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Inhibition of yeast plasma membrane H^+-ATPase by fluoroaluminates

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The mechanism of inhibition of the *Schizosaccharomyces pombe* plasma membrane H^+-ATPase by fluoroaluminates has been investigated. A biphasic inhibitory process was observed at pH 7.5, with a preference for the AlF_4^- species. The dissociation constant found for AlF_4^- is 8.5 μM, Mg^{2+} being an essential cofactor for the inhibition. The rate constant of the rapid inhibition phase is decreased at pH values lower than 7.0 which may reveal a preferential action of AlF_4^- on the E_2 conformation of the enzyme. The slow phase of inhibition was found to be quasi-irreversible and highly dependent on the water activity. This dependence was studied by adding Me_2SO in the solvent and can be explained by the release of five water molecules upon fluoroaluminate binding. It is proposed that inhibition of the H^+-ATPase is due to the formation of a stable E_2-Mg-AlF_4 complex analog to the phosphorylated intermediate of the H^+-ATPase.

Introduction

The yeast plasma membrane H^+-ATPase has been classified as a member of the E_1-E_2-ATPase group. Among other members of this family are the Ca^{2+}-ATPase of sarcoplasmic reticulum and the Na^+/K^+-ATPase. This classification was originally based on similarities in the reaction scheme [1,2] and on the existence of a phosphorylated intermediate [3]. More recently, it has been strengthened by the observation of very large sequence homologies among these enzymes [4].

For the mammalian and fungal ATPases [1], the general reaction scheme includes two extreme conformational states of the enzyme, termed E_1 and E_2. There is evidence that these two conformers have different affinities (and accessibility) of the active site for the transported cation(s). It is proposed that this conformation change is associated with a modification of reactivity of the nucleotide site which can be phosphorylated by ATP in the E_1 form or by P_i in the E_2 form.

For many reasons, the knowledge of the proton ATPase mechanism is by far less advanced than the other ATPases. Some of these reasons are: (i) The transported cation is a proton, which makes labeling experiments impossible. (ii) The affinity for ATP is low, as compared to other ATPases [5,6]. (iii) The phosphorylated intermediate concentration is low because of a high K_m and high speed of hydrolysis [2].

Therefore, there is a need for good substrate analogues to block the reaction intermediates and to provide high-affinity substitute for the natural substrates.

In a previous study [7], we have shown that the complex formed by terbium and formycin triphosphate (FTP-Tb) can act as an analogue of ATP for this ATPase. Vanadate also has been used as an analogue of P_i on the yeast plasma membrane ATPase [7,8].

Here we show that AlF_4^-, a newly developed ATPase inhibitor, can be a very efficient and useful tool to study the H^+-ATPase reaction cycle.

Fluoroaluminates have been used in several NTPases proteins. These complexes seem to work in two different ways. First, they have been shown to act as analogues of the terminal phosphate of NTP for transducin [9,10], tubulin [11], F-actin [12] and mitochon-

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Abbreviations: Hepes, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid]; Mes, 2-[*N*-morpholino]ethanesulphonic acid; Me_2SO, dimethylsulphoxide; SR, sarcoplasmic reticulum.

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drial F_1-ATPase [13]. In this case presumably one fluoroaluminate complex inhibits the enzyme by combining to NDP into the active site. In the very similar case of beryllium it has been shown by Issartel et al. [14] that the possibility of having a ternary complex between the fluoroberyllate and NDP in solution is unlikely.

A second mechanism of inhibition has been reported where the fluoroaluminate complexes act as analogues of P_i in enzymes whose reaction cycle contains a covalent phosphorylated intermediate [15,16].

In this last article, we have shown that AlF_4^- can inhibit the Ca^{2+}-ATPase enzyme through the two mechanisms described above.

In the present paper we demonstrate that fluoroaluminate compounds are potent inhibitors of the yeast plasma membrane H^+-ATPase. This inhibition occurs at neutral pH in the absence of ADP. We propose, therefore, that it functions as an analogue of phosphate, stabilizing a species similar to the E_2-P phosphorylated intermediate of the H^+-ATPase.

Materials and Methods

Purification of the yeast H^+-ATPase. The H^+-ATPase was solubilized and purified from yeast *S. pombe* plasma membranes isolated as described earlier [17]. *S. pombe* 972 h yeasts was grown at 30°C under aerobic conditions in 5.8% (w/v) glucose, 2% (w/v) yeast extract (KAT, Ohli, Hamburg) brought to pH 4.5 with HCl. The cells were harvested in exponential phase of growth. The solubilization of the plasma membrane was carried out using a natural detergent, egg lysolecithin purchased from Avanti Polar Lipids. We used a lysolecithin-to-protein ratio of 4 in presence of 1 mM EDTA at pH 7.5. We did not used ATP in the preparation to eliminate adverse effects on the rest of the experiments. The purified enzyme was obtained after a centrifugation of the lysolecithin extract layered on a linear sucrose gradient ranging from 6 to 30% sucrose (w/w) in 10 mM Tris-acetate (pH 7.5) and 1 mM EDTA sodium salt. The three fractions of the highest ATPase activity were pooled, divided in aliquots, freezed and stored in liquid nitrogen.

The protein concentration was determined by the method of Folin modified by Lowry [18]. We obtained currently a protein concentration of 150–200 μg/ml and an ATPase activity of 20–30 μmol/min per mg.

ATPase assay. ATP hydrolysis was measured by colorimetric determination of the P_i released in solution [17]. The assay was performed at 30°C in 100 μl final volume containing 100 mM Mes-KOH (pH 5.5), 6 mM ATP, 9 mM $MgSO_4$, 10 mM sodium azide and 50 μg/ml lysolecithin. The reaction was started by addition of the enzyme and stopped 8 min later by addition of 300 μl of SDS 1% (w/v). ATPase activity was also measured at different time intervals between 1 and 8 min after beginning of the reaction with ATP.

Inorganic phosphate concentration was evaluated by spectrophotometry at 740 nm after 15 min of a color development carried out with 250 μl of ammonium molybdate reagent and 250 μl of Elon reagent, according to the Fiske and SubbaRow method [19].

Inhibition kinetics. The inhibition kinetics were obtained by incubating H^+-ATPase (40 μg/ml) in the presence of fluoroaluminate complexes in solutions containing 100 mM Mes-Hepes-KOH, Hepes-KOH or Hepes-Tris depending on the pH, 0 or 100 mM KCl, 2 mM $MgCl_2$ for standard conditions or at various concentrations for the study of the effect of magnesium. For certain studies 50 μg/ml lysophosphatidylcholine were added to stabilize the enzyme. The concentrations of the fluoroaluminate complexes were computed with the simulation program TOT [20] using equilibrium constants compiled by Martin [21] and from reference compilation books [22]. The species taken into account in the calculations were as follows: AlF_2^+, AlF_3, AlF_4^-, AlF_5^{2-}, $[Al(OH)_4]^-$, $[Al(OH)_3F]^-$, $[Al(OH)_2F_2]^-$, $[Al(OH)F_2]$, $[Al(OH)_2F]$, $[Al(OH)F_3]^-$, F_2Mg. In addition, the presence of EDTA and Mg^{2+} in the solution was taken into account for the calculation of AlF_4^- concentration. All component concentrations were chosen to avoid the formation of any precipitate. All experiments and solutions were made and stored in plastic vessels to avoid aluminium contamination by glass.

Aluminum contamination of all products used was assessed by atomic absorption spectrometry and the total contamination of a standard medium was below 0.2 μM.

Incubation in fluoroaluminates was performed at 20°C for periods of up to 2 h. During the first 30 min of incubation, 20 μl aliquots were taken every 5 min, then every 15 min. The ATPase activity in the aliquots was determined as described above.

The activities were then expressed as a fraction of the control activity obtained by incubating the H^+-ATPase in the same solution but without $AlCl_3$. The analysis of exponential time-course was performed with the Bio-Kine software from Bio-Logic.

The measurement of the activity were made over a period of 8 min in a medium containing an excess ATP and where the AlF_4^- concentration was diluted 5-fold as compared to that in the incubation medium. Because of this procedure, reversal of inhibition could have partially occurred due to ATP and/or to the AlF_4^- dilution. Several measurement points were made during this period of time and the ATPase activity was found linear from 1 to 8 min of incubation. It is therefore unlikely that any significant reversal could have occurred during this incubation period. On the other hand, any inhibition phase whose reversal rate

constant was faster than a few min^{-1} could not be detected with this method.

Results

Inhibition of the H^+-ATPase activity by fluoroaluminates

Purified H^+-ATPase was incubated in various NaF and $AlCl_3$ concentrations, incubation times being in the range of 0 to 120 min. A large inhibition was observed. Fig. 1 shows the time dependence of the inhibition at pH 7.5. We observed two distinct phases: the fastest of these phases occurred within the first 20 min which was then followed by a very slow process that lasted up to a few hours until total inhibition of the ATPase activity.

This slow secondary phase was difficult to analyze accurately as the control sample also show an important activity decrease over such a long period of incubation.

The data shown on Fig. 1 have been obtained after deduction of the activity measured in the same condition of buffer and incubation time but in the absence of aluminum. Therefore, the inhibition observed is not due to the presence of magnesium fluoride complexes as described elsewhere for transducin [10] or for Ca^{2+}-ATPase [23].

Determination of the fluoroaluminate compound(s) responsible for the rapid process of inhibition

The method of analysis allowing the demonstration of the prominent role of AlF_4^- in the inhibition of the SR ATPase has been detailed in a recent article [16]. A similar method was used here for the yeast H^+-ATPase. The apparent rate constant of inhibition was measured for many Al^{3+} and F^- concentrations. Under each of these conditions we calculated the concentration of most of the AlF_x and $Al(OH)_yF_z$ complexes. All complexes were present in the incubation solution but in variable concentrations depending on Al^{3+} and F^- concentration and on the pH of the solution. We plotted the apparent rate constant of inhibition as a function of the concentration of each complex, as if the inhibition was produced by each of these. Only a few complexes gave a significant correlation between the

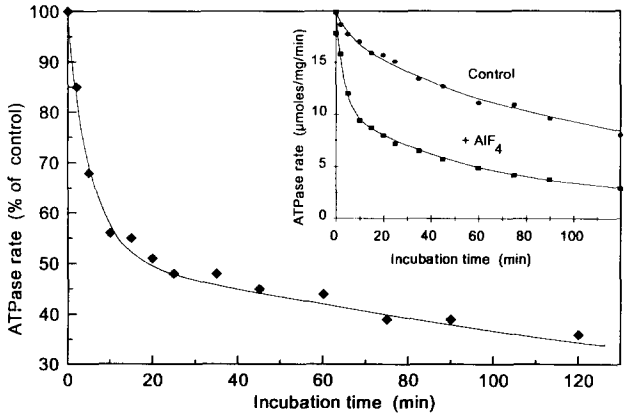

Fig. 1. Time-course of inhibition of H^+-ATPase activity by fluoroaluminates. ATPase activity of the purified enzyme is given as a function of incubation time in a buffer containing: 40 μg/ml ATPase, 50 μg/ml lysolecithin, 100 mM Mes/Hepes/KOH (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 2 mM NaF with 200 μM $AlCl_3$ (corresponding to 10 μM free AlF_4^-). The original ATPase activity data used to calculate this plot are shown in Inset. The activity of the sample incubated in the presence of AlF_4^- represented as a percentage of the control sample but without $AlCl_3$. (The ATPase assays were performed at pH 5.5 as described in Materials and Methods).

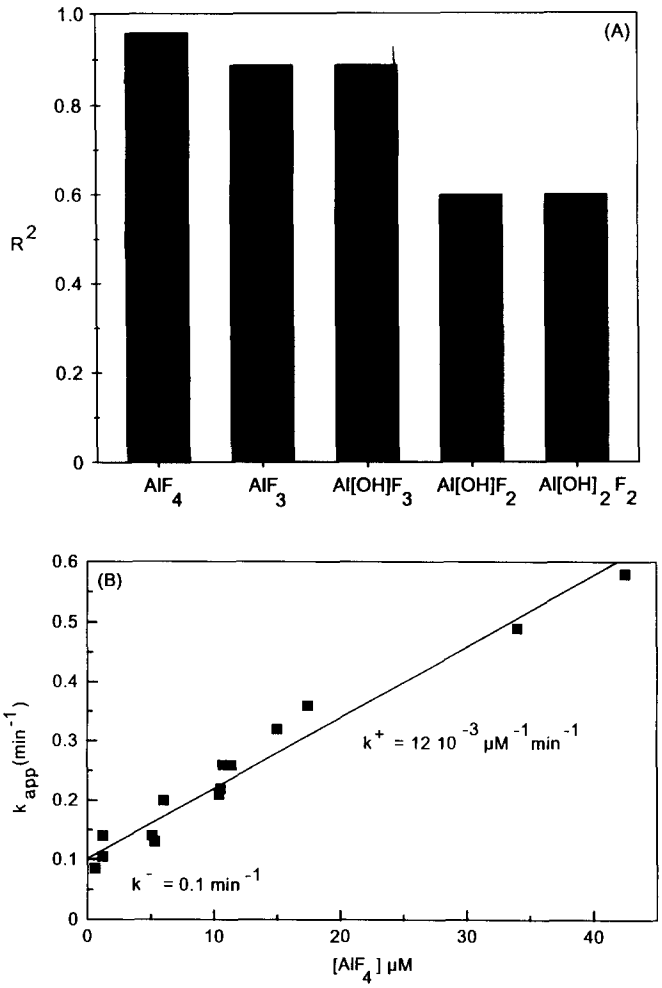

Fig. 2. Determination of the active fluoroaluminate complexes. The experimental conditions were as described in Fig. 1 except that NaF and $AlCl_3$ concentrations were chosen to obtain the indicated AlF_4^- concentration. Values of the rate constants were calculated as described in Materials and Methods. (A) Linear correlation coefficient (R^2) of the curves obtained when plotting the apparent rate constant of the rapid phase versus the calculated concentration of the different fluoroaluminate complexes present in the incubation medium. (B) Apparent rate constants of the rapid phase as a function of AlF_4^- concentration at pH 7.5. The slope of the line gives the k^+ rate, the k^- rate being deduced from the intercept with the y-axis.

rate constant and the concentration; these complexes are given in the Fig. 2A.

A linear correlation coefficient was calculated, the best correlations were obtained for AlF_4^-, AlF_3 and $Al(OH)F_3^-$, Therefore, these complexes are the most realistic candidates for inhibiting this enzyme.

However, the clear-cut results obtained for the SR ATPase could not be entirely reproduced in this present study, due to specific difficulties encountered in the study of the H^+-ATPase. The inhibition was tested by measuring the ATPase activity only. As this measurement was made over a period of several minutes, we could not use too high concentrations of F^- that would have given a too rapid inhibition. Because of this, the range of Al^{3+} and F^- concentration that can be used here were more limited than in the case of SR Ca^{2+}-ATPase whose inhibition was tested by fastest methods available.

The plot of the inhibition rate constant as a function of the AlF_4^- concentration shows the lowest dispersion around a linear dependence. Because of this, we propose that AlF_4^- is probably the most active complex in the H^+-ATPase inhibition, as was found for the SR Ca^{2+}-ATPase.

The simplest and acceptable fit simulating the experimental data points in Fig. 2B is a straight line. This is compatible with a simple bimolecular process where the apparent rate constant is given by:

$$k_{app} = k^- + ([AlF_4]\, k^+)$$

The intercept with the ordinate axis that is clearly different from zero. The value found, $k^{-1} \approx 0.1 \pm 0.05$ min^{-1} indicates that the inhibition at this phase of the reaction is reversible (Fig. 2B). This also implies that a dissociation constant for AlF_4^- can be evaluated, the value obtained is: $k^-/k^+ = 8.5$ μM. This value is very similar to that found for vanadate [7,8]. This result differs from what was found for the Ca^{2+}-ATPase where the 'off-rate' k^- could not be measured as it did not differ significantly from zero [16].

pH dependence of the H^+-ATPase inhibition by fluoroaluminates

Experimental conditions were varied to measure the rate constant of inhibition as a function of pH. Concentration of Al^{3+} and F^- were chosen to take into account the pH dependence of the concentrations of the various fluoroaluminate complexes and to perform the activity assays at a constant concentration of AlF_4^-.

A pH dependence study of the rate of the rapid phase of inhibition is shown on Fig. 3. This plot shows a clear increase of the inhibition rate constant above pH 7.0.

This transition is very similar to that observed for the pH dependence of (i) the H^+-ATPase activity, (ii) the intrinsic fluorescence of this protein, (iii) the fluorescence of bound Terbium-FTP, and (iv) the inhibition by vanadate of the H^+-ATPase [7].

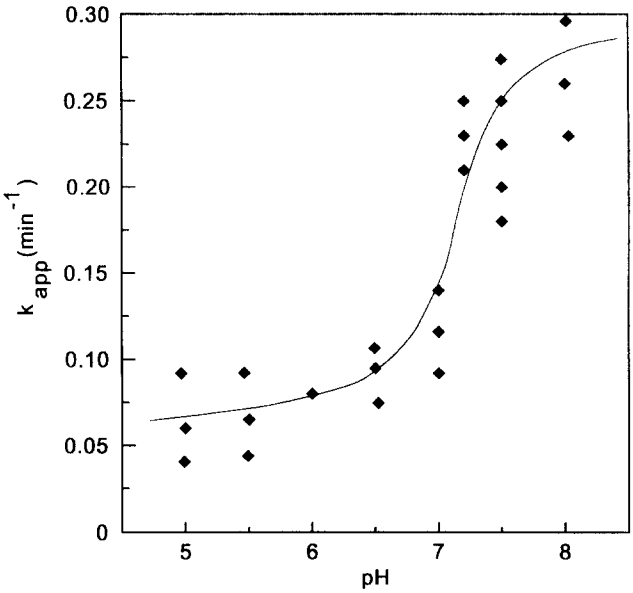

Fig. 3. pH dependence of the apparent rate constant of H^+-ATPase inhibition by fluoroaluminates. The rate constants correspond to the rapid inhibition process. Values of the rate constants were obtained as described in Materials and Methods and plotted as a function of the pH of the incubation medium. $AlCl_3$ and Fluor concentrations were chosen to obtain 10 μM AlF_4^- (NaF was varied from 2 to 4 mM and $AlCl_3$ from 20 to 300 μM).

There is, however, an uncertainty on the real pK value and the amplitude of the rate constant transition in the case of inhibition by AlF_4^-. This uncertainty is due to the possible contribution of other fluoroaluminate complexes: AlF_3 and $Al(OH)F_3^-$ in the H^+-ATPase inhibition as described above.

To check this we measured the same pH dependent inhibition curve by keeping the total concentration $[AlF_4^- + AlF_3 + Al(OH)F_3^-]$ constant. Under these conditions we also found a rate constant of inhibition lower at acidic pH ($k_i = 0.08 \pm 0.02$ min^{-1} at pH ≤ 6.5) as compared to more basic pH ($k_i = 0.15$ at pH 7.5).

Recently, the rates of ATP binding and ATP hydrolysis on purified H^+-ATPase from plasma membranes of *S. cerevisiae* were found to be dependent of the dissociation of a monovalent acid group with a pK of 7.4 [24]. All these effects have been taken as evidences for the existence of a $E_1 \rightleftharpoons E_2$ scheme for the yeast H^+-ATPase [1,7,25]. The fact that the same pH dependence is found here for the rate constant of inhibition by AlF_4^- is a further support for this scheme.

Therefore, AlF_4^- seems to act preferentially on the E_2 form of the enzyme and thus to act as an analogue of P_i, as previously shown for the Ca^{2+}-ATPase [16].

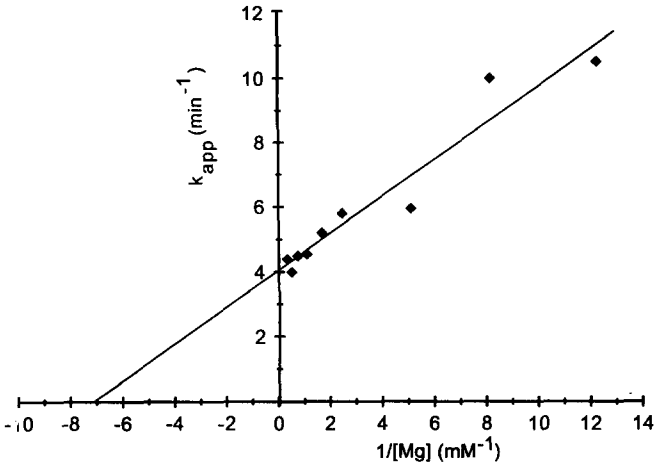

Fig. 4. Effect of Mg^{2+} on the inhibition rate constant. Experimental conditions were as described in Fig. 1, but with varying Mg^{2+} concentrations. pH was fixed at 7.5 and AlF_4^- concentration at 10 μM.

Effect of Mg^{2+} on the rate constant of inhibition

Mg^{2+} is a necessary cofactor for the phosphorylation reaction of all P-type ATPases. It is necessary either for the phosphorylation by ATP in the E_1 state, or for the phosphorylation by P_i in the E_2 state. To confirm the hypothesis that AlF_4^- inhibits the H^+-ATPase by a phosphorylation-like reaction, we have analyzed the rate of inhibition by AlF_4^- as a function of Mg^{2+} concentration.

The effect of Mg^{2+} was tested at concentrations ranging from contaminating Mg^{2+} to 10 mM. The contaminant free Mg^{2+}, that was estimated to 5 to 10 μM in our incubation medium could not be reduced by chelator of any type because this will result in preferential chelation of aluminum. The effect of Mg^{2+} on the rate of inhibition is shown in Fig. 4. Magnesium ions strongly accelerated the rate of inhibition confirming its role as a cofactor of AlF_4^- binding. The apparent affinity displayed by Mg^{2+} in this reaction is 140 μM. Although the role of Mg^{2+} in phosphorylation is well recognized, there are no data available on its apparent affinity in this reaction for the *S. pombe* H^+-ATPase.

A study of the H^+-ATPase of *S. cerevisiae* plasma membranes by Borst-Pauwels and Peters [26] permits some comparison. These authors reported that this enzyme binds Mg^{2+} with a dissociation constant of 400 μM at pH 7.0. This Mg^{2+} binding is involved in vanadate inhibition that stabilizes the enzyme in the E_2 conformation.

On the other hand, higher affinities for Mg^{2+} were measured for the activation by Mg^{2+} of ATP hydrolysis by isolated *S. cerevisiae* H^+-ATPase (k_{app} = 70 μM, [6,27,28]) and by Brooker and Slayman [29] for *Neurospora crassa* H^+-ATPase (k_{app} = 15 μM). These values are not so different from that which has been obtained in the present study for the effect of Mg^{2+} on the inhibition by AlF_4^-. On the other hand most of the variations observed in these studies may be due to differences in the medium pH.

Effect of Me_2SO on the inhibition by AlF_4^-

It has been shown that the rate of phosphorylation of Ca^{2+}-ATPase by P_i is extremely dependent on the water activity of the solvent. In a previous work [16], it has been found that the inhibition by AlF_4^- shows the same water concentration dependence as phosphorylation by P_i. This was a clear demonstration of the similarity between the AlF_4^- and P_i for binding to the E_2 form of the Ca^{2+}-ATPase protein.

Up to now nearly no data exist on a similar role of water activity on the H^+-ATPase. The only report on the role of water activity concerns an effect of Me_2SO on the P_i-ATP exchange catalyzed by the purified *S. pombe* H^+-ATPase. It was found that the rate of exchange increases with the pH and with the presence of Me_2SO [2].

We have tried to measure the effect of Me_2SO on the inhibition of the H^+-ATPase by AlF_4^- at neutral pH, where the enzyme is likely in the E_2 form.

Experiments were carried out in the absence of K^+ because this ion is expected to increase the rate of dephosphorylation as it does for all P-type ATPases [30].

The two phases in the inhibition process, as illustrated in Fig. 1, are characterized by different response to the addition of Me_2SO.

The first phase of the inhibition is Me_2SO independent up to 20% (v/v) concentration. At higher concentrations, we observed a slight reduction of the rate constant of this phase leading to a significant protection against the inhibition by AlF_4^-.

On the opposite, increasing Me_2SO from 0 to 20% produced a 4-fold increase of the rate constant of the slow phase of the inhibitory process. This result may be compared with that found for the Ca^{2+}-ATPase, where a 10-fold activation was observed for 10% Me_2SO only.

Fig. 5 displays an analysis of the rate constant of the slow phase of inhibition as a function of the water concentration. A similar analysis was conducted for the Ca^{2+}-ATPase as reported for the Ca^{2+}-ATPase for the phosphorylation by P_i [31] and for the inhibition by AlF_4^- [16].

For the H^+-ATPase, the slope of the curve shows that the reaction is dependent on the 5th power of the water concentration, as compared to 22 for the Ca^{2+}-ATPase.

Discussion

Our results in this article demonstrate that AlF_4^- inhibits the yeast H^+-ATPase by the formation of a

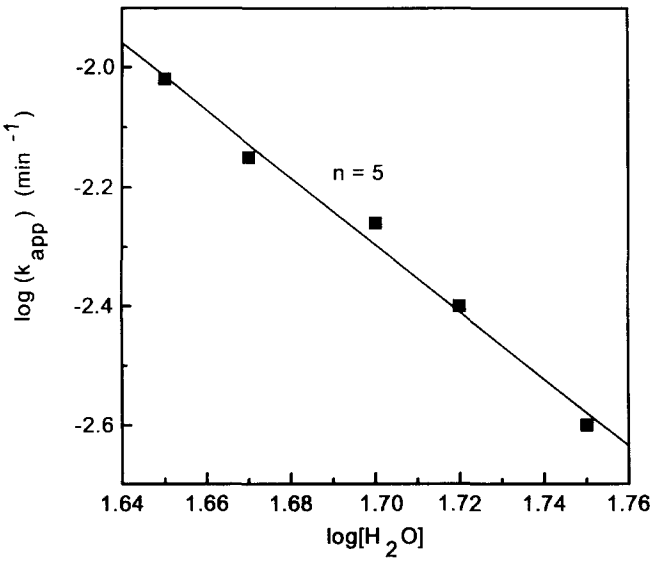

Fig. 5. Effect of water activity on the rate constant of the slow inhibition phase. Experiments were performed in the presence of various concentrations of Me_2SO as described in Fig. 1 except that the buffer used was Hepes/Tris without KCl. The logarithm of the rate constants of the slow phase were plotted as a function of the logarithm of the water concentration. The slope of the straight line drawn through the points gives an estimate of the number of water molecules released from the active site upon AlF_4^- binding.

stable complex similar to a E_2-P form of the enzyme. This effect is similar to that reported in a recent article [16], where we have shown that AlF_4^- is the active complex inhibiting the Ca^{2+}-ATPase.

Accordingly, we have observed that, as for the phosphorylation by P_i, binding of AlF_4^- requires Mg^{2+}. The inhibitory reaction proceeds in two phases: the first phase is reversible ($K_d = 8.5\ \mu M$), is sensitive to pH and Mg^{2+} and insensitive to addition of Me_2SO up to 20% concentration. The slower phase is probably irreversible and is sensitive to the water concentration of the medium as demonstrated by its acceleration by small addition of Me_2SO in the solvent. So we propose that AlF_4^- binding proceeds according to the following scheme:

$$E_2 \cdot Mg + AlF_4 \rightleftharpoons E_2 \cdot Mg \cdot AlF_4 \overset{5H_2O}{\rightleftharpoons} E_2^{\#} \cdot Mg \cdot AlF_4$$

$$\longrightarrow E_2^{\#} \cdot Mg\text{-}(AlF_4)$$

The first step corresponds to a high affinity binding of AlF_4^- to the E_2 state that mimics the formation of the non-covalent phosphate, the second to the dehydration of the active site to for the $E_2^{\#}$ state. This dehydration is then followed by the formation of a stable complex $E_2^{\#} \cdot Mg\text{-}(AlF_4)$. This latter phase is similar to the formation of the covalent E_2-P intermediate of the H^+-ATPase. The strong dependence on water activity of the phosphorylation by phosphate has been first demonstrated for the Ca^{2+}-ATPase enzyme [32].

It is likely that the formation of stable E_2-P or E_2-AlF_4 intermediates are possible processes only because of the occurrence of a concomitant strong dehydration of the active site.

Recently, it has been shown that the hydrophobic character of the protein domain located in the β-strand sector influences the interactions of the enzyme with inorganic phosphate and consequently could play a role in the translocation step [33].

Detection of active-site hydration changes induced by phosphorylation in the case of the yeast H^+-ATPase is of primary interest. It has been proposed [31] that the same mechanism of dehydration of the phosphorylation site would be linked to changes of hydration of the transported cation during the translocation process. In this aspect, the observation of this process with a proton transporting enzyme is of great interest, as hydration properties of proton and calcium are most likely different.

It seems, therefore, very encouraging that the water-activity dependence of the H^+-ATPase inhibition by AlF_4^- is found significantly smaller than that of the same reaction in the Ca^{2+}-ATPase. Thus, if a dehydration/hydration mechanism is involved in the phosphorylation process of the H^+-ATPase, it is of a lesser extent as for the Ca^{2+}-ATPase. Only five water molecules being involved for the former as compared to about 20 for the latter enzyme.

At variance with the Ca^{2+}-ATPase we observed little or no inhibition by the ADP-AlF_4. This was tested at acid pH where the predominant conformation of the enzyme should be the E_1 form. This lack of effect can be attributed to a very low affinity for the complex ADP-AlF_4 that should parallel the millimolar affinity of ATP on the H^+-ATPase protein. Finally, it is likely that the inhibition process observed for AlF_4^- is probably not very different as that occuring in the case of inhibition by vanadate [7,8]. The main advantage of AlF_4^- is its slow binding process that permits easy measurements of kinetics of inhibition and its even slower dissociation rate. The quasi-irreversibility of the inhibition will makes this compound a very useful reaction intermediate analog in particular to study the phosphorylated intermediate with slow biophysical methods.

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