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Pre-Steady State Kinetic Studies on H^+-ATPase from *Candida albicans*[1]

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The mechanism of ATP hydrolysis by plasma membrane H^+-ATPase from *Candida albicans* has been investigated by following the kinetics of H^+ liberation/absorption and the UV difference spectrum in a stopped flow spectrophotometer. A distinct pre-steady state phase of ATP hydrolysis could be defined. While the rapid mixing of P_i and ATPase produced no transient pH changes, the mixing of ADP leads to the release of 1 H^+ per molecule of ATPase. Rapid mixing of ATP with ATPase releases about 2 H^+ per molecule of ATPase, of which around 1.3 H^+ are reabsorbed. The magnitudes of both H^+ release and absorption were found to be independent of ATP concentration. The rate of H^+ release (k_f) shows ATP dependence while the rate of H^+ absorption is independent of ATP concentration. The rate of H^+ liberation with ADP, on a concentration basis, was far less as compared with ATP, indicating a low affinity of the ATPase for ADP. No change in the difference spectrum was observed with ADP. The stoichiometry of ATP binding to PM-ATPase was found to be unity from UV-difference spectrum studies. The k_f values for H^+ release and for the appearance of a difference spectrum following the addition of ATP were found to be similar beyond a 1:1 ratio of ATP:ATPase. The results obtained lead us to propose a 4-step kinetic scheme for the mechanism of ATP hydrolysis.

Key words: ATP hydrolysis, *Candida albicans*, difference spectrum, PM-ATPase.

Proton-translocating ATPase, involved in the maintenance of intracellular pH, accumulation of nutrients, and other important cellular processes (*1, 2*), has been purified from a number of yeasts including the human pathogenic fungus *Candida albicans*. This ATPase comprises a single polypeptide chain of 100 ± 4 kDa. Understanding the fungal H^+-ATPase may provide information broadly applicable to many eukaryotic ion-translocating ATPases such as Na^+-K^+-ATPases and Ca^{2+}-ATPases, because these enzymes are similar in structure and mechanism. Although the steady state kinetics of ATP hydrolysis catalysed by the H^+-ATPase from various fungal species have been investigated (*3*), the detailed molecular mechanisms of these ion-translocating enzymes remains unknown. The results of steady-state phase studies lend themselves to interpretation in terms of a simple kinetic model in which the hydrolysis of ATP can be pictured as follows:

$$E + ATP \longleftrightarrow E \cdot ATP \longleftrightarrow E \sim P + ADP \longleftrightarrow E + P$$

In this work we describe the fast reaction kinetics of ATP hydrolysis by the plasma membrane H^+-ATPase from *C. albicans* followed through the difference spectrum and transient pH changes due to P_i, ADP, and ATP binding to the enzyme. The results obtained can be interpreted in terms of an extended kinetic scheme for ATP hydrolysis by H^+-ATPase.

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MATERIALS AND METHODS

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

Mid-log cells from YEPD medium were harvested and washed twice with sterile distilled water. The washed cells (1 g wet wt.) were suspended in 2 ml of homogenizing buffer (250 mM sucrose, 1 mM PMSF, 10 mM Tris-Cl, pH 7.5). To this suspension of cells, 2 g of glass beads (0.45–0.5 μm size) were added. The cells were then mechanically disrupted in a CO_2-cooled homogenizer (Braun MSK). The suspension was agitated for a total of nine cycles, each 5 s with an interval of 3 s, at 4,000 rpm. The homogenate was centrifuged at $4,000 \times g$ for 5 min at 4°C to remove unbroken cells and glass beads. The pellet was washed once with the same homogenizing buffer. The combined supernatants were then centrifuged at $15,000 \times g$ for 45 min at 4°C, and the crude membrane pellet was resuspended in 0.6 ml suspension buffer (1 mM Tris-Cl, pH 7.5). The preparation obtained routinely contained 4–6 mg/ml protein. The crude membrane preparation contained 0.04 mg ATPase/mg protein. The steady-state activity of the preparation was 0.25–0.3 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 of *o*-vanadate. Thus the contribution of various types of ATPases in our preparation appear 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 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. The traces presented in "RESULTS," therefore, solely reflect the activity of PM-H^+ATPase. Steady-state ATPase assays were performed as described by Gupta *et al.* (*4*).

The pre-steady state H^+ liberation/absorption by PM-ATPase was studied by following the dye absorption pattern at 574 nm in a stopped flow spectrophotometer (Biologic, France) at pH 7.5 (*5, 6*). The pK_a of the dye was 7.8 under our experimental conditions. Two syringes were used in all experiments, one containing the enzyme along with salts and dye and the other containing the substrate (with salts and dye) whose binding to the enzyme was to be studied. Aliquots (100 μl) of solution from each syringe were mixed at a flow rate of 5 μl/ms and the data were acquired through an A/D board on an IBM PS/2 (Tandem) using Biokine rapid kinetics software (ver. 3.14). Solutions were kept under reduced pressure for 12 h to remove dissolved gases, especially CO_2. The pH of the solutions was fixed prior to loading 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 so as to prevent the entry of CO_2 into the cuvette. Quantification of H^+ release/absorption was made employing a pH titration curve of dye prepared under conditions similar to those of the experiments.

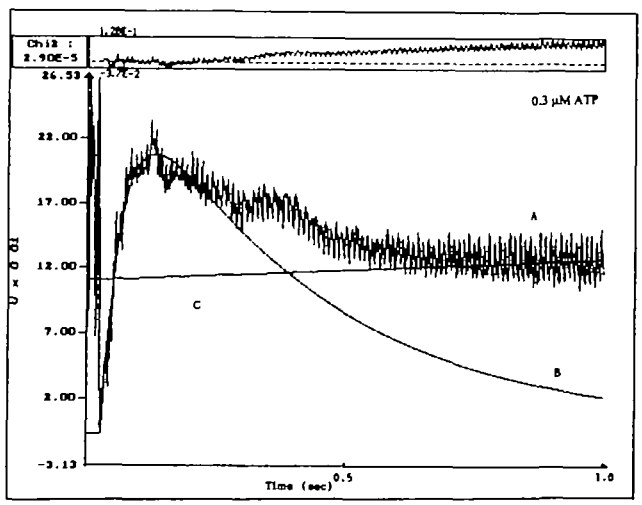

Fig. 1. **Trace A shows the time course of H^+ liberation/absorption at 574 nm following the mixing of PM-ATPase with ATP at 25°C.** An increase in transmission indicates a rise in acidity. Two syringes were used; Syringe S_1, apart from ATP, contained 50 μM *o*-cresolsulphonaphthaleine, 100 mM KCl, 10 mM $MgSO_4$, and 5 mM NaN_3, pH 7.5. The other syringe (S_2) in all cases contained 0.06 μM PM-ATPase along with all the S_1 constituents (except ATP) to avoid dilution. 100 μl from each syringe was mixed at a flow rate of 5 μl/ms; the data acquisition rate is 1 point/ms. The initial portion of the trace shows syringe movement and the signal starts from 30 ms. Trace is an average of 5 records. Trace B is a 1st order, two component fit using t_{origin} as 30 ms. At the top is shown the difference between the fit and the original trace. The top left corner depicts the CHI^2 value for fit calculated as $CHI^2 = \frac{\Sigma(Fit_i - Data_i)^2}{\Sigma_i Fit_i}$ Trace C is base line trace calculated for the period 0.9 to 1 s using linear regression analysis.

To study the rate at which a difference spectrum was formed by PM-ATPase, the change in absorption was followed at 293 nm in SFM-3 at pH 6.5. Syringes were loaded with buffered enzyme and the desired ligands at varying concentrations. The gain of the photomultiplier tube was kept constant throughout the UV-assays for all ligands, thereby permitting the quantification of magnitude.

Baseline calculations were made by linear regression analysis. To calculate rate constants, multiexponential fitting was done using the Biokine software, which uses an algorithm developed by Yeramian and Calverie (*7*). Concentrations and other conditions are noted with the traces.

RESULTS

H^+ Release/Absorption with ATP—All mixing experiments were performed at pH 7.5. The pK_a of the dye (o-cresol) was 7.8 under our experimental conditions and the changes in pH fall on the linear portion of the dye titration curve. The kinetics of H^+ release/absorption were investigated by increasing the ATP concentration over a fixed ATPase concentration of 0.03 μM. Relatively low concentrations of ATP were used to avoid a significant steady-state contribution.

Trace A in Fig. 1 shows a typical original recording of the time course of H^+ liberation/absorption following the mixing of PM-ATPase with ATP. The general pattern obtained was an initial rapid liberation of H^+, as indicated by the rise in transmission, followed by slow H^+ absorption indicated by the decrease in transmission. Towards the end, the absorption traces show a tendency for a rise in transmission. The initial portion of the traces shows the movement of the syringes and the signal starts from 30 ms. Analysis therefore was performed on the section of the traces beyond 30 ms. Quantitation of H^+ release/absorption was made as described in "MATERIALS AND METHODS." The baseline (trace C in Fig. 1) for the time period 0.9 to 1 s yielded variable results. For this reason and because the theoretical calculations showed that the steady-state contribution (from 0.3-0.4 μmol/min/mg of steady-state activity was routinely observed) would be very minimal during 1 s of observation, the steady-state contributions have not been subtracted from the pre-steady-state observations.

TABLE I. **Molar H^+ release and absorption/mole of PM-ATPase with their respective rate constants at various ATP concentrations.**

ATP (μM)	H^+ release	H^+ absorbed	Ratio (H^+ absorbed/H^+ released)
0.3	2.13	1.39	0.65
0.9	2.13	1.39	0.65
1.2	1.65	1.04	0.63
1.5	1.60	0.70	0.44
1.8	2.20	1.47	0.66
2.1	2.20	1.83	0.83
2.4	2.13	1.55	0.72
2.7	1.99	1.48	0.74
3.0	1.72	0.94	0.55
Average	1.97	1.28	0.65

Table I shows H^+ release and absorption per mole of PM-ATPase, along with their ratio, at various ATP concentrations. H^+ release/mol of ATPase does not show any systematic change and appears to be independent of ATP concentration, showing a phase of ATP hydrolysis distinct from the steady-state phase. The initial ATPase reaction thus appears to be a stoichiometric interaction of ATP with ATPase. The average H^+ release/molecule of ATPase is around 1.97. The amount of H^+ absorbed per mole of ATPase after the peak acidity is achieved during the initial phase of ATP hydrolysis by the PM-ATPase, also shows no systematic change and varies slightly, possibly because the peak acidity was variable. For this reason, the ratio of H^+ absorbed to H^+ released was calculated for each ATP concentration. This ratio shows consistency and the average H^+ absorption for each H^+ release is 0.65. Thus out of 1.97 mol of protons released per mol of ATPase, 1.28 mol of protons per mol of ATPase are reabsorbed. The figures have been rounded off to 2 mol of protons released and 1.3 mol of protons absorbed per mole of ATPase for discussion.

H^+ Release/Absorption with P_i and ADP—Figures 2 and 3 show typical original records of P_i and ADP mixing with ATPase, respectively, under conditions similar to those of ATP mixing. The trace for ADP has been inverted for the purpose of fit. No significant change in the transmission pattern of o-cresol was observed beyond 30 ms, the time for mixing until the end of observation, for any P_i concentration employed (up to 22.5 μM). It appears that no H^+ release/absorption takes place following P_i and ATPase interaction. Following the mixing of ADP with ATPase the traces showed an increase in acidity (rise in transmission). However, in contrast to ATP, the mixing of ADP with PM-ATPase showed no reversal of trend indicating that the ADP-ATPase complex is non-productive, at least in the observed time scale. H^+ release showed no systematic change with increase in ADP concentration, and the mean value was 0.91. The results, therefore, indicate that around 1 H^+ is released following the binding of ADP to ATPase.

Rate of H^+ Release/Absorption (k_f) with ATP and ADP—The traces showing H^+ release/absorption following the mixing of various ATP concentrations with ATPase were fitted to a two-component first order exponent. k_f (H^+ release) shows a linear dependence on ATP concentration (Table II) whereas k_f (H^+ absorption) was found to be fairly constant for all concentrations of ATP; the average value is 2.39. It must be noted that dx/dt is obtained from the very initial part of the trace where the turn around from acidity to alkalinity is shown, and for this reason, the second component of the fit, for most part, does not follow the original trace. It appears that H^+ absorption at later stages of the trace is aided by more than one agency. Further, the steady state would arrive after a molecule of ATP is hydrolysed by a certain population of enzyme molecules in which a fraction of H^+ would be released due to equilibration of released $H_2PO_4^-$ depending upon the pH. The results, therefore, appear to be cumulative. k_f (H^+ release) following the mixing of ADP and ATPase showed a concentration dependency, and the figures for 2.5 and 22.5 μM ADP were, respectively, 1.36 and 164.9 s^{-1}. On a concentration basis these figures are markedly low compared to the k_f (H^+ release) with ATP, indicating a low affinity for ADP.

Difference Spectrum with ATP and ADP—The formation of a difference spectrum after mixing various ATP concentrations with PM-ATPase from *C. albicans* was followed by stopped flow spectrophotometry at a fixed wavelength of 293 nm. A typical original recording is shown in Fig. 4 for 1.2 μM ATP mixed with 1 μM PM-ATPase.

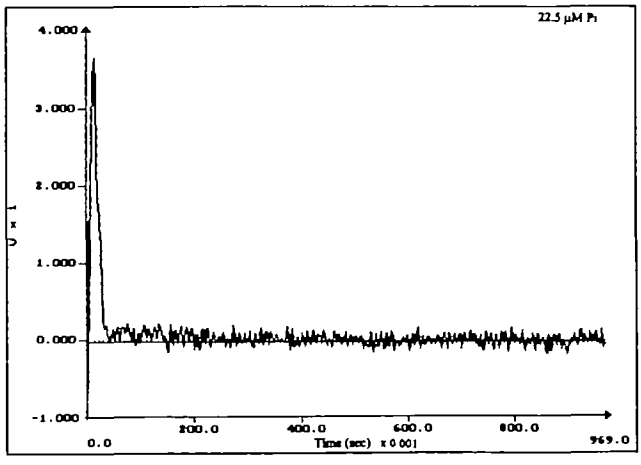

Fig. 2. **Trace shows the absorption pattern of dye o-cresol at 574 nm following the mixing of 0.06 μM PM-ATPase with P_i.** Experimental conditions are same as in Fig. 1. Trace is an average of 4 records.

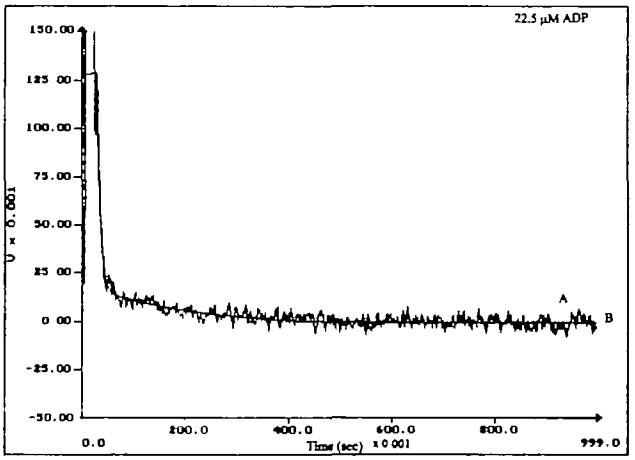

Fig. 3. **Trace A shows the time course of H^+ liberation at 574 nm following the mixing of 0.06 μM PM-ATPase with ADP.** Experimental conditions are same as in Fig. 1. The trace has been inverted for the purpose of fitting and is an average of 4 records.

TABLE II. **Rate of H^+ release/absorption (s^{-1}) following the mixing of ATP with PM-ATPase.**

ATP (μM)	k_f (H^+ release)	k_f (H^+ absorption)
0.3	23.50	2.72
0.9	39.37	1.89
1.2	47.0	2.43
1.5	54.3	2.20
1.8	58.0	1.83
2.1	64.7	2.54
2.4	79.34	2.37
2.7	100.8	2.80
3.0	109.3	2.76
Average		2.39

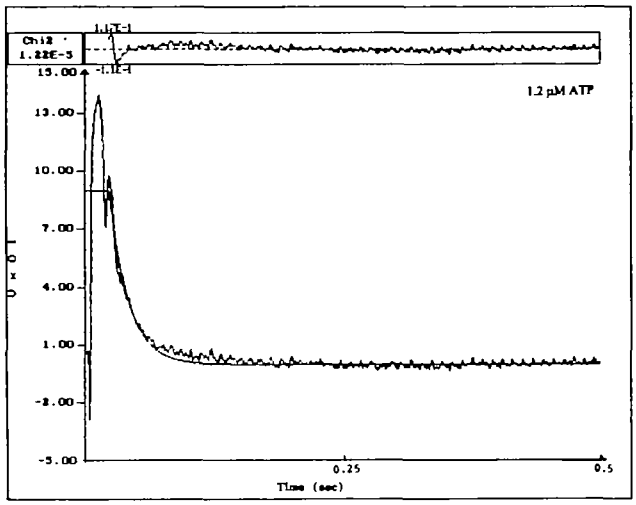

Fig. 4. **Trace shows the time course of transmission changes at 293 nm following the mixing of PM-ATPase with ATP at 25°C.** The trace has been inverted for the purpose of fitting; in the actual recording a rise in transmission was observed. Two syringes were used: syringe S_1, apart from ATP, contained 3 mM $MgCl_2$, 100 mM KCl, 5 mM NaN_3, and 20 mM MES, pH 6.5. The other syringe (S_2) in all cases contained 2 μM PM-ATPase along with all S_1 constituents (except for ATP) at same pH to avoid dilution. 100 μl from each syringe was mixed at a flow rate of 5 μl/ms: the data acquisition rate is 1 point/0.5 ms. Mixing time was 30 ms. Trace is an average of 4 records. The bold line between the trace is a one component, first order simulation starting from 30 ms.

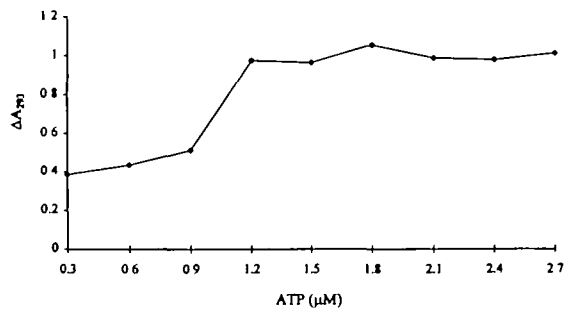

Fig. 5. **Amplitude change as a function of ATP concentration at fixed PM-ATPase (1 μM).**

The mixing time was 30 ms in all cases and, therefore, the signal has been considered to be the peak observed after 30 ms. The trace has been inverted for the purpose of fitting. In the actual experiments a rise in transmittance was observed. The change in amplitude at 293 nm (ΔA_{293}) reached a maximum value after a rapid increase in transmission and no reversal was observed. Figure 5 shows ΔA_{293} as a function of ATP concentration. The minimum concentration of ATP needed to give maximum ΔA was around 1.2-fold the concentration of the PM-ATPase used. The stoichiometry of ATP binding to ATPase thus appears to be 1. No difference spectrum was observed when ADP in the concentration range (2.5-22.5 μM) was mixed with 1 μM PM-ATPase under conditions similar to those of the ATP binding experiments.

Figure 6 shows the rate value (k_f) for the formation of a difference spectrum as a function of ATP concentration

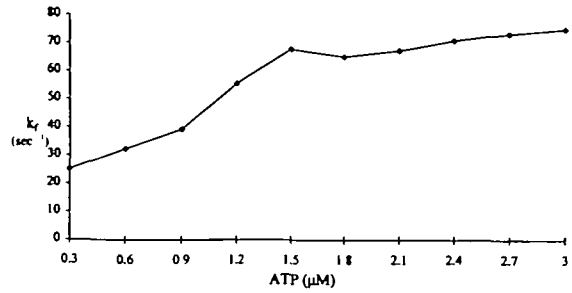

Fig. 6. **Rate of change of the difference spectrum as a function of ATP concentration.**

determined from one-component, first order fit. An Initial rapid increase in k_f was observed up to an ATP/ATPase ratio of 1.4, as determined from the intersection of the two widely differing slopes. At higher concentrations, although the rate of difference spectrum formation shows a dependency on ATP concentration, the slope is about 5 times less.

DISCUSSION

Results can be interpreted in terms of a 4-step kinetic scheme for PM-ATPase (Fig. 7). Since one proton is liberated when ADP binds to the enzyme and the total magnitude of H^+ release following ATP binding was found to be two, it appears that one proton is released to accommodate ATP at the active site (step 1). The second proton possibly comes from transphosphorylation of P_i from ATP to Enzyme (step 2). The argument for the release of H^+ concomitant to the formation of the E~P complex is supported by the observations of other investigators that ATPase has acidic pH optima and that the rate of E~P complex formation is faster at low pH (*8*). Step 3, the release of ADP, is shown to account for 1 out of the total 1.3 H^+ absorbed back. Since the binding of ADP leads to the release of one H^+ it appears logical to conclude that a proton will be absorbed back concomitant with the release of ADP. The release of ADP prior to P_i from the enzyme's active site has been established from steady-state kinetic studies (*9*). Step 4, the dissociation of P_i, is shown to absorb another H^+, because the 0.3 H^+ fraction is still unaccounted for. Since the transphosphorylation step is presumed to account for the release of one H^+, it would appear that aspartate is again protonated once the P_i dissociates. Since only 1.3 H^+ are absorbed, there is only a fractional contribution of step 4 within the time period of observation. Only one molecule of ATP is hydrolysed during the initial phase of our study. Once the population of E~P dissociates, the released phosphate ($H_2PO_4^-$) would equilibrate to release a fraction of H^+ into the solution. Beyond this time, transient pH studies, therefore, do not allow any meaningful conclusion. The fact that externally added P_i showed no H^+ absorption/liberation indicates that the H^+ binds to a site different from where the terminal P_i of ATP binds. This clearly demonstrates, that the E~P complex arrived at by ATP dissociation (by the enzyme) is different from the E•P complex arrived at by externally added P_i. The difference spectrum change results confirm the stoichiometry of ATP:ATPase binding as 1:1, as the signal showed no significant change when the ATP concentration was raised beyond a 1:1 ATP:ATPase ratio. The k_f for H^+ release shows a linear

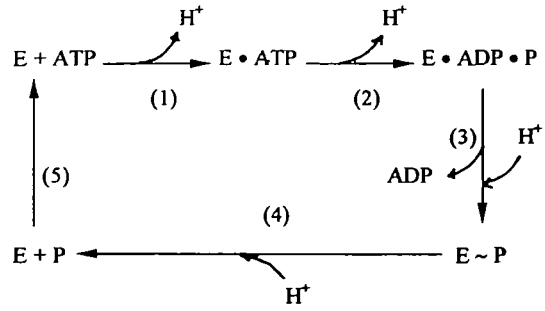

Fig. 7. **A tentative scheme showing the cyclic process of ATP hydrolysis.**

ATP dependence and the magnitude is one order greater compared to that of ADP implying that step 1 of the scheme is slow compared to step 2. The fact that the step involving ATP binding to the enzyme is slow compared to the later step has been corroborated by other investigators (*10*). Comparable k_t values for H^+ release and changes in the different spectrum following ATP binding beyond a 1:1 ratio of ATP:ATPase indicate that tryptophan perturbation (change in difference spectrum) occurs when the enzyme accepts a phosphate from ATP. The absence of any difference spectrum signal following the mixing of ADP and ATPase further supports the argument that perturbation in tryptophan is observed only when the terminal P_i of ATP finds a place on the enzyme active site, and not merely because of the binding of the nucleotide. The rate of H^+ absorption had an average value of 2.39 and showed no ATP concentration dependence, implying that E~P complex formation is fast compared to its dissociation.

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REFERENCES

1. Goffeau, A. and Slayman, C.W. (1981) The proton translocating ATPase of the fungal plasma membrane. *Biochim. Biophys. Acta* **639**, 197-223
2. Monk, B.C., Kurtz, M.B., Marrinan, J.A., and Perlin, D.S. (1991) Cloning and characterization of the plasma membrane H^+-ATPase from *Candida albicans*. *J. Bacteriol.* **173**, 6826-6836
3. Koland, J.G. and Hammes, G.G. (1986) Steady state kinetic studies of purified yeast plasma membrane proton-translocating ATPase. *J. Biol. Chem.* **261**, 5936-5942
4. Gupta, P., Mahanty, S.K., Ansari, S., and Prasad, R. (1991) Isolation, purification and kinetic characterization of plasma membrane H^+-ATPase of *Candida albicans*. *Biochem. Int.* **24**, 907-915
5. Tokiwa, T. and Tonomura, Y. (1965) The pre-steady state of myosin-adenosine triphosphate system: II. Initial rapid absorption and liberation of hydrogen ion by a stopped flow method. *J. Biochem.* **57**, 616-625
6. Koretz, J.F., Hunt, T., and Taylor, E.W. (1972) Studies on mechanism of myosin and actomyosin ATPase. *Cold Spring Harbor Sym. Quant. Biol.* **37**, 179-184
7. Yeramian, E. and Calverie, P. (1987) Analysis of multiexponential functions without a hypothesis as to the number of components. *Nature* **326**, 169-174
8. Dufour, J.P., Boutry, M., and Goffeau, A. (1980) Plasma membrane ATPase of yeast: Comparative inhibition studies of the purified and membrane-bound enzymes. *J. Biol. Chem.* **255**, 5735-5741
9. Bowman, B.J. and Bowman, E.J. (1986) H^+-ATPases from mitochondria, plasma membranes and vacuoles of fungal cells. *J. Membr. Biol.* **94**, 83-97
10. Wach, A., Ahlers, J., and Graber, P. (1990) The H^+-ATPase of the plasma membrane from yeast. Kinetics of ATP hydrolysis in native membranes, isolated and reconstituted enzymes. *Eur. J. Biochem.* **189**, 675-682