The Yeast Plasma Membrane H\(^+\)-ATPase

AN ESSENTIAL CHANGE OF CONFORMATION TRIGGERED BY H\(^+\)

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The plasma membrane of *Schizosaccharomyces pombe* contains an H\(^+\)-ATPase similar to the cation transport ATPases of other eukaryotic organisms. The fluorescence excitation and emission spectra of the purified H\(^+\)-ATPase are characteristic of tryptophan residues. pH reduction from 7.5 to 5.7 produces a 4% decrease in fluorescence intensity, while a further reduction to pH 5.0 leads to an increase of fluorescence. A close correlation is observed between the pH dependence of the intrinsic fluorescence and the pH dependence of (i) ATPase activity, (ii) the fluorescence of Thiolformycin triphosphate bound to the active site, and (iii) inhibition by vanadate of ATPase activity. It is proposed that the effect of pH on intrinsic fluorescence reveals the existence of an H\(^+\) induced conformational change of the H\(^+\)-ATPase similar to the E\(_1\) ↔ E\(_2\) transition of the other plasma membrane cation transport ATPases.

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 ATPases, 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). At the same time the 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.

A similar molecular mechanism has been postulated for the H\(^+\)-ATPase from the plasma membrane of fungi (1). In *Neurospora crassa*, evidence of conformational changes has been obtained from the analysis of trypsin-treated plasma membrane vesicles incubated in the absence or presence of several ATPase substrates or effectors (5). In the yeast *Schizosaccharomyces pombe*, P ↔ HOH exchange kinetics (1) as well as enzyme phosphorylation (3) and P ↔ ATP exchange data (2) have strengthened the concept that the H\(^+\)-ATPase mechanism involves at least two isomeric forms of the enzyme.

In the case of the mammalian Ca\(^{2+}\)- and Na\(^+\),K\(^+\)-ATPases, direct evidence for the E\(_1\)-E\(_2\) conformational change has come from intrinsic fluorescence changes induced by substrate binding (6). This approach associated with rapid kinetic methods has yielded very complete details of the enzyme reaction scheme. In the present study, we have examined the intrinsic fluorescence of purified H\(^+\)-ATPase from the plasma membrane of *S. pombe* and have detected a proton-induced change of fluorescence intensity. A strong correlation was observed between the pH dependence of this change and that of the ATPase activity, inhibition by vanadate, and the fluorescence yield of Tb-FTP\(_{10}\) bound to the ATP site. It is concluded that the binding of the translocated cation (H\(^+\)) induces a conformational change of the H\(^+\)-ATPase and that this change is similar to the E\(_1\) ↔ E\(_2\) transition of the other plasma membrane transport ATPases.

EXPERIMENTAL PROCEDURES

Materials

ADP, ATP (disodium salt, grade II), TbCl\(_3\)·6H\(_2\)O, Mops, and egg lysoskeleton were purchased from Sigma. Mes, pyruvate kinase (in glycerol) and phosphoenolpyruvate were from Boehringer Mannheim. MgSO\(_4\)·7H\(_2\)O, NaN\(_3\), and sodium dodecyl sulfate were from Merck. Orthovanadate was from BDH Ltd. p-Methylaminoanilone sulfonate (Elon) was from Kodak. Formycin triphosphate (FTP, tetralithium salt) was from Behring Diagnostics. Other reagents were of the purest commercially available grade.

Purification of the Plasma Membrane ATPase—*S. pombe* 972h\(_{+}\) was grown at 30 °C in 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.

Isolation of plasma membranes and solubilization of ATPase were carried out as described by Dufour et al. (7). Before use, the purified ATPase was passed through a Sephadex G-50 column, as described by Fenolosky (6), to set the enzyme in the right buffer and to eliminate sucrose from the preparation.

Adenosine Triphosphatase Assays—Unless stated otherwise, ATPase assays were carried out at 30 °C in 100 μl of the following medium: 10 mM Mops, 10 mM Mes, 50 mM acetate (brought to pH 6.0 with HCl), 100 μg/ml lysoskeleton, 15 mM NaN\(_3\), 9 mM MgSO\(_4\), purified ATPase 10 μg/ml. After 2 min of preincubation, the reaction was started with 6 mM Na\(_2\)ATP and ended by 300 μl of sodium dodecyl sulfate

1 The abbreviations used are: FTP, formycin triphosphate; Mops, 3-(N-morpholino)propanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid.
sulfate 1% after 6–20 min. The released inorganic phosphate was measured as described by Fiske and Subbarow (9). For \( K_m \) and \( K_i \) determinations, an ATP-regenerating system was added, consisting of 60 IU/ml pyruvate kinase and 2.5 mM phosphoenolpyruvate. In those conditions and at all ATP concentrations, the rate of hydrolysis was constant for more than 20 min.

**Fluorescence Recording**—All fluorescence data were recorded on a high sensitivity fluorimeter manufactured by Bio-Logic Co. (Claix, France). The reaction was followed at 20 °C in a 1 × 1-cm fluorescence cuvette (2-ml final volume) under stirring. Additions to the cuvette were made with Hamilton constant rate syringes through special openings. The sample was excited through a monochromator with a 150-watt Xenon arc lamp. Emitted light and light scattering were simultaneously recorded at right angles from excitation through collecting lenses and interference filters. All the data were corrected for dilution signals and for unspecified optical signals originating in the interaction of fluorescent species with component of the medium in absence of ATPase.

**Measurements of Tb-FTP Fluorescence**—Tb-FTP fluorescence was excited at 305 nm, and the emitted light was filtered with a Belzers K-55 broad band filter (580 nm), or a narrow band-pass filter centered at 546 nm (the strongest Tb\(^{3+}\) emission line) to increase the signal-to-noise ratio. Light scattering changes were simultaneously monitored through a narrow band-pass filter centered at 570 nm (between two Tb\(^{3+}\) emission lines). The medium (2 ml) contained 5 \( \mu \)g/ml purified ATPase, 50 mM Mes (brought to pH with HCl or NaOH) and 50 \( \mu \)g TbCl\(_3\). Enzyme was preincubated at 20 °C during 5 min before adding FTP. FTP concentration of stock solutions was estimated from the absorbance at 295 nm \( (\epsilon_{295} = 11.5 \times 10^4 \text{M}^{-1} \text{cm}^{-1}) \), pH 6.0).

**Measurements of Intrinsic Fluorescence**—The sample was excited at 295 nm and the emitted light at 320 nm was recorded at right angles from excitation with filters 0–54 and 7–54 from Corning. The medium (2 ml) contained 10 \( \mu \)g/ml purified ATPase in 50 mM KCl, 5 mM Mes, 5 mM Mops, 5 mM acetate (brought to the adequate pH with KOH).

**Protein Concentration Determination**—The concentrations of protein were determined by the method of Bradford (10) or by the Folin protein assay using bovine serum albumin as standard. A molecular mass of 100,000 daltons was used for the calculation of the molar concentration of the purified H\(^{+}\)-ATPase, assuming a 80% pure preparation.

**RESULTS**

**pH Effects on the Overall Turnover (\( k_{cat} \)) for ATP Hydrolysis**—In a preliminary set of experiments, we have determined the pH range in which the purified H\(^{+}\)-ATPase is stable under our conditions (Fig. 1A, squares). The purified ATPase was preincubated in assay medium at the indicated pH for several time intervals and then brought back to the optimal pH (6.0) for testing residual activity. As illustrated in the figure, a preincubation of 6 min produced no irreversible loss of activity from pH 5.4 to pH 7.3. At lower or higher H\(^{+}\) concentrations, the enzyme was rapidly denatured, leaving only 75% residual activity at pH 6.0 or 4.8.

In another set of experiments, ATPase activity was tested directly at the indicated pH, all other conditions being the same (Fig. 1A, triangles). Therefore, these data reflect global effects (reversible and irreversible) of pH on ATPase activity. Comparing the pH curves from the two sets of experiments, reversible H\(^{+}\) effects on the catalytic mechanism can be distinguished from irreversible denaturation at extreme values of pH. An optimal pH near 5.8–6.0 was found, in agreement with Dufour et al. (11). For lower values of pH, there was a rapid decrease of activity. Since this decrease occurred in a range where the rate of denaturation is high, the contribution of irreversible, and unspecific loss of active ATPase molecules cannot be assessed. This part of the curve was not evaluated further. For H\(^{+}\) concentrations lower than 10\(^{-7}\) M, there was also an important decrease of the turnover rate of ATP hydrolysis \( (k_{cat}) \), which was clearly distinct from trivial denaturation. As shown in Fig. 1B, this part of the data fits well a titration curve in the following equation.

\[
1/k_{cat} = 1/k_{cat}^0 + K_a/(k_{cat}^0 [H^+])
\]  

This is obtained for a protonable residue with a dissociation constant \( K_a \) which activates ATPase activity to a maximal value of \( k_{cat}^0 \), at very low pH. Experimental values of 1.17 × 10\(^{−7}\) M for \( K_a \) (pK\(_a\) = 6.9) and 42 s\(^{-1}\) for \( k_{cat}^0 \) were determined from the slope and the intercept with the y axis in Fig. 1B.

**pH Dependence of the Tryptophanyl Fluorescence of ATPase**—The variation of intrinsic fluorescence is a well established way to probe protein structural changes (13). This technique has been used here to monitor the effects of pH on the conformation of the H\(^{+}\)-ATPase. Fig. 2 illustrates the fluorescence emission (A) and excitation (B) spectra of purified ATPase. These spectra are characteristic of tryptophanyl fluorescence with a broad peak centered at 330 nm for emission and at 285 nm for excitation. Because the fluorescence quantum yield of the ATPase is high, only small amounts of protein were necessary for measuring its fluorescence properties.

We observed that a variation of pH modifies the fluorescence intensity of the ATPase tryptophan(s) without inducing any detectable wavelength shift (emission or excitation). Fig. 2C illustrates a typical trace of the intrinsic fluorescence change corresponding to a pH shift from 7.5 down to 7.2. The fluorescence change is fast and can be reversed by bringing the pH back to its initial value. The variations of intrinsic fluorescence intensity were recorded from pH 7.5 to 5.0 (Fig. 3). From pH 7.5 to around 5.7, the acidification of the medium was accompanied by a 4% decrease in intrinsic fluorescence of the H\(^{+}\)-ATPase whereas further acidification of the me-
ADP, or free Mg$^{2+}$. In none of these cases could a significant modification of the intrinsic fluorescence signal be observed. At the highest ATP concentration tested, measurements were difficult because of the high absorbance signals produced by the concentrated solutions of ATP or ADP used. However, an intrinsic fluorescence change similar to that induced by Mg-ATP on the Ca$^{2+}$-ATPase would have been observed easily.

**pH Effects on the Nucleotide-binding Site of the H$^+$-ATPase**—In a previous paper, Ronjat et al. (15) have shown that formycin-triphosphate is an analog of ATP which is readily hydrolyzed by the H$^+$-ATPase. In the presence of terbium ions, FTP hydrolysis is inhibited, and its binding to the nucleotide site can be evaluated. Because of a fluorescence transfer phenomenon from FTP to Tb$^{3+}$, fluorescence of the complex is very sensitive to its physicochemical environment. We have therefore used FTP as a probe to investigate H$^+$ effects on the nucleotide site of H$^+$-ATPase. Fig. 4A illustrates the increase of Tb-FTP fluorescence transfer rate produced by successive increments of FTP at several pH values. Obviously the affinity for the substrate was not affected by pH (Fig. 4B), a pH-independent dissociation constant ($K_D$) of 1.2 $\mu$m was found, in agreement with competition studies reported by Ronjat et al. (15).

However, the maximal fluorescence change observed for binding of saturating concentrations of FTP ($\Delta F_{FTP}$) was highly sensitive to pH, as shown in Fig. 4, A and C. The fluorescence intensity was low at acidic pH, increased up to pH 5.9, and then decreased again for higher pH values. This pH curve, with a $pK_a$ of 6.85 (Fig. 4D), is quite similar to the one observed for ATPase activity (Fig. 1A).

Taken together, the results indicate that the affinity of the ATPase for the metal-nucleotide complex is the same at all pH values, but the fluorescence intensity of the bound Tb-FTP complex is highly dependent on the pH of the medium. It is likely that the physicochemical properties of the active site are modified by H$^+$, either directly in the substrate pocket and/or through a conformational change. This latter would correlate well with the one observed by tryptophanyl fluorescence analysis and with the activating effect of protons on H$^+$-ATPase activity.

**H$^+$ Sensitivity of ATPase Inhibition by Vanadate**—The results above support the classification of the H$^+$-ATPase as a member of the $E_1$-$E_2$ class. To further test this idea, we have used vanadate-Mg. Vanadate-Mg is a very specific inhibitor of this class of ATPases, binding to an intermediate conformation able to be phosphorylated by inorganic phosphate (16-19).

In the experiment of Fig. 5, H$^+$-ATPase was preincubated with vanadate in the assay medium at 2 °C for 30 min before adding ATP, leading to maximum inhibition without significant thermal denaturation. A Dixon plot of the data was nonlinear at vanadate concentrations higher than 10 $\mu$m (not shown), as already reported (18). Although inhibition by such high vanadate concentrations was reduced at high pH, investigations in this range were not pursued further. For low vanadate concentrations, simple inhibition patterns (noncompetitive versus Mg-ATP), were observed at all pH values. The apparent $K_i$ was 8 $\mu$m at pH 6.0 in agreement with values previously reported (18), the apparent inhibition constant for vanadate being decreased for increasing pH values. This observation suggests that vanadate binds preferentially the $E_2$ conformation that is present at low proton concentrations.

Surprisingly, for the H$^+$-ATPase from S. cerevisiae, Borst-Pauwels et al. (20) have reported a stimulation of vanadate inhibition when the pH is decreased. However, these authors
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**FIG. 4.** Fluorescence increase of Tb-FTP upon binding to the purified ATPase. A, 5 µg/ml purified ATPase were incubated in 2 ml containing 50 mM Mes (brought to the indicated pH with HCl or KOH) and 50 µM TbCl\(_3\). After 5 min of incubation at 20 °C, aliquots of 1 µM FTP were progressively added and the fluorescence change was recorded as described under “Experimental Procedures.” For all FTP concentrations a blank was performed without ATPase and subtracted from the signal. B, effect of the pH on the dissociation constant (K\(_d\)) for Tb-FTP. C, effect of the pH on the maximal fluorescence changes observed for saturating concentrations of Tb-FTP. D, double-reciprocal representation of the same data.

It did not test inhibition at pH values below 7.0 where the transition between the E\(_1\) and E\(_2\) state is supposed to take place.

It appears from Fig. 5 that H\(^+\) and vanadate are mutually exclusive on the ATPase (competitive inhibition). Protonation reduces the number of sites available for vanadate, either directly in the site or through a change of conformation. Absolute K\(_d\) of 1.3 µM vanadate and K\(_d\) of 1.3 × 10\(^{-7}\) M proton (pK\(_{a}\) 6.9) were calculated from the slopes and the intersecting ordinate.

**Effect of Simultaneous Binding of Vanadate and Tb-FTP—**

Since vanadate was shown to act competitively with protons and since protons were shown to induce a strong increase of Tb-FTP fluorescence, we have investigated the binding of Tb-FTP to the ATPase in the presence of vanadate. Aliquots of 1 µM FTP were progressively added to ATPase preincubated with different concentrations of vanadate. As shown in

**FIG. 5.** pH effects on vanadate inhibition of the H\(^+\)-ATPase (Dixon plots). 1.3 µg of purified plasma membrane was first preincubated at 4 °C in a volume of 90 µl containing 11.1 mM Mes, 11.1 mM Mops, 55.5 mM acetate, 16.6 mM NaN\(_3\), 3.3 mM MgSO\(_4\), 0.22–11.1 µM orthovanadate, 2.7 mM phosphoenolpyruvate and 66 UI/ml pyruvate kinase (ATP regenerating system). The pH was brought to the indicated value with HCl or NaOH. After a 30-min preincubation, 10 µl of Mg-ATP 100 mM were added, and the reaction was allowed to proceed for 20 min at 30 °C. Inorganic phosphate was then determined as described under “Experimental Procedures.”

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**DISCUSSION**

Intrinsic fluorescence has been used to study a number of transport proteins: Ca\(^{2+}\)-ATPase (6), the Na\(^+\),K\(^+\)-ATPase (21), and the mitochondrial nucleotide carrier (22). In each case, it varies with the binding of the substrate and/or the turnover of the carrier. In the present paper we show that the fluorescence quantum yield of the H\(^+\)-ATPase varies with the proton concentration of the medium (Fig. 3). This variation indicates changes of polarity in the vicinity of one or more tryptophanyl residues, which result most probably from changes of conformation of the protein. Indeed, the intrinsic fluorescence of Ca\(^{2+}\)-ATPase has been shown to be a reliable...
index of the conformational change related to Ca\textsuperscript{2+} binding at the high affinity site (13).

The specificity of the change observed by intrinsic fluorescence was confirmed by the use of an extrinsic fluorescent probe, Tb-FTP, a non-hydrolyzable analog of Mg-ATP (15). We have shown that the binding of FTP is H\textsuperscript{+} insensitive, the same dissociation constant (K\textsubscript{d}) being observed at all pH values (Fig. 4B). However, the fluorescence intensity of bound Tb-FTP is sensitive to protons, indicating that the cation-induced conformational change monitored by tryptophan fluorescence also affects the nucleotide site. Analogous results have been obtained with Ca\textsuperscript{2+}-ATPase using Tb-FTP (23) and fluorescein isothiocyanate (25).

In the present study, a pK\textsubscript{a} near 6.9 was found from quantitative analysis of pH effects on tryptophanyl or Tb-FTP fluorescence and on ATPase activity.

Since the same pK\textsubscript{a} value was found under steady-state and equilibrium conditions, the possibility of a misleading kinetic pK\textsubscript{a} (12) dominated by a kinetic constant is ruled out. Therefore, the catalytic intermediate accumulating under steady-state conditions must have the same pK\textsubscript{a}, as the free enzyme. This is in agreement with the P-HOH experiments leading to the conclusion that E\textsubscript{2} accumulates during turnover (1). It is not possible to completely rule out the existence of H\textsuperscript{+} interactions with general groups on the substrate and/or the protein, which are not directly related to a meaningful event in the transport process. However, several lines of evidences suggest that the pH effects observed in this work are directly related to an important catalytic step of the H\textsuperscript{+}-ATPase.

(i) Ionization of the substrate is ruled out since some experiments were performed on the free enzyme.

(ii) H\textsuperscript{+} has strong effects on intrinsic (tryptophanyl) or extrinsic (Tb-FTP) fluorescence and on H\textsuperscript{+}-ATPase activity. These effects are specific to protons since they are not seen with Ca\textsuperscript{2+} nor with Na\textsuperscript{+}. On the contrary Ca\textsuperscript{2+}-ATPase or Na\textsuperscript{+},K\textsuperscript{-}-ATPase fluorescence and activities have been shown to be highly sensitive to those ions, respectively (13, 14).

(iii) No direct H\textsuperscript{+} effect was reported for Ca\textsuperscript{2+}-ATPase or Na\textsuperscript{+},K\textsuperscript{-}-ATPase in the pH range investigated in the present study (13, 14), although the primary structure of H\textsuperscript{+}-ATPase is highly homologous to the other P-type ATPases (4).

(iv) In contrast, as reported in this article, several mechanistic features of the yeast H\textsuperscript{+}-ATPase have been shown to involve a proton-sensitive site with an apparent pK\textsubscript{a} of 6.7.

Altogether, these results point to a meaningful change of conformation of the ATPase related to proton binding and ATPase activation.

The lack of fluorescence change induced by the addition of Mg-ATP remains to be explained. In fact, several lines of evidence suggest that only a minor fraction of the H\textsuperscript{+}-ATPase seems to exist in the E-ATP or E-P form during turnover. (i) As shown by kinetic studies under single turnover conditions (27), by the high variability of the K\textsubscript{m} of Mg-ATP hydrolysis (11, 26), and by the fact that the K\textsubscript{m} depends upon the pH of the medium (27), the phosphoryl transfer reaction appears to be much faster than ATP binding and releasing steps, preventing the accumulation of the E-ATP intermediate. (ii) During turnover, only a few percent of the enzyme is phosphorylated (3) indicating that the dephosphorylation step may be faster than the aforementioned steps. Consequently, the slowest step of the hydrolytic reaction is probably related to Mg-ATP binding, with most of the enzyme remaining in the free E\textsubscript{1} and/or E\textsubscript{2} form even in the presence of Mg-ATP. This would explain the apparent insensitivity of the intrinsic fluorescence to the addition of Mg-ATP. It would also explain why the pK\textsubscript{a} for H\textsuperscript{+} binding is the same for the free enzyme at equilibrium and for the accumulating catalytic intermediate at steady-state. (iii) Finally, the absence of any fluorescence change induced by Mg-ATP might be attributed to the fact that either the binding of Mg-ATP does not involve a significant protein conformational change, or that the conformational change does not affect the fluorescent center. Indeed structural analysis based on nucleotide sequence (4) predicts that among the 12 tryptophanyl residues of the yeast H\textsuperscript{+}-ATPase, only 2 are located in the cytoplasmic a60 structure that is supposed to be part of the nucleotide-binding site. Use of lipophilic tryptophanyl quenching probes has also shown high heterogeneity of tryptophanyl response from hydrophilic loops or membrane spans of the Ca\textsuperscript{2+}-ATPase (28, 29).

A final observation is that vanadate inhibition of the H\textsuperscript{+}-ATPase is strongly increased at high pH values and that inhibition is competitive with protons again with a pK\textsubscript{a} of 6.9. This competition explains previous results describing an acidic shift of the optimal pH of yeast H\textsuperscript{+}-ATPase activity in the presence of vanadate (18). This has also to be compared with the observed competition between high affinity cation binding and vanadate for the Ca\textsuperscript{2+}-ATPase (17, 30) and Na\textsuperscript{+},K\textsuperscript{-}-ATPase (19).

In conclusion, the present work shows that H\textsuperscript{+} plays an important and specific role in the reaction cycle of the plasma membrane H\textsuperscript{+}-ATPase. In the minimal scheme of Fig. 7 in which all the substrates and inhibitors used in this paper are represented, interesting features of the protonation step involve: (i) initiation of the Mg-ATP hydrolysis cycle, (ii) occurrence of a concomitant conformational change that can be detected by intrinsic fluorescence and that affects the nucleotide pocket (Tb-FTP fluorescence), and (iii) reduction of apparent affinity for vanadate binding.

From the evidence discussed here and by homology with the other P-type ATPases we propose that this protonable site with an apparent pK\textsubscript{a} of 6.9 is the high affinity binding site for the translocated cation.

REFERENCES

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