressed cells causes rapid activation of plasma membrane $H^+$-ATPase (Serrano, 1983; Sychrová & Kotyk, 1985). Non-metabolizable sugar analogues had no effect (Serrano, 1983). Since the activation persists upon plasma membrane isolation, it seems to be the result of covalent modification. Phosphorylation of yeast $H^+$-ATPase has been demonstrated by several groups...
(McDonough & Mahler, 1982; Yanagita et al., 1987) and data have been presented which suggest that dephosphorylation leads to deactivation of the enzyme (Kolarov et al., 1988). Recently, a correlation between activation and phosphorylation of specific sites on the protein has been demonstrated (Chang & Slayman, 1991). Addition of glucose to derepressed S. cerevisiae cells also induces a transient increase in the cAMP level which triggers a protein phosphorylation cascade (Thevelein, 1988). This suggests that H+-ATPase activation after glucose addition might be the result of a phosphorylation process, possibly catalysed by cAMP-dependent protein kinase. Conflicting evidence, however, has been presented with respect to cAMP involvement in the regulation of plasma membrane H+-ATPase (Mazon et al., 1989; Ulaszewski et al., 1989). In this paper, we report that glucose-induced activation of H+-ATPase is also observed in derepressed cells of the fungus Fusarium oxysporum var. lini.

It has been suggested previously that the difference in variation of the extracellular pH between F. oxysporum var. lini cultures grown in media containing either glucose or lactose as carbon source could be explained by a different degree of activation of the plasma membrane H+-ATPase by the two sugars (Brandão & Nicoli, 1988). In this paper, we demonstrate that the activation of plasma membrane H+-ATPase of F. oxysporum var. lini depends on the substrate used. In addition, evidence is presented suggesting a role for the transient sugar-induced increase in the cAMP level as the triggering event for activation.

Methods

Micro-organism and its maintenance. Fusarium oxysporum var. lini ATCC 10960 was maintained on potato dextrose agar (PDA). It was incubated at 30 °C for 7 d and then kept in a refrigerator at 4 °C for a maximum 15 d.

Culture conditions. Cells were grown in a gyratory incubator (200 r.p.m.) at 30 °C for 20 h. The culture medium contained 2% (w/v) peptone, 0.025% MgSO₄, 7H₂O, 0.16% NH₄Cl dissolved in a saline mixture (Fermor & Wood, 1981). The mycelium was harvested by filtration and washed with distilled water. Subsequently, the cells were transferred to a flask containing Vogel's saline medium (Vogel, 1956), in which KH₂PO₄ (37 mM) was replaced by KCl (37 mM). The cells were incubated at 30 °C for 5 h under shaking to allow derepression to occur. After being filtered and washed the cells were weighed and divided into individual samples. For the experiment on the growth kinetics of F. oxysporum in medium containing glucose as carbon source (Fig. 2), the cells were incubated in a New Brunswick fermenter as described before (Brandão et al., 1988). The culture medium contained 2% (w/v) glucose, 0.2% peptone, 0.1% yeast extract, 0.15% KH₂PO₄ and 0.075% MgCl₂.

Activation conditions. In experiments on the activation of H+-ATPase, samples of 0.5–1.0 g (wet wt) were added to flasks containing 25 ml of 25 mM-MES buffer, pH 6.0, or 25 mM-glycine buffer, pH 5.0, for about 10 min at 30 °C before addition of organic substrates (2% g/vol. or 4% g/vol. in the case of lactose) or protonophores [2,4-dinitrophenol (DNP) 0.25–1 mM; carbonyl cyanide m-chlorophenylhydrazone (CCCP) 0.1–0.5 mM]. DNP and CCCP were added in ethanol to give final ethanol concentrations of, respectively, 0.96% and 6% in the cell suspension. In the control experiments 6% ethanol was added; it had no effect on H+-ATPase activity or cAMP levels. To measure cAMP levels, samples of 1.65 g (wet wt) were incubated for 10 min at 30 °C in flasks containing 50 ml of the buffers described above before addition of sugars or protonophores. Samples of 2.5 ml were taken from the suspension and the cells collected as quickly as possible by vacuum filtration. The filters with the cells were immediately frozen in liquid nitrogen and subsequently used to determine cAMP levels.

Determination of H+-ATPase activity. The procedures used to obtain F. oxysporum plasma membranes and to determine H+-ATPase activity for the time curves of H+-ATPase activity in vitro and for determinations in vitro of vanadate sensitivity, Kₐ values and pH optimum were as described previously (Brandão & Nicoli, 1988). The buffer used for determination of the optimum pH was 50 mM-MES adjusted with Tris (Serrano, 1983).

cAMP determination. This was done as described previously (Thevelein et al., 1987a).

Other determinations. Glucose consumption and dry weight were determined as described previously (Brandão & Nicoli, 1988). Protein was determined by the Lowry method with bovine serum albumin as standard.

Reproducibility of results. All experimental points are means of the values obtained in two independent experiments. The two independent experiments gave similar results.

Fig. 1. Effect of sugar addition on plasma membrane H+-ATPase activity in F. oxysporum cells. The sugars were added at time zero and samples were taken at the times indicated. ○, O, 2% (w/v) glucose with (O) or without (O) pre-incubation with 70 μM-cycloheximide for 10 min. ●, Glucose removed after 5 min incubation; △, 2% (w/v) galactose; ■, 4% (w/v) lactose; ▲, control (buffer without sugar).
Results

Addition of different sugars to cells of *F. oxysporum* var. *lini* caused a varying degree of activation of the plasma membrane H⁺-ATPase (Fig. 1). As observed before with *S. cerevisiae* (Serrano, 1983; Sychrová & Kotyk, 1985), glucose showed the greatest activating effect. Pre-incubation of cells with 70 μM-cycloheximide before addition of glucose did not prevent the activation. If the cells were removed 5 min after glucose addition, washed and incubated again without glucose, the H⁺-ATPase activity decreased to the initial level in about 20 min.

Activation by glucose changed several properties of the H⁺-ATPase. H⁺-ATPase activity in plasma membranes of *F. oxysporum* var. *lini* cells obtained immediately before and 5 min after glucose addition was tested for pH optimum, sensitivity to vanadate and *Km* value for ATP. Activation by glucose changed the pH optimum of the enzyme from 6-0 to about 7.5 (Fig. 2a), made the enzyme more sensitive to inhibition (non-competitive in both cases) by vanadate (*Km* 145 μM for the activated enzyme and 226 μM for the non-activated enzyme in exp. 1; in exp. 2 110 μM and 170 μM, respectively) (Fig. 2b), and lowered its *Km* value for ATP.

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Fig. 2. Properties of *F. oxysporum* plasma membrane H⁺-ATPase. (a) pH optimum, (b) inhibition by vanadate and (c) *Km* of plasma membrane H⁺-ATPase activity. ○, Activated enzyme; □, non-activated enzyme.
(from 3.2 mM to 0.26 mM in exp. 1 and from 2.0 mM to 0.27 mM in exp. 2) (Fig. 2c). The $V_{\text{max}}$ did not change appreciably [19.8 nmol Pi min$^{-1}$ (mg protein)$^{-1}$ in exp. 1 and 22.4 nmol Pi min$^{-1}$ (mg protein)$^{-1}$ in exp. 2).

The growth kinetics of *F. oxysporum* var. *lini* cells in a medium containing glucose as the main carbon source are shown in Fig. 3. An increase in H$^+$-ATPase activity occurred during the first 3 h with a peak corresponding to the end of the lag phase. Up to 12 h of culture, rapid growth and intense acidification of the extracellular medium occurred simultaneously. Afterwards, growth slowed and the pH of the medium stabilized at about 2.8 for up to 50 h of culture.

Addition of glucose also caused an increase in the cAMP level (Fig. 4). A cAMP increase caused by lactose and galactose could not be demonstrated reliably. Only the difference between glucose and the other sugars clearly fits with the effect on H$^+$-ATPase activation (see Figs. 1 and 4). As opposed to previous results with *S. cerevisiae* (Mazon et al., 1982; Purwin et al., 1986; Thevelein et al., 1987b; Van der Plaat, 1974) the cAMP increase observed in *F. oxysporum* is clearly lower. Therefore, we also studied the effect of the protonophores DNP and CCCP on the intracellular level of cAMP in *F. oxysporum*. Both DNP and CCCP caused pronounced cAMP increases with optimal concentrations of 0.75 mM and 0.25 mM, respectively (Fig. 5a). The effect of DNP and CCCP was strongly dependent on pH. Even with optimal concentrations, an effect was observed only at pH 5-0 (25 mM-glycine buffer) and not at all at pH 4.0 (25 mM-glycine buffer) or pH 6.0 (25 mM-MES buffer) (results not shown). Both DNP and CCCP also caused a strong and rapid activation of H$^+$-ATPase (Fig. 5b). The optimum concentrations (0.75 mM for DNP and 0.25 mM for CCCP) were similar to those for the effect of DNP and CCCP on the cAMP level (results not shown).
Although these results show that both glucose and protonophores cause increases in the intracellular level of cAMP as well as activation of the plasma membrane H+

-ATPase in *F. oxysporum* var. *hi* cells, the data did not allow conclusions as to whether there is a relationship between enzyme activation and cAMP increase. In an attempt to gain more evidence for such a relationship, we studied the effect of acridine orange on the increase in the cAMP level and the activation of H+

-ATPase induced by addition of DNP. In *S. cerevisiae*, acridine orange is a potent inhibitor of *in vivo* cAMP synthesis (Thevelein & Beullens, 1985). As shown in Fig. 6(a), previous addition of 10 μM-acridine orange for 10 min partially inhibited the DNP-induced cAMP increase, whereas pre-incubation with 40 μM-acridine orange completely eliminated it. DNP-induced H+

-ATPase activation was affected by acridine orange in a very similar way (Fig. 6b). An increase in enzyme activity could still be observed when 10 μM-acridine orange was used but the activation was completely suppressed at 40 μM. Control experiments showed that acridine orange, in the concentrations used in the *in vivo* experiments, did
not have an inhibiting effect in vitro on the H⁺-ATPase activity present in *F. oxysporum* var. *lini* plasma membranes (data not shown).

**Discussion**

Many sugar-induced regulatory phenomena have been described in fungi, and have been studied in great detail in *S. cerevisiae* in particular. In general, with respect to the effectiveness of inducing these regulatory phenomena, one can draw a rough line between rapidly fermentable sugars such as glucose, fructose, mannose and sucrose, and other sugars which are more slowly metabolized such as galactose, maltose, lactose and trehalose. This difference is also observed for plasma membrane H⁺-ATPase activation both in *S. cerevisiae* (Serrano, 1983, Sychrová & Kotyk, 1985) and *F. oxysporum* (Fig. 1). Sugar-induced plasma membrane H⁺-ATPase activation in *Fusarium* also resembles activation of the enzyme in *Saccharomyces* in other respects. The sugar-induced shifts in the pH optimum, *Kₐ* value and vanadate sensitivity (Fig. 2) are very similar to those reported for *S. cerevisiae* (Serrano, 1983; Sychrová & Kotyk, 1985). Since these changes remain present after plasma membrane isolation, they most probably reflect covalent modification of the enzyme. The same conclusion was reached by Serrano (1983). In general, the glucose-induced response might be quite similar between *Fusarium* and *Saccharomyces*, since glucose-induced inactivation of the high-affinity glucose uptake system observed in derepressed *S. cerevisiae* cells (Bisson & Fraenkel, 1984), was also described for derepressed cells of *F. oxysporum* var. *lini* (Brandão & Loureiro-Dias, 1990).

Contradictory results have been published concerning the possible involvement of cAMP-dependent protein phosphorylation in the regulation of the plasma membrane H⁺-ATPase in *S. cerevisiae*. According to Ulaszewski et al. (1989), incubation of temperature-sensitive mutants defective in cAMP synthesis leads to a decrease in plasma membrane H⁺-ATPase activity. The inhibition is prevented by addition of exogenous cAMP in strains containing an additional mutation which allows suppression of the temperature-sensitive phenotype by external cAMP. In addition, it was shown by Eraso & Gancedo (1987) that incubation of yeast cells at low pH causes activation of the plasma membrane H⁺-ATPase. It is well known that intracellular acidification causes large increases in the cAMP level in yeast (Mazon et al., 1982; Purwin et al., 1986; Thevelein et al., 1987a; Trevilhyan & Pall, 1979). In *Fusarium*, stimulation of cAMP synthesis by protonophores was observed only in a very narrow pH range (pH ±0.5) (Fig. 5a). This is probably due to the sensitivity of the system to intracellular acidification, the efficiency of the protonophores in lowering the pH in *Fusarium* cells and also the fact that when the pH drops too low a general toxic effect may be expected. Recent experiments in our laboratory have shown that the protonophores CCCP and DNP also cause large increases in plasma membrane H⁺-ATPase activity in *S. cerevisiae* (J. Becher dos Passos, unpublished results). On the other hand, using different yeast mutants affected in the RAS/cAMP pathway M. J. Mazon’s group (Portillo & Mazon, 1986; Mazon et al., 1989) came to the conclusion that cAMP was not involved in the regulation of H⁺-ATPase activity. Mutants in the RAS-activating protein CDC25, however, were deficient in activation of the ATPase, which these authors concluded identified a new function for CDC25.

The glucose-induced transient increase in cAMP level reported for *Fusarium* cells in this paper (Fig. 4) extends the range of fungi in which such rapid cAMP spikes have been observed. Similar sugar-induced cAMP responses have been observed for vegetative cells of *Neurospora crassa* (Pall, 1977) and *S. cerevisiae* (Van der Plaat, 1974), and spores of *Mucor rouxii* (Dewerchin & Van Laere, 1984), *Phycomyces blakesleeanus* (Van Mulders & Van Laere, 1984), *Pilobolus longipes* (Bourret, 1986) and *S. cerevisiae* (Thevelein, 1984). At the time of the initial discovery of protonophore-induced cAMP increases it was thought that plasma membrane depolarization was responsible for the observed effect (Pall, 1977; Trevilhyan & Pall, 1979). Subsequent work, however, has clearly shown that intracellular acidification is the mediator of the protonophore effect on the cAMP level (Caspani et al., 1985; Purwin et al., 1986; Thevelein et al., 1987a). Intracellular acidification might also be the mediator of the stress-induced effects on the cAMP level reported by Pall (1977) for *Neurospora* [see Thevelein (1991) for a recent extensive discussion].

Our results on sugar- and protonophore-induced activation of plasma membrane H⁺-ATPase in *Fusarium* also show a correlation with increased intracellular cAMP levels (Figs. 4 and 5). The parallel inhibition of H⁺-ATPase activation and increased cAMP levels appears to confirm the existence of a causal relationship between the two phenomena (Fig. 6). The specificity of acridine orange as an inhibitor of cAMP synthesis is not well established, however. In addition, because of the relatively small sugar-induced cAMP increases observed in *Fusarium*, it was not very meaningful to carry out the acridine orange inhibition experiment for sugar-induced activation of plasma membrane H⁺-ATPase.

Experiments with a wide range of yeast mutants affected in cAMP metabolism and/or protein phosphorylation could probably give a decisive answer to the
question of whether sugar-induced activation of plasma membrane H+-ATPase is mediated by cAMP as a second messenger. It cannot be excluded that the H+-ATPase is regulated by more than one mechanism. The effect observed by Ulaszewski et al. (1989) on H+-ATPase activity was much weaker than the effect observed on the cAMP level in the same mutants. In addition, Mazon et al. (1989) also observed reduced H+-ATPase activities at the restrictive temperature in temperature-sensitive mutants defective in cAMP synthesis. They had also shown that phorbol esters are able to stimulate plasma membrane H+-ATPase activity (Portillo & Mazon, 1985). Our results with F. oxysporum, although consistent with cAMP involvement, do not exclude the possibility of additional activation of the H+-ATPase by another pathway.

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