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Effect of Glucose, Its Analogs and Some Amino Acids on Pre-steady State Kinetics of ATP Hydrolysis by PM-ATPase of *Pathogenic Yeast (Candida albicans)*

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Fast kinetics of transient pH changes and difference spectrum formation have been investigated following mixing of ADP/ATP with partially purified plasma membrane PM-ATPase of the pathogenic yeast *Candida albicans* in the presence of five nutrients: glucose, glutamic acid, proline, lysine, and arginine and two analogs of glucose: 2-deoxy D-glucose and xylose. Average H^+-absorption to release ratio, indicative of population of ATPase undergoing complete hydrolytic cycle, was found to be 0.27 for control. This ratio varied between 0.25 (proline) to 0.36 (arginine) for all other compounds tested, except for glucose. In the presence of glucose, H^+-absorption to release ratio was exceptionally high (0.92). While no UV difference spectrum was observed with ADP, mixing of ATP with ATPase led to a large conformational change. Exposure to different nutrients restricted the magnitude of the conformational change; the analogs of glucose were found to be ineffective. This suppression was maximal in the case of glucose (80%); with other nutrients, the magnitude of suppression ranged from 40-50%. Rate of H^+-absorption, which is indicative of E~P complex dissociation, showed positive correlation with suppression of conformational change only in the case of glucose and no other nutrient/analog. Mode of interaction of glucose with plasma membrane H^+-ATPase thus appears to be strikingly distinct compared to that of other nutrients/analogs tested. The results obtained lead us to propose a model for explaining glucose stimulation of plasma membrane H^+-ATPase activity.

Plasma membrane H^+-ATPase of *Candida albicans* actively extrudes H^+ out of the cell to generate an electrochemical gradient, which provides energy for secondary transport systems (Serrano, 1988). ATP hydrolytic activity and H^+-extrusion are regulated by some nutrients, most notably, glucose (Serrano, 1983). Stimulation of ATPase activity by glucose 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 stimulated 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 (Venema and Palmgren, 1995). 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 (de la Fuente et al., 1997). A distorted mushroom-like form for the enzyme mass is supported by cryoelectron microscopy studies at the 8 Å level for both Ca^{2+}-ATPase and H^+-ATPase (Auer et al., 1998). This map reveals ten membrane spanning alpha-helices in the membrane domain, four major cytoplasmic domains and clearly indicated N-terminal and an inhibitory C-terminal. 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 (Eraso and Portillo, 1994; Portillo et al., 1989). Pre-steady state kinetic studies suggest a four-step kinetic scheme of ATP hydrolysis for *Candida albicans* PM-H^+-ATPase (Manzoor et al., 1999). 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 which

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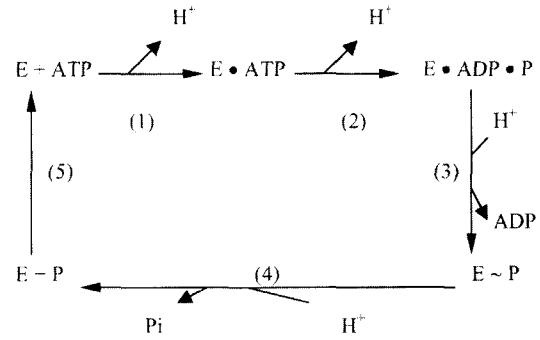
lead to release of $1H^+$ each. Step-3 and Step-4 depict release of ADP from E-P·ADP complex and dissociation of E-P complex, respectively. The last two steps lead to absorption of $1H^+$ each. The scheme for hydrolytic cycle of PM-ATPase is given below:



In the present study we have investigated the effect of five nutrients, namely, glucose, glutamic acid, lysine, arginine and proline and two analogs of glucose: 2-deoxy D-glucose and xylose, on pre-steady state kinetics of ATP hydrolysis. The results have been interpreted in terms of effects of these compounds on various steps of ATP hydrolytic cycle.

Materials and Methods

Materials

All biochemicals and enzymes were obtained from Sigma, whereas all inorganic chemicals were of analytical grade and purchased from E. Merck (India). *Candida albicans* (ATCC 10261) was obtained from Dr. Rajendra Prasad, Jawaharlal Nehru University, New Delhi.

Purification of H^+-ATPase from Candida albicans and determination of its activity

PM-ATPase was isolated as described earlier (Manzoor et al., 1999). The preparation obtained routinely contained 4-6 mg/ml protein. The crude membrane preparation contained 0.04 mg ATPase/mg of protein. The steady-state activity of the preparation was 0.25-0.30 μmol/min/mg protein at pH 7.5 in the presence of 5 mM NaN_3. About 70% of the ATPase activity was lost in the presence of 1 mM o-vanadate in the absence of NaN_3, whereas 90-95% of the activity was lost in the presence of NaN_3 and o-vanadate. Thus, the contribution of various types of ATPase in our preparation appears to be 70% PM-H^+-ATPase, 20-25% mitochondrial ATPase and the remaining 5-10% due to either vacuolar ATPase or non-enzymatic ATP hydrolysis. The experiments with ATPase were performed in the presence of NaN_3, and 90-95% of the pH transient signal obtained reflects the PM-H^+-ATPase activity. Control experiments were performed in the presence of inhibitors of PM-ATPase and mitochondrial ATPase. The signal obtained in control experiments has been subtracted from the signal obtained in test experiments in the presence of NaN_3. Steady-state ATPase activity, protein and enzyme estimation were performed as described by Gupta et al. (1991).

Determination of H^+ liberation/absorption ratio

The pre-steady state H^+-liberation/absorption by PM-ATPase was studied by following the absorption pattern of a pH sensitive dye, o-cresol sulphonaphthalein (o-cresol red) at 574 nm in a stopped flow spectrophotometer (Biologic) at pH 7.5 (Koretz et al., 1972; Tokiwa and Tonomura, 1965). The pK_a of the dye was 7.8 under our experimental conditions. Two syringes (S_1 and S_3) were used in all experiments. Along with the desired concentration of ATP, S_1 contained 50 μM o-cresol red, 100 mM KCl, 10 mM $MgSO_4$ and 5 mM NaN_3, pH 7.5. Syringe S_3 in all cases contained 0.05 μM PM-ATPase along with all constituents of S_1 (except for ATP) to avoid dilution. one hundred μl from each syringe was mixed at a flow rate of 5 μl/msec. Nutrients/analogs (10 mM) were loaded along with the enzyme in S_3. The data were acquired through an A/D board on an IBM PS/2 (Tandem) using Biokine rapid kinetics software (ver. 3.14). Prior to loading, the solutions were kept under reduced pressure for 12 h to remove dissolved gases, especially CO_2. The pH of solutions was fixed under N_2 atmosphere. The syringe compartment of the SFM was flushed with CO_2-free N_2 gas that was continuously bubbled at the exit port of the syringe compartment to prevent the entry of CO_2 into the cuvette. Quantification of H^+ release/absorption ratio was made employing a pH titration curve of dye prepared under conditions similar to that of test experiments.

Determination of UV difference absorption spectra of PM-ATPase

To study the rate of formation of difference spectrum by PM-ATPase, the change of absorption was followed at 293 nm in a stopped flow spectrophotometer at pH 6.5 following mixing of PM-ATPase with varying ATP concentrations (3-30 μM) at 25°C. This wavelength was employed as the difference in spectra due to ATP and ADP binding is maximum for other ATPases (Motita, 1969). Syringe S_1 along with ATP contained 3 mM $MgCl_2$, 100 mM KCl, 5 mM NaN_3 and 20 mM MES buffer, pH 6.5. Syringe S_3 in all cases contained 0.05 μM PM-ATPase along with all constituents of S_1 except for ATP. 100 μl from each syringe was mixed at a flow rate of 5 μl/msec. To see the effect of nutrients/analogs on the difference spectrum formation, the compounds were loaded along with the enzyme in S_3. Gain of the photomultiplier tube was kept constant throughout UV-

assays for all ligands thereby permitting quantification of magnitude. Signal change obtained in the absence of nutrients (control) was taken as 100%. Baseline calculations were done using a linear regression analysis. To calculate rate constants, biokine software, which uses alogrithm developed by Yeramian and Calverie (Yeramian and Calverie, 1987), was employed for multiexponential fitting. Concentrations and other conditions are noted along with the traces.

Results

Transient kinetics of H^+ release/absorption

In this set of experiments the effect of five nutrients (glucose, glutamic acid, lysine, arginine and proline) and two analogs of glucose (2-deoxy D-glucose and xylose) on kinetics of H^+ release/absorption following binding of ADP and ATP to PM-ATPase has been investigated. It has been reported that mixing of ADP with PM-ATPase of *Candida albicans* leads to release of one H^+ per molecule of ATPase (Manzoor et al., 1999). Mixing of ADP with PM-ATPase in the presence of 5 mM concentrations of all five nutrients and two analogs led to the same general pattern, i.e., initial rapid H^+-release and no reversal of the trend. No systematic variation in magnitude of H^+-release was observed for varying ADP concentrations (from 3 μM to 30 μM) for 0.025 μM PM-ATPase in the presence of all nutrients/analogs. Mean value of H^+-release in the absence of nutrients (control) was 0.95 per molecule of ATPase. Within experimental limits (+/-5%), similar H^+-release magnitude was observed in the presence of all nutrients/analogs. It appeared that none of the nutrients/analogs tested interfered with the binding of ADP to ATPase.

The typical pattern of transient pH changes obtained when ATP was mixed with 0.025 μM PM-ATPase in the absence and presence of nutrients/analogs was that of rapid H^+-release followed by its slow absorption. Fig. 1A and 1B give typical original recordings of transient pH changes when ATP was mixed with ATPase in the absence of any nutrient/analog (control) and in the presence of glucose, respectively. No systematic change in magnitude of H^+-release/absorption was obtained with increasing concentration of ATP from 3 to 30 μM either in the absence or presence of nutrients/analogs. This indicated that the enzyme was saturated with substrate with respect to pre-steady state signal. Since the magnitude of the signal for proton release/absorption was independent of ATP concentration and the values were close to each other, they have been averaged. Table 1 gives the magnitude of average H^+- released and absorbed per molecule of PM-ATPase. Compared to ADP binding where there was release of 1 H^+/molecule of PM-ATPase, the binding of ATP led to release of approximately 2 H^+/molecule of PM-ATPase in the

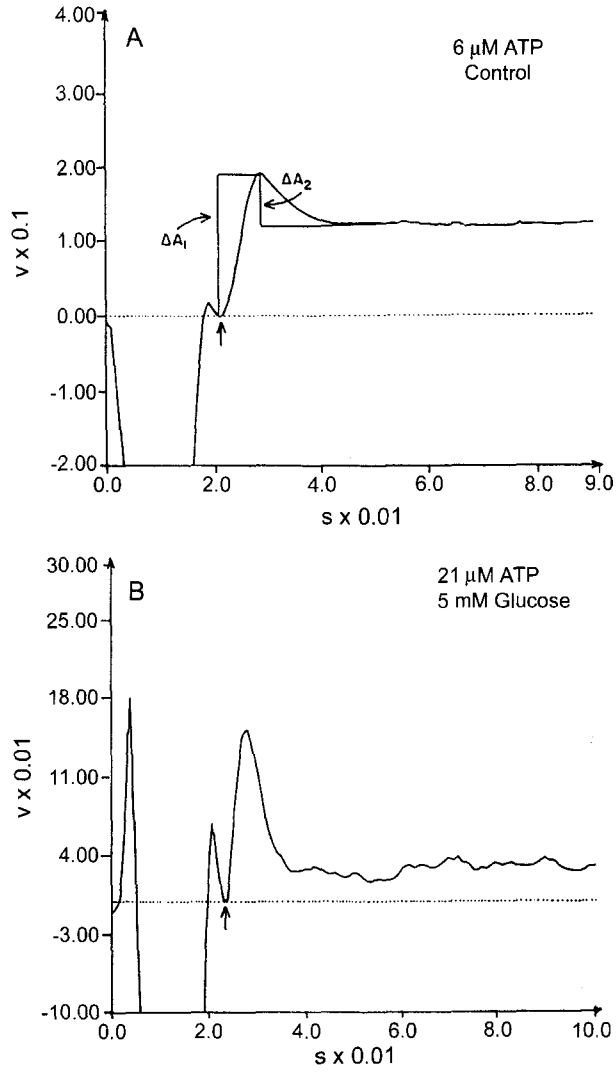


Fig. 1. Time course of H^+ liberation/absorption at 574 nm following mixing of PM-ATPase with indicated ATP concentrations at 25°C in the absence of any nutrient (A) and in the presence of 5 mM glucose (B). Increase in transmission (volts) indicates rise in acidity. ΔA_1 is acidity signal and ΔA_2 is alkalinity signal. Initial portion of the traces show syringes movement and stop artifacts, signal starts from around 20 ms and is depicted by an arrow (↑). Traces are average of 6 and 8 recordings, respectively.

absence of nutrients/analogs. Of all the nutrients tested, magnitude of H^+-release for glucose (1.80) and glutamic acid (1.84) were close to that of control implying that both glucose and glutamic acid did not significantly interfere with the events of ATP binding or E~P complex formation. Other nutrients/analogs affected either one or both of these processes, as the magnitude of H^+-released was markedly low, varying from 0.31 for lysine to 0.84 for proline. H^+-absorption evidently comes from the events of release of ADP from E-P·ADP complex and P_i from E~P complex. The results of steady state indicate that the dissociation of E~P complex is slow as compared to

Table 1. Average molar H^+ release/absorption per mole of H^+-ATPase and ratio of H^+ absorbed to H^+ released in the absence and presence of 5 mM nutrients/analogs

Incubation with	Average H^+ released (moles/mole of ATPase)	Average H^+ absorbed (moles/mole of ATPase)	Ratio (H^+ absorbed/released)
Control	1.9	0.51	0.27
Glucose	1.8	1.67	0.92
2-Deoxy D-glucose	0.43	0.12	0.28
Xylose	0.73	0.20	0.27
Glutamic acid	1.84	0.54	0.29
Proline	0.84	0.21	0.25
Lysine	0.31	0.08	0.26
Arginine	0.69	0.25	0.36

Variation was less than mean±5%

dissociation of ADP from E-P·ADP complex. Lower recorded value of proton absorption as compared to proton release, therefore, would mainly emanate from the slow dissociation of E~P complex and not E-P · ADP complex. Fractional release of H^+ obtained in the presence of nutrients/analogs suggests that only a part of the enzyme population has reached upto E-P · ADP and it would be this population which would be absorbing H^+ to complete the reaction cycle. To bring into focus this population with the objective to see the effect of nutrients/analogs on the ATP hydrolytic cycle we have tabulated H^+ absorbed/released ratio. Table 1 also gives the data and hence the magnitude of E~P complex dissociation on the scale of 1. Ratio of 1 means that whatever population of enzyme binds to ATP completes the whole reaction cycle. Values between zero and one would imply that part of the enzyme molecules gets stuck at some stage in the reaction cycle, probably at E~P stage. Except for glucose, all nutrients have this ratio very close to that of without nutrient condition, implying that all these nutrients have no effect on E~P complex dissociation. Interestingly, glutamic acid which showed proton release of 1.84 has absorption to release ratio of only 0.29, suggesting that mere higher E~P complex formation has no significant effect on the overall ATP hydrolysis rate. Results obtained with glucose are exception in this series as it has an absorption to release ratio of 0.92, implying that almost all the E~P complexes formed in the presence of glucose is dissociated within the time scale of observation. Only glucose therefore appears to promote E~P dissociation. Table 2 shows relative rate of H^+ absorption following mixing of ATP with ATPases in presence and absence of nutrients. Rate of H^+ release following mixing of ATP with ATPase showed almost a linear increase in the absence of nutrients/analogs. Similar trend was obtained in the presence of nutrients/analogs. For the purpose of comparing rate of H^+ release in the presence of nutrients/analogs, the rate of each concentration has been calculated for 1 μM ATP and then averaged.

Table 2. Relative H^+ absorption rate following mixing of ATP with ATPase in the absence and presence of 5 mM nutrients/analogs

Incubation with	Average rate (dx/dt) of absorption mv/ms/μM of ATP
Control	1
Glucose	1.9
2-Deoxy D-glucose	0.24
Xylose	0.12
Glutamic acid	0.95
Proline	0.36
Lysine	0.19
Arginine	0.28

Variation was less than mean±5%

Difference absorption spectra of H^+-ATPase in the presence of ATP or ADP

The kinetics of difference spectrum formation after mixing various concentrations of ATP with PM-ATPase of *C. albicans* in stopped flow spectrophotometer was followed at a fixed wavelength of 293 nm in the absence and presence of various nutrients/analogs. Two typical original recordings are given as Fig. 2A (control) and 2B (glucose). PM-ATPase concentration (0.025 μM) and gain of photomultiplier were constant throughout these sets of experiments. A decrease in transmission signal was observed both in the absence and presence of nutrients/analogs. No systematic increase or decrease in signal change was obtained with an increase in concentration of ATP from 3 to 30 μM. It has been reported that in the absence of any nutrient/analog, the signal change at 293 nm can be attributed to conformational change of enzyme concomitant to E~P complex formation (Manzoor et al., 1999). Table 3 gives the percentage conformation change in the presence of nutrients/analogs with respect to conformational change in control, which is taken as 100%. No difference spectrum was observed when ADP in the concentration range 3-

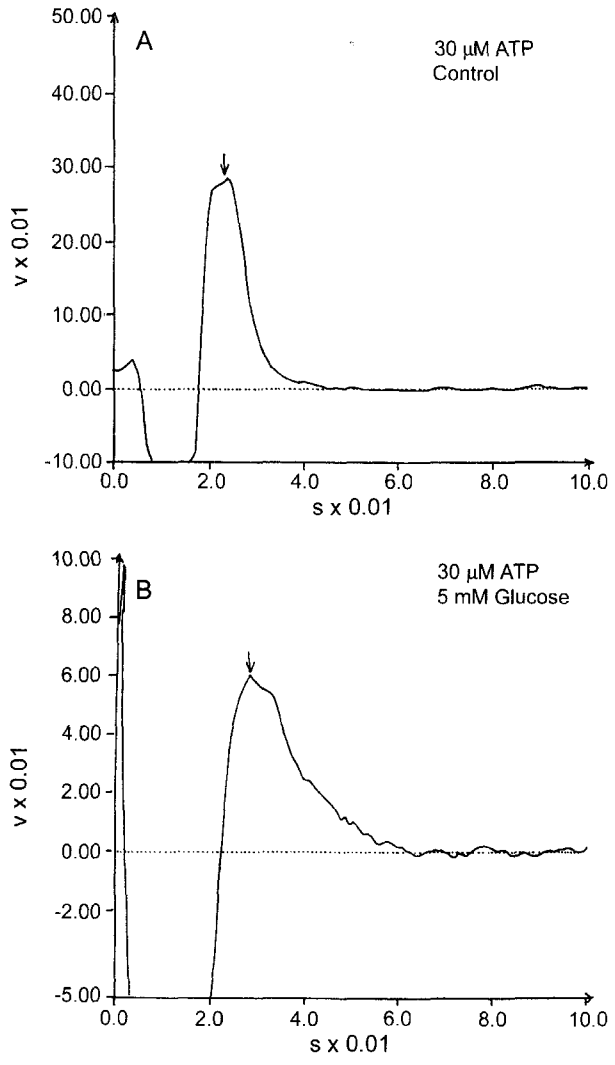


Fig. 2. Time course of transmission changes (Volts) at 293 nm following mixing of PM-ATPase with indicated ATP concentrations at 25° C in the absence of any nutrient (A) and in the presence of 5 mM glucose (B). Initial portion of the traces shows syringe movement and stop artifacts. Signal starts from around 20 ms and is depicted by an arrow (↑). Traces are average of 5 and 7 recordings, respectively.

30 μM was mixed with 0.025 μM PM-ATPase under conditions similar to that of the ATP binding experiment both in the absence and presence of nutrients/analogs. 2-deoxy-D-glucose, xylose and to a large extent lysine did not prevent the conformational change which was obtained in the absence of any nutrient/analog (control). Significant prevention of conformational change was shown by glutamic acid, proline and arginine. Most striking suppression of conformational change was observed, however, in the presence of glucose. From the comparison of conformational change with the rate of H^+-absorption (Table 2) which is a reflection of E~P dissociation, it becomes evident that only in the case of glucose the rate is greater than that of control. In the presence of rest of

Table 3. The percentage conformational change taking control as 100% following mixing of ATP with ATPase in the absence and presence of 5 mM nutrients/analogs

Incubation with	% Conformational change
Control	100
Glucose	20
2-Deoxy D-glucose	100
Xylose	100
Glutamic acid	38
Proline	40
Lysine	90
Arginine	50

Variation was less than mean±5%

the nutrients/analogs, the rate of H^+-absorption is below that of control indicating negative effect of these nutrients/analogs. Thus the mode of interaction of glucose with PM-ATPase is entirely different as compared to other nutrients/analogs.

Discussion

Stimulation of PM-ATPase activity of yeast cells exposed to glucose is well documented (Serrano, 1983). It has been suggested that the carboxyl terminus of yeast PM-ATPase interacts with its stalk region and ATP binding domain. The former interaction limits the access of H^+ to their transport site (Clarke et al., 1989) and the latter ATP to its binding site. These two interactions can be indirectly mediated by conformational coupling between them (Serrano and Portillo, 1990). Similar model has been proposed for the Ca^{2+}-ATPase as well in which the carboxyl terminus inhibits the activity of enzyme by interacting with the active site and that this interaction is released by phosphorylation. Comparable H^+ release observed in the absence (1.9 moles H^+/mole of ATPase) and presence (1.8 moles H^+/mole of ATPase) of glucose suggests that glucose does not directly affect either the ATP binding or E-P complex formation. Large conformational change observed during ATP hydrolytic cycle in the absence of any nutrient could be related to movement of carboxyl-terminus. This movement, which could apparently be a consequence of E-P complex formation, inhibits dissociation of the E-P complex. This would result in decreased affinity of ATPase for ATP as observed by other investigators. The strikingly less conformational change observed in the presence of glucose compared to that of control suggests that glucose might be interacting with the regulatory carboxyl domain preventing the conformational change which would otherwise slow down E~P dissociation and ATP hydrolysis. This argument is supported by observation that glucose that accords the highest suppression of carboxyl terminus movement shows the highest H^+ absorption rate.

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