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Kinetic analysis of nutrient stimulated H^+ efflux by PM-ATPase of *Candida albicans*

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ABSTRACT. Rate of H^+ efflux and its stimulation by nutrients/analogs have been quantified in cells and spheroplasts of *Candida albicans*. In the absence of any nutrient, yeast cells showed an average H^+ efflux rate of 22.3 nmoles/min/mg yeast cells. Addition of 5mM Glucose to the medium resulted in striking stimulation of H^+ efflux by 7.5 fold. Supplementation of medium with 2-deoxy-D-glucose, Arginine & Glutamic acid lead to a minimal stimulation of 1.37, 1.50 & 1.42 fold, respectively, over control. Xylose, Lysine and Proline were non-effective. Control Spheroplasts had an H^+ efflux rate of 2.0 nmoles/min/mg yeast cells. Glucose stimulation of H^+ efflux was markedly reduced (2.22 fold). H^+ efflux did not significantly differ from control following supplementation of spheroplasts with either analogs of glucose (2-deoxy-D-glucose, xylose) or amino acids (Arginine, Lysine, Proline, Glutamate). These results have been compared with effect of nutrient/analogs on ATP hydrolytic cycle of isolated PM-ATPase reported earlier by this lab. Percentage of enzyme population going over to E.ADP.P complex stage following mixing with glucose and glutamate (94% & 96%, respectively) is comparable to Control (100%). Mixing of PM-ATPase with ATP in the presence of other nutrients/analogs leads to very significant reduction in population of E.ADP.P. Proportion of E.ADP.P complex which completes formation and dissociation of E~P complex (Power-Stroke) is similar to control in the presence of all nutrients/analogs except for glucose. Population of PM-ATPase molecule completing full ATP hydrolytic cycle is highest in presence of glucose (86.4%). This figure is only 27% for control, 27.8% for glutamate, and is significantly less in presence of other nutrients/analogs (ranging from 4.2% for Lysine to 20.5% for Xylose). Glucose positively affects both legs of hydrolytic cycle, while other nutrients/analogs negatively effect first leg of cycle and are neutral with respect to second leg. Exceptional Glucose stimulation of H^+ efflux by *Candida* cells can therefore be correlated with the stimulation of second leg of the ATP hydrolytic cycle.

Key words: *Candida albicans*, H^+- efflux, H^+- ATPase, stopped flow, spheroplasts.

RESUMEN. En este trabajo se presenta el efecto cuantitativo de la adición de nutrientes/análogos en la tasa de eflujo de H^+ en células y esferoplastos de *Candida albicans*. En ausencia de cualquier nutriente, las células de levadura presentan un eflujo promedio de H^+ de 22.3 nmoles/min/mg de células. La adición de glucosa 5mM al medio estimuló el eflujo de H^+ en 7.5 veces. Al suplementar el medio con 2-deoxi-D-glucosa, arginina o ácido glutámico se ocasionó una mínima estimulación de 1.37, 1.50 ó 1.42 veces, respectivamente, sobre el control. La adición de xilosa, lisina y prolina careció completamente de efecto. Los esferoplastos control presentaron una tasa de eflujo de H^+ de 2.0 nmoles/min/mg de células. La estimulación del eflujo de H^+ por glucosa resultó notablemente reducida (2.22 veces). El eflujo de H^+ no difirió notablemente respecto al control al suplementar los esferoplastos con análogos de glucosa (2-deoxi-D-glucosa, xilosa) o aminoácidos (arginina, lisina, prolina, glutamato). Estos resultados han sido comparados con el efecto de la adición de nutrientes/análogos en el ciclo hidrolítico del ATP de la PM-ATPasa reportada con anterioridad por este laboratorio. El porcentaje relativo de la enzima comprometida en la forma de E.ADP.P posterior a la adición de glucosa o glutamato (94 y 96%, respectivamente) es comparable con el control (100%). Al mezclar PM-ATPasa con ATP en presencia de otros nutrientes/análogos condujo a la reducción significativa de la forma E.ADP.P en la población. La proporción del complejo E.ADP.P, el cual completa la formación y disociación del complejo E~P (Power-Stroke) es similar al control en presencia de todos los nutrientes/análogos excepto para glucosa. La población de moléculas de PM-ATPasa que culminan el ciclo hidrolítico completo de ATP es mayor en presencia de glucosa (86.4%), comparado con el control (27%), con la adición de glutamato (27.8%) y con la de otros nutrientes/análogos (4.2% a 20.5%). La adición de glucosa afecta positivamente ambas vertientes del ciclo hidrolítico, mientras que otros nutrientes/análogos sólo afectan la primera vertiente del ciclo siendo neutrales con la segunda. La excepcional estimulación del eflujo de H^+ por glucosa en células de *Candida* puede por lo tanto correlacionarse con la estimulación de la segunda vertiente del ciclo hidrolítico del ATP.

Palabras clave: *Candida albicans*, eflujo de H^+, H^+- ATPasa, flujo detenido, esferoplastos.

INTRODUCTION

The plasma membrane ATPase (PM-ATPase) of the opportunistic pathogen *Candida albicans* generates electrochemical gradient of protons that drives the active transport of nutrients by H^+- symport.[1] ATP hydrolytic activity and H^+-extrusion are regulated by some nutrients, most notably, glucose.[2] Stimulation of ATPase activity by glu-

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cose metabolism results from its combined effect on the K_m, V_{max}, optimum pH and vanadate binding state of this enzyme. The fact that H^+-accumulation is stimulalated to a higher degree by glucose suggests that H^+-pumping can be regulated independently of ATP hydrolysis. Glucose may alter the H^+-/ATP stoichiometry of the plasma membrane H^+-ATPase or promote coupling of ATP hydrolysis to H^+-translocation. The initial rate of H^+-translocation suggests that the glucose activated H^+-ATPase translocates more H^+ per ATP consumed than the enzyme isolated from the glucose-deprived cells.[3] In other studies it has been found that glucose triggers transcriptional and post transcriptional mechanisms that increase the level and activity of *S. cereviseae* plasma membrane H^+-ATPase. It is proposed that glucose triggers degradation of an inhibitory protein resulting in enzyme activation.[4] Molecular mechanism of the stimulation is not known but it appears to be based on elimination of an inhibitory interaction of the C-terminus with the active site of the enzyme.[5,6] Pre-steady state kinetic studies suggest a four step kinetic scheme of ATP hydrolysis for *Candida albicans* PM-H^+-ATPase.[7] Step-1 is binding of ATP to the enzyme, whereas Step-2 is conversion of E·ATP complex to E-P·ADP complex, both of these steps lead to a release of $1H^+$ each. Steps-3 and Step-4 depict release of ADP from E-P·ADP complex and dissociation of E-P complex, respectively. Last two steps lead to absorption of $1H^+$ each. The scheme for hydrolytic cycle of PM-ATPase is given below:

Earlier results from this lab have shown that Glucose, glutamic acid, lysine, arginine and proline and two analogs of glucose: 2-deoxy D-glucose and xylose affect pre-steady state kinetics of ATP hydrolysis.[8] In the present study we have quantified H^+ efflux by cells and spheroplasts of *Candida* in the presence of glucose, its two analogs and four amino acids. Results obtained have been correlated with the effect of these nutrients/analogs on the pre-steady state kinetics of ATP hydrolysis by PM-ATPase.

MATERIALS AND METHODS

All biochemicals and enzymes were obtained from Sigma Chemical, USA whereas all inorganic chemicals were of analytical grade and were procured from E. Merck (India).

Yeast and Cultivation

Stock cultures of *Candida albicans* (ATCC 10261) were maintained on slants of nutrient agar (yeast extract 1%, peptone 2%, D-glucose 2% and agar 2.5%) at 4ºC. To initiate growth for experimental purposes, one loop full of cells from an agar culture were inoculated into a 25 ml of YEPD nutrient medium and incubated at 30ºC for 24 h i.e. up to stationary phase (primary culture). The cells from primary culture (10^8 cells ml^{-1}) were reinoculated into 100 ml fresh YEPD medium and grown for 8-10 h i.e., upto mid-log phase (10^6 cells ml^{-1}).

Proton extrusion measurements

Mid-log phase cells harvested from YEPD medium were washed twice with distilled water and routinely 100 mg cells were suspended in 5ml solution containing 0.1 M KCl and 0.1 mM $CaCl_2$. Suspension was kept in a double-jacketed glass container with constant stirring. The container was connected to a water circulator at 25ºC. H^+ extrusion rate was calculated from the volume of 0.01 N

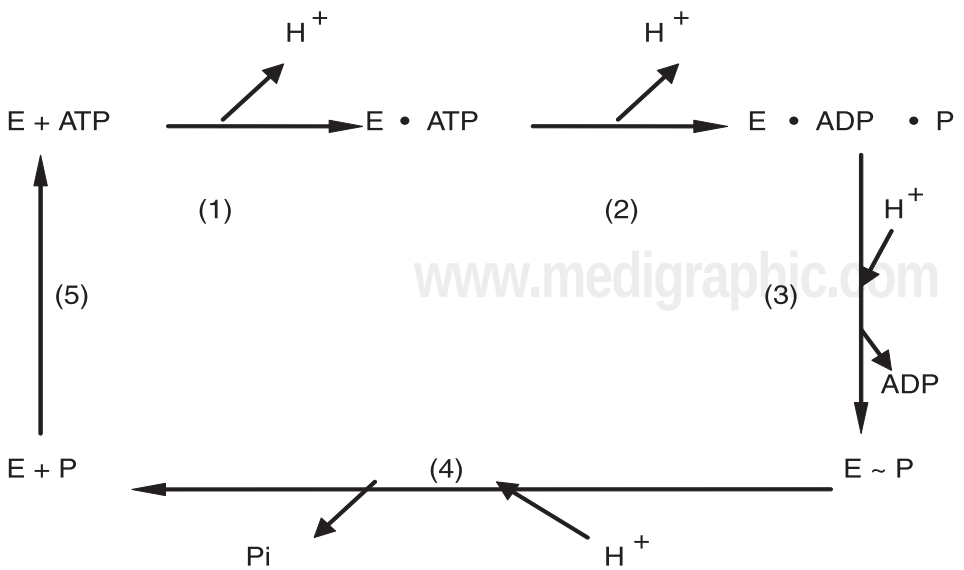

Figura 1. *kinetic scheme of ATP hydrolysis by H^+ ATPase.*

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NaOH consumed in automatic titration at pH-stat mode of the Autotitrimeter (Radiometer, Denmark) over a period of 10 min.[9] Increments and rate of delivery of titrant was adjusted according to demand of the experiments and were routinely 100µl and 40ml/min. Initial pH was adjusted to 7.0 using 0.01 N HCl/NaOH.

ATP determination

Concentration of ATP was determined by measuring the decrease in absorbance at 340 nm that results when NADH is oxidized to NAD^+ in the following enzyme coupled system consisting of glyceraldehydes-3-phosphate dehydrogenase and phosphoglycerate kinase. The mid-log phase cells (100 mg) were washed twice with distilled water and suspended in 10ml solution containing 0.1 M KCl and 0.1 m*M* $CaCl_2$ (control). For the effect of glucose, cells were suspended in solution containing 5 m*M* glucose in addition to 0.1 M KCl and 0.1 m*M* $CaCl_2$. Cells were incubated for 10 min and centrifuged at 5000 rpm for 5 min in cold. The pellet was extracted with 0.6 ml of 2 M $HClO_4$ for 5 min in cold and centrifuged for 5 min at 1500 g. The supernatant thus obtained was neutralized with a mixture of 0.6 ml of 1.8 M KOH and 0.4 M $KHCO_3$ in cold. After 5 min it was centrifuged again and supernatant was used for ATP determination. For ATP estimation of spheroplast, both in absence and presence of glucose, same incubation mixture was used except that it contained in addition 0.6 M sorbitol (control) or 5 m*M* glucose + 0.6 M sorbitol (Test). After incubation spheroplasts were lysed by addition of 1.2 ml distilled water and centrifuged at 5,000 rpm in cold and supernatant thus obtained was used for ATP estimation. Care was taken that time elapsed between end of incubation and estimation was same in all cases.

RESULTS

Table 1 gives the effect of nutrients/analogs on the rate of H^+- efflux by *Candida albicans* cells measured through pH stat assay. Control (without nutrients/analogs) had an average H^+ efflux rate of 22.3 nmoles/min/mg yeast cells. Cells show a striking stimulation of 7.5 fold when 5mM glucose is added to the incubation medium. Exposure of cells to 2-deoxy-D-glucose, Arginine and Glutamic acid gave minor stimulation of 1.37, 1.50 and 1.42 fold, respectively, over control. H^+ efflux in the presence of 5 mM Xylose, Lysine and Proline did not show significant variation with control.

Table 2 gives the effect of nutrients/analogs on the rate of H^+- efflux by *Candida albicans* spheroplasts. The control spheroplasts had an H^+ efflux rate of 2.0 nmoles/min/mg yeast cells. In the context of spheroplasts, mg yeast cells means spheroplasts derived from 1 mg yeast cells. Spheroplasts in general showed very less H^+- efflux as compared to cells. Stimulation by glucose is found to be minimal (2.22 fold) over control. H^+- efflux by spheroplasts in presence of other nutrients/analogs did not show much variation with control spheroplasts.

Table 3 shows population of PM-ATPase which goes on to form E.ADP.P complex in the ATP hydrolytic cycle by releasing H^+.[8] A very high percentage of enzyme molecules complete first leg of cycle and form E.ADP.P complex in the presence of glucose (94%) and glutamate (96%) as compared to control (100%). Analogs of glucose

Table 1. Effect of nutrients on the rate of H^+- efflux by *Candida* cells at pH 7.0. Control means cells were present in 0.1 m*M* $CaCl_2$ and 0.1 M KCl at 25°C.

Incubation with	Range of relative H^+- efflux rate (nmoles/min/mg yeast cells)
Control	1*
Glucose (5m*M*)	7.45-7.65 (7.50)
2-Deoxy D-Glucose (5m*M*)	1.32-1.42 (1.37)
Xylose (5m*M*)	0.85-0.93 (0.90)
Arginine (5m*M*)	1.42-1.56 (1.50)
Lysine (5m*M*)	1.13-1.21 (1.15)
Proline (5m*M*)	0.98-1.02 (1.00)
Glutamic Acid (5m*M*)	1.30-1.50 (1.42)

The control had an average (of four independent recordings) proton efflux rate of 22.3 nmoles/min/mg yeast cells. Rests of the records are for 4-6 experiments. Values in parenthesis are average.

Table 2. Effect of nutrients on the rate of H^+- efflux by *Candida* spheroplasts at pH 7.0. Control means cells were present in 0.1 m*M* $CaCl_2$ and 0.1 M KCl at 25°C.

Incubation with	Range of relative H^+- efflux rate (nmoles/min/mg yeast cells)
Control	1*
Glucose (5m*M*)	2.20-2.25 (2.22)
2-Deoxy D-Glucose (5m*M*)	1.32-1.38 (1.36)
Xylose (5m*M*)	1.20-1.28 (1.25)
Arginine (5m*M*)	1.12-1.18 (1.15)
Lysine (5m*M*)	1.28-1.32 (1.30)
Proline (5m*M*)	1.23-1.63 (1.27)
Glutamic Acid (5m*M*)	0.95-1.05 (1.00)

The control had an average (of 4 independent recordings) proton efflux rate of 2.0 nmoles/min/mg yeast cells. Rests of the records are for 4-6 experiments. Values in parentheses are average.

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(2-deoxy-D-glucose and xylose) and rest of the amino acids (Arginine, Lysine and Proline) greatly reduced the population of enzyme molecules forming E.ADP.P complex by affecting the first leg of hydrolytic cycle. Population of E.ADP.P complex which absorbs H^+ and complete second leg of cycle by undertaking formation and dissociation of E~P (or 'Power-Stroke' analogous to myosin-ATPase) for control is 27%. Population undergoing power-stroke is almost same in the presence of all nutrients and analogs, except for glucose. Only glucose had a high Power-stroke population of 92%. Table 4 lists the population of enzyme molecules, which complete full hydrolytic cycle of ATP. This population is 27.0% for control and 27.8 for glutamate. Significantly less population of enzyme molecules complete full ATP hydrolytic cycle in the presence of analogs of glucose (2-deoxy-D-glucose: 06.4%, Xylose: 20.5 %) and other aminoacids (Arginine: 12.9%, Lysine: 04.2%, Proline: 11.0%). In the presence of glucose, exceptionally high population of enzyme molecules, 86.4% complete full ATP hydrolytic cycle.

DISCUSSION

ATP hydrolytic cycle of PM-ATPase consists of two distinct phases.[7,8] A H^+ release phase consisting of ATP binding and translocation of terminal phosphate residue leading to formation of E.ADP.P complex, and a H^+ absorption phase consisting of formation and dissociation of E~P complex. Population of enzyme forming E.ADP.P complex is significantly less in the presence of nutrients/analogs, except for glucose and glutamate. This suggests direct interaction of these compounds with ATPase. Table 1 & Table 2 however, does not show corresponding decrease in H^+ efflux by yeast cells or spheroplasts in the presence of these compounds. This clearly shows that in intact cells the portion of PM-ATPase exposed to extracelluar environment does not interact with any of the tested nutrients/analogs. Small stimulation shown by nutrients, else than glucose, may be attributed to non-specific causes. Up to 80% of H^+- efflux was abolished in the presence of 50µg/ml diethylstilbestrol a specific inhibitors of PM-ATPase.[10] This indicates that the extruded H^+ also comes from sources other than PM-ATPase activity. Mutation studies have shown that nutrient induced H^+- efflux may not solely come from PM-ATPase activity.[10] The facts that, strikingly low stimulation of H^+- efflux by glucose is observed in the case of spheroplasts and that there is near equal H^+- efflux rates by cells and spheroplasts in presence of nutrients/analogs else than glucose indicate that minor stimulation of H^+- efflux shown by nutrients/analogs other than glucose may not be via PM-ATPase. Profound effect of nutrient/analogs else than glucose and glutamate on Reaction cycle of ATP hydrolysis may be a regulatory mechanism as high concentration of nutrients inside the cell indicates that cell need not import any more, so the high H^+-gradient requirement is not required.

High turnover shown by glucose and glutamate in the formation of E.ADP.P complex indicates that these two compounds do not inhibit ATP binding or phosphate translocation. In the absence of any nutrient (control) only 27% E.ADP.P complex undertakes power-stroke. In the presence of analogs of glucose and amino acids almost a similar percentage, ranging from 25% to 36% undertakes power-stroke. Only glucose has a very dramatic effect on Power-stroke, almost all of the E.ADP.P complex goes over the power stroke. Table 4 brings out unique role of glucose with respect to ATP hydrolytic cycle; overall PM-ATPase cycling

Table 3. Average Population of PM-ATPase forming E.ADP.Pi Complex and completing power-stroke in the absence and presence of 5 mM nutrients/analogs.

Mixing with	PM-ATPase Population Forming E.ADP.Pi Complex (%)	E.ADP.Pi Population completing 'Power-Stroke'(%)
Control	100	27
Glucose	94	92
2-Deoxy-D-Glucose	23	28
Xylose	38	27
Arginine	36	36
Lysine	16	26
Proline	44	25
Glutamic Acid	96	29

Variation was less than mean ±5%

Table 4. Average percentage population of PM-ATPase completing full ATP hydrolytic cycle in the absence and presence of 5 mM nutrients/analogs.

Mixing with	Population of PM-ATPase Completing full ATP hydrolytic cycle (%)
Control	27.0
Glucose	86.4
2-Deoxy-D-Glucose	06.4
Xylose	20.5
Arginine	12.9
Lysine	04.2
Proline	11.0
Glutamic Acid	27.8

Variation was less than mean ±5%

population following mixing of glucose is 86.4%. This Figure for control is 27% and is significantly less in presence of all nutrients/analogs. It is evident that all other nutrients/analogs except glucose inhibit enzyme's hydrolytic cycle. Molecular mechanism of glucose activation of PM-ATPase is not well established but it may involve several kinases,[12] ubiquitin-proteasome proteolytic pathway.[13] Phosphatidyl ionositol signaling[14] and G-proteins.[15] Signal transduction pathways, however, eventually leads to phosphorylation of Ser/Thr within C-terminal of the PM-ATPase.[16]

From the present study it is evident that glucose interacts with ATPase and affects E~P complex formation and dissociation. Low glucose activation of H^+ extrusion in spheroplasts suggests that integrity of envelope is important for this process. ATP concentrations in control cells and spheroplasts were found to be, respectively, 0.35 nmoles/mg cells and 0.33 nmoles/mg cells. For glucose incubated cells intracellular ATP values were 0.245 nmoles/mg cells and 0.50 nmoles/mg cells for cells and spheroplasts, respectively. Spheroplasts do not show much glucose stimulation even at high ATP, it is thus clear that high ATP is not the causative agency for glucose stimulation of PM-ATPase.

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