

An H⁺-ATPase Assay: Proton Pumping and ATPase Activity Determined Simultaneously in the Same Sample¹

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ABSTRACT

A continuous spectrophotometric assay of H⁺-ATPase activity was developed by combining two well-known methods for measuring proton pumping and ATPase activity. Proton uptake into plasma membrane vesicles from *Avena sativa* L. (cv Rhiannon) was monitored as the absorbance decrease at 495 nm of the ΔpH probe acridine orange. Simultaneously, ATPase activity was measured by following the absorbance decrease at 340 nanometers by coupling ATP hydrolysis enzymatically to the oxidation of NADH. This H⁺-ATPase assay is convenient for determining the relative relationship between ATP hydrolysis and proton pumping.

H⁺-ATPases are enzymes that couple the hydrolysis or synthesis of ATP to a transmembrane movement of protons. H⁺-ATPases are found in nearly all cells and are divided into three distinct classes: the plasma membrane type, the vacuolar or lysosomal type, and the F₀F₁ type, which is present in mitochondria and chloroplasts (13, 14).

In the case of Na⁺/K⁺- and Ca²⁺-ATPases unidirectional ion fluxes can be measured using radioisotopes, but in the case of H⁺-ATPases such measurements are not possible. Proton fluxes may be followed indirectly by use of metachromatic dyes that change absorbance or fluorescence properties in response to the formation of pH gradients in membrane vesicular systems (6). When studying H⁺-ATPases using such an indirect approach it is important to avoid the artefacts that have recently been ascribed to metachromatic dyes (9, 16). This can be achieved by measuring ATP hydrolysis and apparent proton pumping simultaneously under identical conditions. This is also a way to examine the kinetic correlation between proton pumping and ATP hydrolysis.

The purpose of the present work was to develop a method to study the H⁺-ATPase reaction in a more direct way, particularly the coupling between ATP hydrolysis and H⁺ pumping. ATPase activity can be monitored spectrophotometrically by use of enzyme systems that couple ATP hydrolysis to the oxidation of NADH (8). In the present communication it is shown that it is possible by a combination of two well-known methods to make parallel measurements in the

same cuvette of ATPase activity and H⁺ pumping. The assay described was developed for the plant plasma membrane H⁺-ATPase, but should also be applicable to the other types of H⁺-ATPases.

MATERIALS AND METHODS

Chemicals

Lactate dehydrogenase (solution in glycerol), pyruvate kinase (solution in glycerol), NADH, PEP², and ATP were from Boehringer. ATP (disodium salt) was converted to BTP salt on an Amberlite IR-120 (BDH Chemicals) anion exchange column, eluted with Mops-BTP and buffered with BTP.BSA (essentially fatty acid and globulin-free), valinomycin, nigericin, and acridine orange were from Sigma. All other chemicals were of highest grade commercially available.

Plant Material

Oat (*Avena sativa* L. cv Rhiannon) was grown hydroponically in the dark and the roots harvested after 8 d as described previously (10).

Plasma Membrane Vesicles

Plasma membranes consisting of a mixture of right side-out and inside-out vesicles were purified from a microsomal fraction (10,000 to 30,000g pellet) in an aqueous polymer two-phase system (5, 10).

H⁺-ATPase Assay

ATPase activity was determined simultaneously with proton pumping (10). The assay medium consisted of 10 mM Mops-BTP (pH 7.0), 2 mM ATP, 4 mM MgCl₂, 140 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mg mL⁻¹ BSA, 20 μM acridine orange, 0.25 mM NADH, 1 mM PEP, 50 μg mL⁻¹ pyruvate kinase, 25 μg mL⁻¹ lactate dehydrogenase, and 50 μg mL⁻¹ plasma membrane protein in a total volume of 1 mL. Stock solutions of ATP, EDTA, BSA, and PEP, all pH 7.0 with BTP, were stored in frozen vials and thawed just prior to use. A mixture containing Mops-BTP, ATP, KCl, EDTA, DTT, BSA, and acridine orange was equilibrated at room temperature (or 20°C) and a portion of 860 μL was transferred to a disposable 1 mL cuvette, 1 cm light path. Then, 50 μL membrane suspension was added, followed by 50 μL of a

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² Abbreviations: PEP, phosphoenolpyruvate; BTP, 1,3-bis(tris(hydroxymethyl)-methylamino)propane.

freshly prepared solution of NADH mixed with PEP, and 25 μL of a mixture of pyruvate kinase and lactate dehydrogenase. After incubation for 4 min, automatic recording in 12 s intervals at 340 nm and 495 nm was initiated using a spectrophotometer (Shimadzu UV-160) that can automatically switch between the two wavelengths. The reference cuvette contained distilled water. MgCl_2 was added to start the reaction and mixed into the assay solution by stirring for a few seconds with the tip of the pipette. In this assay ATP hydrolysis is coupled enzymatically to oxidation of NADH, and the rate of ATP hydrolysis was quantified from the rate of NADH oxidation measured at 340 nm after 30 s (8). Proton uptake into the vesicles was monitored simultaneously as the absorbance decrease at 495 nm of the ΔpH probe acridine orange. Proton pumping was determined from the initial slope of acridine orange absorbance quenching.

BSA is present in the reaction mixture since it extracts fatty acids that might be present in the plasma membranes where they function as uncouplers (12). Since Tris buffers also might function as uncouplers (4), they should for the same reason be avoided. The reaction can be started by addition of either ATP or Mg^{2+} , but here ATP is present already in the incubation medium because ATP protects the plasma membrane H^+ -ATPase from inactivation during incubation (11).

Protein

Protein was measured essentially as in (1), with BSA as standard.

RESULTS AND DISCUSSION

The H^+ -ATPase Assay

Several cationic dyes, commonly employed as probes for determination of pH gradients across energy-transducing and model membranes, were studied for their potential use in a spectrophotometric H^+ -ATPase assay in combination with measuring NADH absorption. The requirements for the probe to be used in this assay were (a) marked absorbance quenching in response to proton pumping and (b) spectral properties as different from NADH as possible. A dye that fulfilled both criteria was acridine orange. In the H^+ -ATPase assay described, ATPase activity is coupled to the oxidation of NADH that can be followed as absorbance decrease at 340 nm, and proton pumping activity is coupled to quenching of the A at 495 nm of acridine orange. The absorption spectra of NADH and acridine orange do not overlap (Fig. 1G). Furthermore, the quenching of acridine orange absorbance at 495 nm was not accomplished by any measurable A changes at 340 nm (Fig. 1B), and, similarly, when following the disappearance of NADH at 340 nm no A changes were observed at 495 nm (Fig. 1C). Since the measurement of the two optical probes did not interfere with each other, ATPase activity and proton pumping could be followed continuously and simultaneously in the same sample (Fig. 1, D-F).

ATP is regenerated from ADP as long as any PEP remains in the assay. PEP and lactate, which accumulates during the assay, could be added to a concentration of up to 5 mM without affecting H^+ -ATPase activity. However, NADH could not be added to a final concentration of more than 0.25 mM

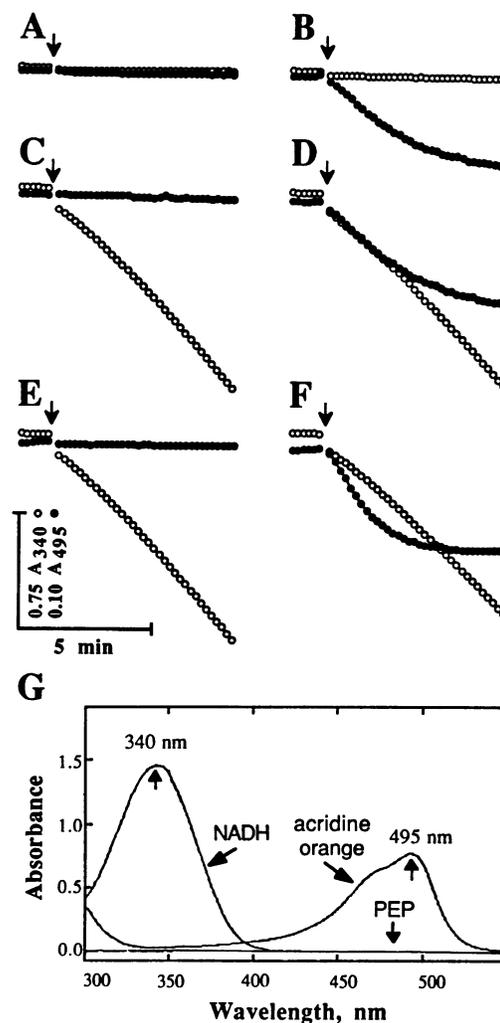


Figure 1. Simultaneous measurement of MgATPase activity and MgATP-dependent proton pumping in plasma membrane vesicles. ATPase activity (○) was coupled to NADH oxidation which was followed as absorbance decrease at 340 nm. Proton transport (●) was measured by the quenching of acridine orange absorbance at 495 nm. The H^+ -ATPase assay mixture described in "Materials and Methods" was used except that acridine orange, pyruvate kinase, and lactate dehydrogenase were initially omitted, and that the protein concentration was 25 $\mu\text{g mL}^{-1}$. MgCl_2 was added as indicated by arrows. A, Without any further additions. In subsequent experiments the following reagents had been added to the reaction mixture; B, 20 μM acridine orange; C, 50 $\mu\text{g mL}^{-1}$ pyruvate kinase, 25 $\mu\text{g mL}^{-1}$ lactate dehydrogenase; D, 20 μM acridine orange, 50 $\mu\text{g mL}^{-1}$ pyruvate kinase, 25 $\mu\text{g mL}^{-1}$ lactate dehydrogenase; E, 0.5 $\mu\text{g mL}^{-1}$ nigericin, otherwise as in D; F, 0.5 $\mu\text{g mL}^{-1}$ valinomycin, otherwise as in D; G, visible absorbance spectra of acridine orange, NADH, and PEP.

because of its strong *A* properties. Therefore, when all the NADH present initially has been consumed, the *A* decrease at 340 nm stops abruptly, even though ATP hydrolysis and ATP regeneration continue to proceed.

Kinetics of Acridine Orange Quenching and ATP Hydrolysis

The acridine orange signal was linearly related to changes in the ATPase activity. Thus, the observed kinetics for ATPase activity and quenching of acridine orange absorbance at 495 nm using the H⁺-ATPase assay were identical with respect to dependency upon ATP concentration (Fig. 2) and pH (data not shown), consistent with the idea that the same enzyme is responsible for ATP hydrolysis and proton pumping.

A correlation between ATP hydrolysis and quenching of acridine orange absorbance was not always found. ATP hydrolysis continued to proceed after the establishment of a steady state proton gradient (Fig. 1, D and F). In the presence of nigericin, an ionophore which catalyzes the electroneutral exchange of H⁺ for K⁺ (17), no proton gradient was able to build up. Addition of valinomycin, an ionophore that makes the membrane permeable to K⁺ (17), and thus allows the export from the vesicle of a positive charge upon the entry of a proton, almost doubled the rate of proton accumulation (Fig. 1F). ATPase activity, however, was largely unaffected by both ionophores (Fig. 1, E and F). These observations indicate that changes in acridine orange quenching can be independent of changes in ATP hydrolysis, since the rate of proton accumulation is not dependent solely on the activity of the H⁺-ATPase itself, but is related also to the 'leakiness' of the membrane (the passive efflux of protons) and, in the case of the electrogenic H⁺-ATPases, to factors preventing the formation of a membrane potential such as the increased permeability of the membrane to cations and anions (3, 19).

Coupling between Acridine Orange Uptake and H⁺-ATPase Activity

Several attempts have been made to relate quantitatively the quenching of acridine orange to Δ pH. Provided the intra-

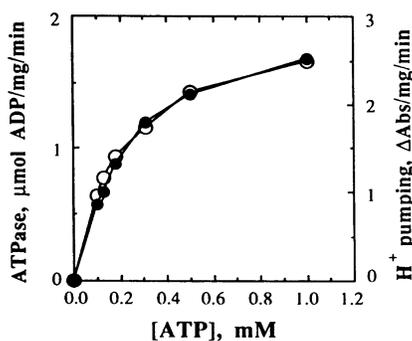


Figure 2. Effect of ATP concentration on H⁺ accumulation (●) and ATPase activity (○). For each concentration of ATP, intravesicular acidification and ATP hydrolysis were measured in the same sample using the H⁺-ATPase assay described in "Materials and Methods."

vesicular volume is known, one method is to plot the ratio of the intravesicular and extravesicular concentration of the probe as a function of the imposed pH gradient (6). It is then assumed that the observed decrease in absorbance or fluorescence represents the amount of protons within the vesicles. In a membrane-free system, anions and low temperature induce dimerization of acridine orange, and thus cause the same spectral changes of the dye as observed during the assay (9). Anions are assumed to follow the movement of protons during proton pumping by electrogenic H⁺-ATPases (19), and this may also be the case when artificial pH-jumps are introduced depending on the concentration of anions inside and outside the vesicles. In both cases anions accumulating within the vesicles would be expected to function as amplifiers of the acridine orange quenching signal by decreasing the concentration of acridine orange monomers (9). Particularly in the case of ATP-dependent H⁺ pumping, the degree of accumulation of anions is not known, and therefore the degree of amplification cannot be calculated. It is therefore questionable whether quenching of acridine orange can be quantitatively correlated with proton fluxes, as has been done in order to determine the H⁺/ATP stoichiometry of H⁺-ATPases (2, 15).

The initial rate of acridine orange quenching was linearly related to the concentration of acridine orange in the assay mixture up to 20 μM (Fig. 3). Similarly, the total extent of quenching increased linearly with increasing acridine orange concentration and saturated at the same concentration (Fig. 3). This indicates a direct correlation between initial rate of quenching and total extent of quenching. This observation is contrary to other results (7), where such a relationship was not found when the protein concentration of the assay medium was varied, but is consistent with data by Briskin (3).

Acridine orange, being a weak base, can move freely across membranes in its unprotonated form and, if a pH gradient is established, would be expected to accumulate in its protonated form on the acid side of the membrane (6). That the degree of acridine orange quenching is concentration dependent supports this assumption. It also implies, that at low concentrations of acridine orange (below 20 μM), which are commonly used in proton pumping assays, the degree of absorbance quenching does not necessarily represent proton pumping only, since it may equally well reflect the depletion of acridine orange from the extravesicular medium.

As an alternative approach to quantitate proton pumping it was investigated whether there was a direct correlation between the uptake of acridine orange and H⁺-ATPase activity. For each μM of acridine orange present in the reaction mixture (at these low concentrations) the absorbance after initiating the H⁺-ATPase reaction could be quenched to a maximal degree of 0.015 *A* units (Fig. 3B). Assuming that all of the acridine orange molecules (at low dye concentrations) are taken up by the membrane vesicles, the decrease of 0.015 *A* units should thus reflect the uptake of 1 nmol mL⁻¹ acridine orange. The optimal initial rate by which acridine orange absorbance could be quenched in that example was 0.075 *A* units min⁻¹ mL⁻¹ (Fig. 3B). The initial rate by which acridine orange was accumulated in the membrane vesicles can thus be calculated to be about 5 nmol of acridine orange min⁻¹ mL⁻¹ or about 0.1 μmol min⁻¹ mg protein⁻¹. The ATPase

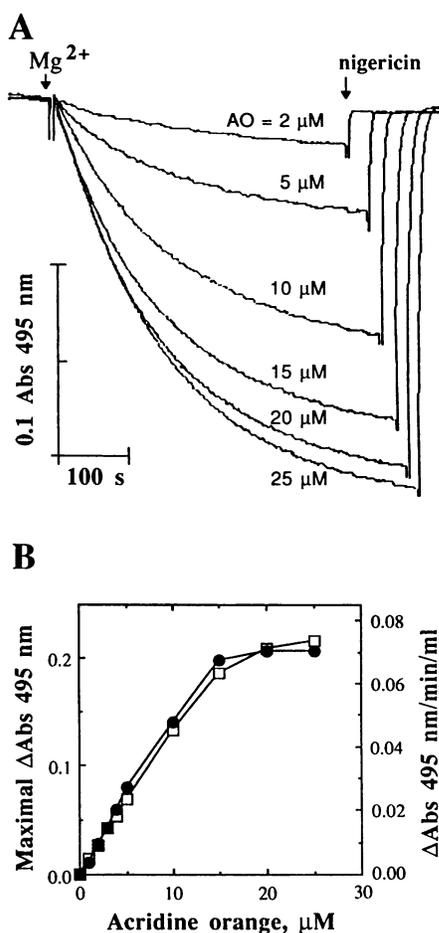


Figure 3. Effect of acridine orange concentration on the degree of absorbance quenching at 495 nm during the H^+ -ATPase assay. The H^+ -ATPase assay mixture described in "Materials and Methods" was used except that the initial concentration of PEP was 4 mM. A, A decrease at 495 nm as a result of Mg ATP-dependent proton uptake into the plasma membrane vesicles. $MgCl_2$ and nigericin (0.5 μ g/ml) were added as indicated by arrows; B, comparison of the maximal A decrease (\bullet) with the initial rate of quenching of acridine orange (\square) at 495 nm during the H^+ -ATPase assay. Data were from an experiment similar to that in A.

activity in the example was $1.4 \mu\text{mol ATP hydrolysed min}^{-1} \text{mg protein}^{-1}$. This indicates a very low degree of coupling between ATP hydrolysis and initial accumulation of acridine orange. The relationship between hydrolysis of ATP on the one hand and the unidirectional flux of protons and acridine orange on the other hand may be artificially low because the membranes are leaky to protons (10) and/or lipophilic ion pairs produced by protonated acridine orange and anions (16).

Measuring Acridine Orange Quenching

A direct correlation between initial rate of quenching and total extent of quenching was not always found. The maximal degree to which acridine orange absorbance could be quenched was the same in the presence and in the absence of valinomycin (Fig. 1, D and F), whereas the initial rate of

absorbance quenching in the presence of valinomycin was stimulated two-fold as compared to the control (Fig. 1, D and F). In the presence of an ATP regenerating system the maximum level of acridine orange absorbance quenching became more pronounced, as compared to the level in the absence of one of its constituents, *e.g.* pyruvate kinase (Fig. 4C), whereas the initial rate of quenching was the same in the presence and in the absence of pyruvate kinase (Fig. 4C).

The maximal degree to which acridine orange absorbance can be quenched is referred to as the steady state where the rates of protons pumped in and protons passively leaking out of the membrane vesicles equals each other (3). Thereby, the total level of the pH gradient should reflect the rate of proton pumping. However, since this steady state is only reached after considerable time this might not always be the correct interpretation, as illustrated by the following. (a) Reduced acridine orange quenching may reflect the depletion of acridine orange from the extravesicular medium as discussed above. (b) There may be a maximum level for proton or acridine orange accumulation within the vesicles. Such a maximum level could have been obtained after the addition of valinomycin in Figure 1F. (c) Reduced acridine orange quenching may also reflect ATP depletion. At low ATP concentrations and in the absence of an ATP regenerating system the absorbance of acridine orange at 495 nm, after being maximally quenched, slowly increased and returned to its original level as the reaction mixture was depleted of ATP

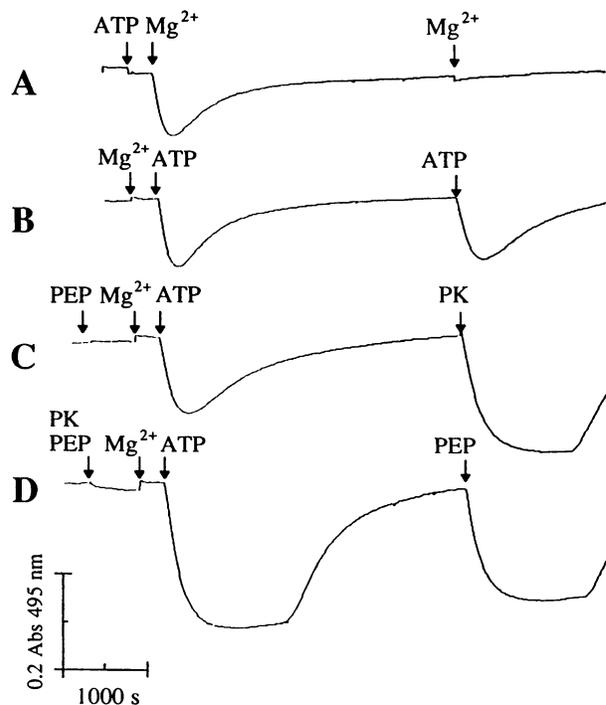


Figure 4. Effect of an ATP regenerating system (PEP and pyruvate kinase) on the acridine orange signal. The H^+ -ATPase assay mixture described in "Materials and Methods" was used except that ATP (here 0.1 mM), PEP, and pyruvate kinase (PK) were initially omitted, and that the protein concentration was $100 \mu\text{g mL}^{-1}$. In different experiments ATP, $MgCl_2$, PEP, and pyruvate kinase were added in varying order as indicated by arrows.

(Fig. 4). Further addition of ATP, but not Mg^{2+} , caused the *A* to be quenched again (Fig. 4, A and B). Addition of pyruvate kinase in combination with PEP also reinitiated acridine orange quenching (Fig. 4C), but the maximal degree to which acridine orange was quenched became more pronounced (Fig. 4, C,D). The presence of PEP and pyruvate kinase prevents depletion of ATP by regenerating ATP from ADP. (d) Both inorganic phosphate and ADP released as products from the ATPase reaction may act as inhibitors as they are accumulated and slow down the H^+ -ATPase reaction (18). This problem is partly solved by an ATP-regenerating system that removes ADP. In the absence of an ATP regenerating system, it is therefore questionable to use the maximal degree of acridine orange quenching as a measure for the rate of proton pumping.

CONCLUSION

When measuring acridine orange absorbance quenching, the initial rate is probably the best measure of activity as in most enzymatic studies. The H^+ -ATPase assay described should be of use in the study of H^+ -ATPases, and has already been very useful for characterizing relative changes in the coupling between ATP hydrolysis and proton pumping induced by H^+ -ATPase effectors (10, 12), and to verify the homogeneity of H^+ -ATPase preparations by kinetic analysis (10).

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