Biochimica et Biophysica Acta, 1153 (1993) 283–288

BBAMEM 76171

ATP activation of plasma membrane yeast H^+-ATPase shows complex kinetics independently of the degree of purification

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(Received 9 June 1993)

Key words: ATPase, H^+-; Transport ATPase; ATP; ATP activation; (Yeast)

ATP stimulation of plasma membrane H^+-ATPase activity from a wild baker's yeast (*Saccharomyces cerevisiae*) was followed under conditions of progressive degrees of purification. A particular emphasis was put to cover a wide range of concentrations which went from 2 μM up to 3000 μM ATP. The preparations used were (i) crude membrane fraction, (ii) untreated plasma membrane fraction obtained by differential centrifugation, (iii) residual plasma membrane treated with Triton X-100, (iv) enzyme solubilized with either Zwittergent 3–14 alone or after Triton X-100 treatment. Under all conditions the fitting of the dose–response curves required an equation composed by the sum of two Michaelian terms. Depending on the treatment, the K_m values and V_{max} values varied. The fitted curves displayed a high affinity-low V_{max} (K_m values of 7–60 μM and V_{max} values of 0.03–0.50 μmol P_i/mg per min) and a low affinity-high V_{max} component (K_m values of 408–1960 μM and V_{max} values of 0.26–5.82 μmol P_i/mg per min). The complex ATP activation curve of the yeast plasma membrane H^+-ATPase is in line with similar behavior found for the H^+-ATPase of higher plants and all known animal cation transport ATPases.

Introduction

The H^+-ATPase enzyme present in the plasma membrane fraction of fungi and higher plants belongs to the group of the so-called P-ATPases. Its specific role is to expel protons from the intracellular towards the extracellular milieu allowing the creation of a proton gradient that drives nutrients into the cells [1–3]. The presence of free Mg^{2+} ions is essential for activity and the optimal [$MgCl_2$]/[ATP] ratio is about 1–1.5 [1–5]. It has been claimed that MgATP is the actual substrate [5–7]; however, due to the simultaneous presence of three species (free Mg^{2+}, free ATP and the MgATP complex) the answer is practically impossible to find; this is particularly true from steady state kinetics [8,9]. Most of the previous works on ATP activation in these enzymes have concluded that only one ATP site of relatively low affinity (K_m between 200 μM and 2000 μM) was present. Nevertheless, in a few cases complex ATP activation curves were reported. The first appeared in 1979 when Bowman and Slayman [10] described in *Neurospora crassa* a sigmoid relationship of activity vs. [ATP]; the authors proposed a two-site model with positive cooperative to account for their data. The second observation was obtained in *Saccharomyces cerevisiae* by Koland and Hammes [5] who reported two K_m values for MgATP with values of 590 μM and 4900 μM. On the other hand, using the same yeast species and analyzing an [ATP] range of 50–10 000 μM, Wach et al. [7] found single Michaelian kinetics in plasma membrane fractions, solubilized and reconstituted H^+-ATPase. Likewise, experiments on *Schizosaccharomyces pombe* [11,12] and *Candida tropicalis* [13] produced single Michaelian kinetics. A possible explanation for a single ATP site in these protons ATPases was that, as a difference with other P-ATPases, the high affinity nucleotide sites had been lost [2]. In studies carried in our laboratory, covering a range of [ATP] from 2 μM up to 5000 μM, we found that the untreated plasma membrane fraction of higher plants exhibits a H^+-ATPase activity with a definite complex response to ATP concentration. Under otherwise optimal hydrolysis conditions a two-Michaelian fit produced one K_m of about 10–20 μM and another of

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*′,*N*′,*N*′,*N*′-tetraacetic acid; GTED20, 20% glycerol/10 mM Tris-HCl (pH 7.6 at room temperature)/1 mM EDTA-Tris/1 mM dithiothreitol; GTED50, 50% glycerol/10 mM Tris-HCl (pH 7.6 at room temperature)/1 mM EDTA-Tris/1 mM dithiothreitol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; psi, pounds per square inch; SDS, sodium dodecyl sulfate; Zwittergent 3–14, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonic acid.

about 300–500 μM [14]. It is possible that in some of the reported experiments with H^+-ATPase the low K_m for the nucleotide was missed because the lowest [ATP] investigated were not low enough. On the other hand, it is conceivable that the single Michaelian behavior resulted from the fact that in many instances the enzyme was subjected to drastic purification procedures. These considerations led us to reexamine the problem in *S. cerevisiae* working with a wide range of [ATP] and different purification procedures.

Materials and Methods

The work was performed with a baker's yeast (*Saccharomyces cerevisiae*). Commercial fresh yeast was washed twice with bidistilled water and incubated for 10 min (100–200 mg wet weight/ml) in a medium containing 100 mM Mes-Tris (pH 6.5 at 30° C) and 100 mM glucose [15]. Immediately thereafter the cells were frozen in a dry ice/methanol mixture and stored at −75°C for 24 h. All following procedures were carried out at 4°C. The cells were subjected to mechanical disruption in a solution of 50 mM Tris-HCl (pH 8.0 at room temperature), 5 mM EDTA-Tris, 0.2 mM PMSF. To the volume of disrupted cell a similar volume of the GTED20 (20% glycerol, 10 mM Tris-HCl (pH 7.6 at room temperature), 1 mM EDTA-Tris and 1 mM dithiothreitol) was added. The crude and plasma membrane fractions were obtained by differential centrifugation and discontinuous sucrose gradients (43.5 and 53.5% w/w) as described by Serrano [15]. They were stored in GTED20 at −20°C without any loss of enzymatic activity. For Triton X-100 treatment the plasma membrane fractions (2 mg/ml) were incubated for 10 min at 0°C in GTED20 with 500 mM KCl at a total protein/Triton X-100 ratio of 1:2. The membranes were recovered in the pellet after centrifugation for 45 min at 35 000 rpm in a Beckman T 50 rotor, suspended in GTED20 and stored as usual [16]. For solubilization with Zwittergent the method of Serrano [16] with modifications was followed. The plasma membrane fraction, native or previously treated with Triton X-100, was present at a total protein concentration of 4 mg/ml. To 1 ml of that suspension 0.65 mg phosphatidyl serine and 3.2 mg Zwittergent 3–14 were added. After gentle mixing it was sonicated for 1 min and then centrifuged in the rotor 30 A-100 of a Beckman Airfuge for 10 min at 30 psi. The enzyme was recovered from the supernatant and centrifuged in a glycerol discontinuous gradient (GTED20 and GTED50) for 5 h at 45 000 rpm in a T 50 Beckman rotor. The enzyme was recovered from the pellet, suspended in GTED20 and stored.

SDS-PAGE procedures were performed on a vertical cube (Mini-Protean II Bio-RAD) by the method of Laemmli [17] using an 8% polyacrylamide gel. Samples of 10 μg total protein were pretreated for 20 min at 37°C in the buffer sample with 5 mM PMSF and 0.25 mg/ml chymostatin. Gibco-BRL prestained protein molecular weight standards of high molecular weight range were used. The densitometry of dry gels previously stained with Coomassie Brilliant Blue R-250 was performed in a Beckman DU 70 spectrophotometer using the automatic baseline to estimate the areas under the peaks.

Protein content was measured with the method of Bradford [18] using BSA as standard. ATPase activity was assayed at 30°C. The enzyme and all ligands (except ATP) were preincubated for 5 min at 30°C and the reaction started with the addition of ATP. The amounts of ATP hydrolyzed were estimated on the basis of the radioactivity released from [γ-^{32}P]ATP of known specific activity [19]. Preliminary experiments had shown that, for the conditions used, the reaction was linear with time and protein concentration; therefore, in order to keep hydrolysis below 5–8%, in some cases time (between 5 and 15 min), and in others total protein, were varied. The incubation solutions routinely contained 50 mM Mes as Tris salt (pH 6.0 at 30°C)/0.1 mM EGTA as Tris salt/20 mM K_2SO_4/30 mM $NaNO_3$/0.16 mM ammonium molybdate/5 mM sodium azide/1 mM free Mg^{2+}, variable ATP (from 2 μM to 3000 μM) with and without vanadate. The nominal concentration of free Mg^{2+} was estimated as in Ref. 20. We considered specific H^+-ATPase activity the ATP hydrolysis sensitive to vanadate in the presence of azide, molybdate and nitrate. The usual vanadate concentration was 200 μM. However, in our hands soluble enzyme was less sensitive to vanadate; therefore, with those preparations we insured maximal inhibition with 500 μM vanadate. Duplicate samples were run in each case, and the differences between duplicate never exceeded 5%. Curve fitting was performed with a non-linear regression computing program using the chi-squared criterion (Scopfit, National Biomedical Simulation Resource, Duke University Medical Center, USA). More details are given in the figure legends.

Results and Discussion

The present work analyzes the dose–response curves of H^+-ATPase activity from yeast (*S. cerevisiae*) over a wide range of ATP concentrations (from 2 μM up to 3000 μM) in enzyme preparations with different degrees of purity. In general, the various fractions used (crude membrane, untreated and Triton X-100-treated plasma membrane and Zwittergent 3–14 solubilized enzyme) displayed characteristics (average activity, sensitivity to inhibitors, degree of purity) comparable to those described by others under similar conditions [7,16]. This is particularly important for the assessment of the activity–[ATP] relationships. Table I summarizes

TABLE I

Characterization of the different fractions obtained after purification and / or solubilization of H^+-ATPase from Saccharomyces cerevisiae

The enzymatic activities were estimated from the release of ^{32}P from [γ-^{32}P]ATP. The assay conditions were (mM): Tris-Mes (pH 6.0 at 30°C), 50; EGTA (Tris salt), 0.1; K_2SO_4, 20; $NaNO_3$, 30; $MgSO_4$, 5; ATP (Tris salt), 3; ammonium molybdate, 0.16; sodium azide, 5, with and without 200–500 μM vanadate. When present, lysolecithin was 0.025 mg/ml and sonicated soybean phospholipids 0.4 mg/ml. The 104 kDa peak bands were obtained from the scan of Coomassie blue-stained gels and are expressed as percentage of the total area above the automatic base line. Each entry is the mean ± S.E. of at least three determinations except for the gel scanning that includes the range of two. See Materials and Methods for details.

Fraction	Recovered protein[a] (mg)	ATPase activity (μmol P_i /mg per min)		Percentage of 104 kDa band (range)
		V-insensitive	V-sensitive	
Crude membrane	203 ±5.4	0.095 ± 0.006	0.28 ± 0.02	3.6– 4.4
Plasma membrane	7.4 ±1.2	0.059 ± 0.008	0.95 ± 0.07	10.7–16.0
			0.42 ± 0.02[b]	
Triton X-100 residual	1.5 ±0.3	0.019 ± 0.001	3.14 ± 0.21	15.4–27.2
			0.48 ± 0.03[b]	
Zwittergent soluble	0.3 ±0.17	0.025 ± 0.020	2.10 ± 0.08	15.3–21.0
Triton X-100 Zwittergent soluble	0.13 ± 0.09	0.156 ± 0.101	7.79 ± 0.18	40.6–51.0

[a] From 20 g of fresh baker's yeast.

[b] Without lysolecithin and soybean phospholipids in the incubation media.

the different enzymatic fractions with some of their principal properties. As purification proceeds, together with the expected reduction in the total protein, we observe the following. (i) A reduction in the vanadate-insensitive (considered by us as unspecific) ATPase activity, which in the Zwittergent soluble preparation is

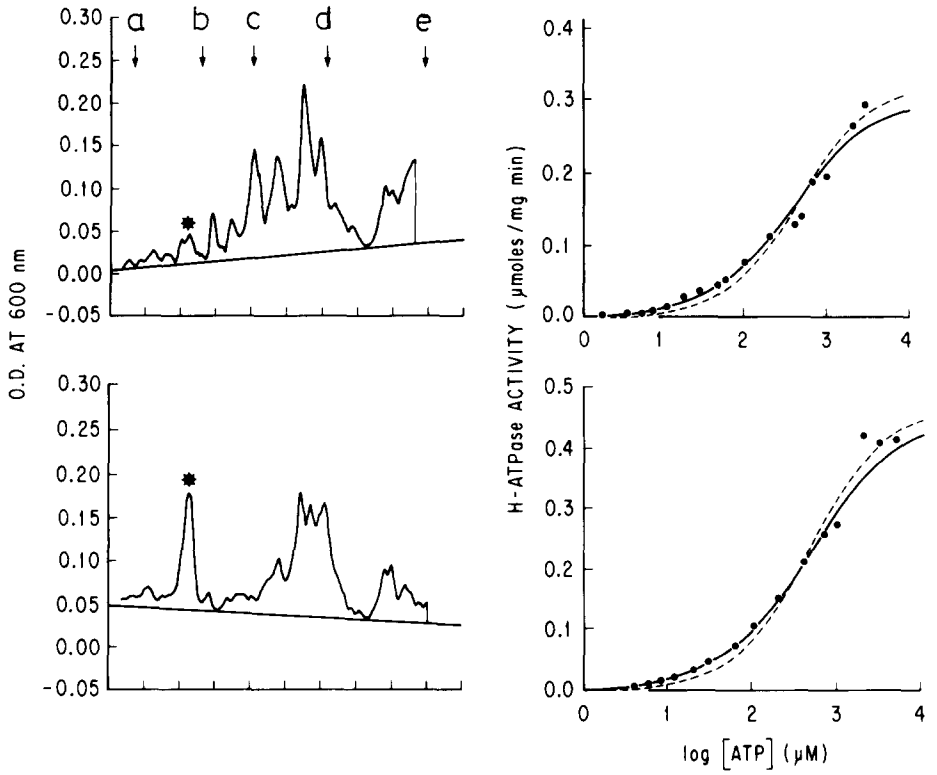

Fig. 1. Typical densitometer PAGE scans (left panels) and ATP stimulation curves of H^+-ATPase activities (right panels) of crude (top) and plasma membrane (bottom) fractions from *S. cerevisiae*. In the scans the arrows indicate the position of the following molecular mass standards: (a) 200 kDa; (b) 97.4 kDa; (c) 68 kDa; (d) 43 kDa; (e) 25.6 kDa. The band corresponding to the H^+-ATPase is marked with an asterisk and represents the 3.6% (top) and 16% (bottom) of the total protein. In the estimation of ATP hydrolysis the ionized [Mg^{2+}] was kept constant at 1 mM. Each point is the mean of duplicate determinations where the difference between duplicates never exceeded 5%. The lines through the points are the best fit to a single Michaelian (broken line) or to the sum of two Michaelians (solid line). The mean and standard error of the fitting parameters for the two sites model are given in Table II. See text for details.

not significantly different from zero. (ii) An increase in the vanadate-sensitive (specific) H^+-ATPase activity. In the case of crude and Triton X-100-treated plasma membrane fraction, a sizeable increase in the specific ATP hydrolysis is seen when lysolecithin and soybean phospholipids are included in the incubation media; it is conceivable that lysolecithin somehow opens up the existing vesicles exposing more enzyme units to the required ligands [16,21]. (iii) There is no difference between the recovered Triton X-100-treated fraction and that solubilized without Triton X-100 treatment; nonetheless, the fraction soluble in Zwittergent increased its specific activity when it had been previously exposed to Triton X-100. For some reason the combination of both detergents leads to a more active preparation. However, we found it more unstable during storage, particularly when the total protein concentration is below 2–3 mg/ml. (iv) The degree of purification, expressed as the percentage of the 104 kDa band over the total scanned area of the gels above the automatic baseline, goes from 4% in crude membrane up to 47% in Triton X-100-treated Zwittergent solubilized enzyme. (v) As has been already described, treatment with Triton X-100 increases hydrolytic activity, perhaps not only by removing unspecific proteins but by unmasking catalytic units as well [12] or by a direct effect on the enzyme [22]. On the other hand, the detergent has also an inhibiting effect, possibly due to delipidation of the membrane [2,3], thus introducing difficulties for an strict evaluation of activity vs. purity. At any rate, whereas the specific protein (expressed as the percentage of the 104 kDa band) increases about 11-fold from crude membrane to solubilized enzyme, the specific H^+-ATPase activity shows a 28-fold increment when the same preparations are compared. (vi) In addition, an important feature of the Triton X-100-treated Zwittergent solubilized enzyme is that it is not affected by azide, molybdate and nitrate (not shown).

Figs. 1–3 illustrate typical cases where the densitometer pattern of the SDS-PAGE and the dose–response curves for ATP were run in parallel for each preparation. In the gel scans the peaks corresponding to the specific enzyme protein (marked with an asterisk) exhibited the fractional increase in the

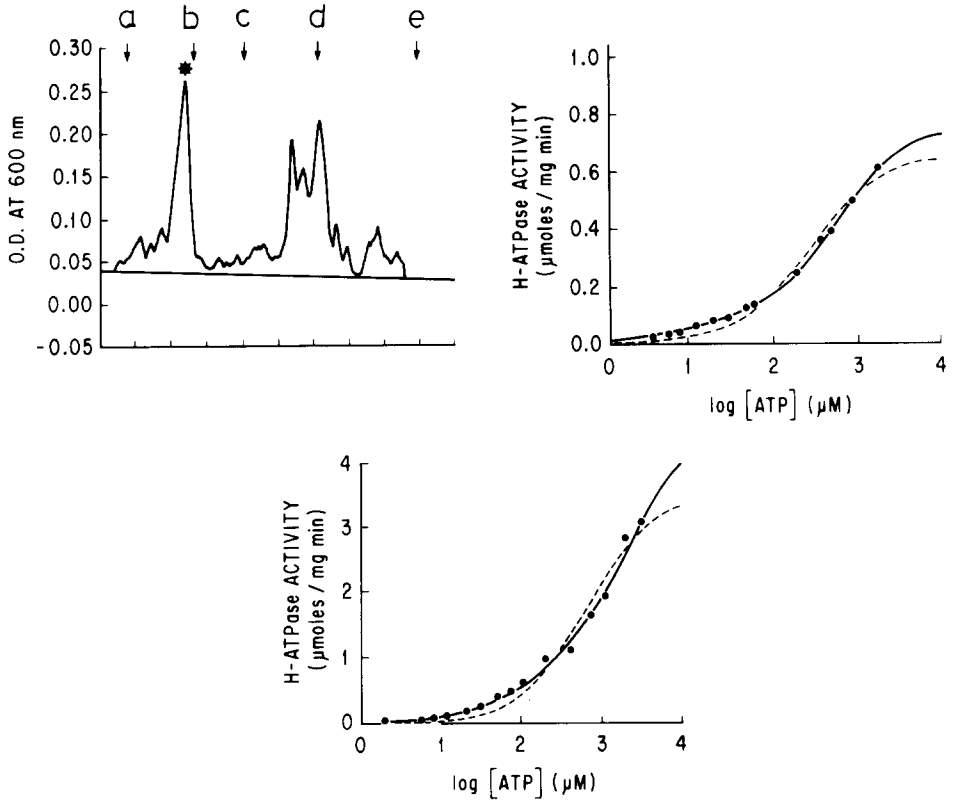

Fig. 2. Typical densitometer PAGE scans (upper left panel) and ATP stimulation curves of H^+-ATPase activities plasma membrane fractions of *S. cerevisiae*-treated with Triton X-100 and assayed for activity without (upper right panel) and with (lower panel) 400 μg/ml soybean phospholipids and 300 μg/ml BSA. In the scan the arrows indicate the position of the following molecular mass standards: (a) 200 kDa; (b) 97.4 kDa; (c) 68 kDa; (d) 43 kDa; (e) 25.6 kDa. The band corresponding to the H^+-ATPase is marked with an asterisk and represents the 27% of the total protein. In the estimation of ATP hydrolysis the ionized $[Mg^{2+}]$ was kept constant at 1 mM. Each point is the mean of duplicate determinations where the difference between duplicates never exceeded 5%. The lines through the points are the best fit to a single Michaelian (broken line) or to the sum of two Michaelians (solid line). The mean and standard error of the fitting parameters for the two-site model are given in Table II. See text for details.

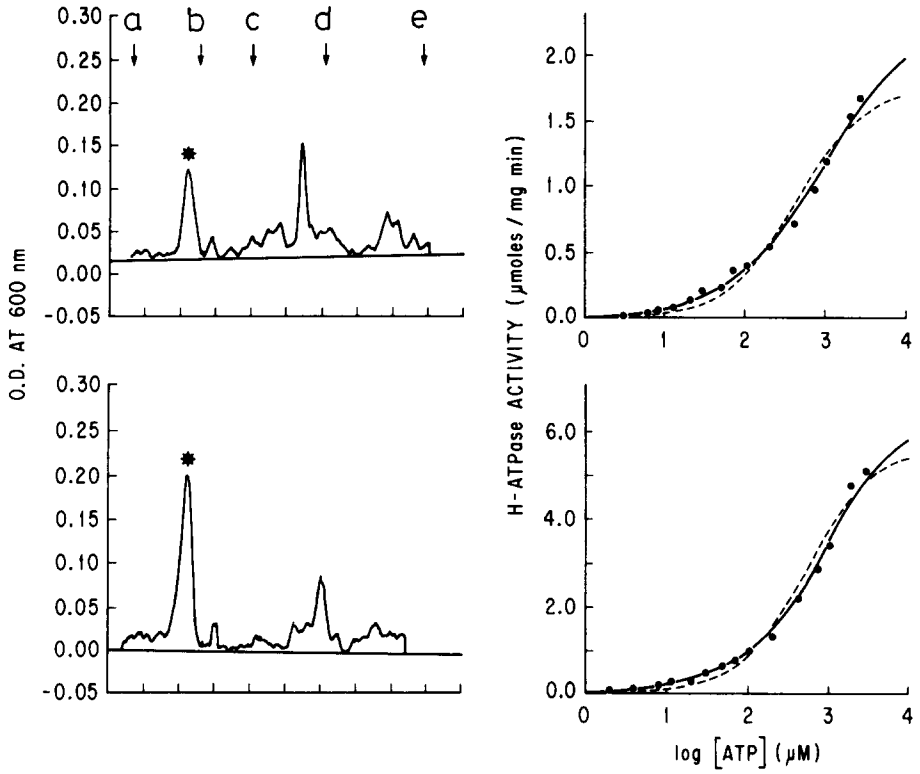

Fig. 3. Typical densitometer PAGE scans (left panels) and ATP stimulation curves of H^+-ATPase activities (right panels) of Zwittergent solubilized (top) and Triton X-100-treated before Zwittergent solubilization (bottom) H^+-ATPase from *S. cerevisiae*. In the scans the arrows indicate the position of the following molecular mass standards: (a) 200 kDa; (b) 97.4 kDa; (c) 68 kDa; (d) 43 kDa; (e) 25.6 kDa. The band corresponding to the H^+-ATPase is marked with an asterisk and represents the 21% (top) and 40% (bottom) of the total protein. In the estimation of ATP hydrolysis the ionized [Mg^{2+}] was kept constant at 1 mM. Each point is the mean of duplicate determinations where the difference between duplicates never exceeded 5%. The lines through the points are the best fit to a single Michaelian (broken line) or to the sum of two Michaelians (solid line). In all cases the ATPase assay media contained 400 μg/ml soybean phospholipids and 0.025 mg/ml lysophosphatidyl choline. The mean and standard error of the fitting parameters for the two sites model are given in Table II. See text for details.

specific enzyme protein as purification proceeded. In the ATP activation the experimental points show the best fit obtained with a single hyperbola (dotted line) and with the sum of two Michaelian functions. An extremely important result is that, under all conditions investigated, the sum of two Michaelians provided a better fit for the data. This can be seen even by eye, for in all cases the data points at ATP concentrations

TABLE II

Kinetic parameters (K_m and V_{max}) of the two components of the H^+-ATPase activity from S. cerevisiae as influenced by the degree of purification and / or solubilization

The Snedecor's *F* test applied to the differences between experimental and theoretical points favored the two-Michaelian models on the basis of the following values. Microsomal fraction: $F = 9.1$, df = 32 and 30, $P < 0.01$. Plasma membrane fraction: $F = 9.5$, df = 28 and 26, $P < 0.01$. Triton-treated membrane fraction assayed with soybean phospholipids and BSA in the media: $F = 10$, df = 32 and 30, $P < 0.002$. Triton-treated membrane fraction assayed without soybean phospholipids and BSA in the media: $F = 29$, df = 32 and 30, $P < 0.001$. Zwittergent solubilized H^+-ATPase from native plasma membrane: $F = 23$, df = 32 and 30, $P < 0.001$. Zwittergent solubilization following pretreatment of the plasma membrane fraction with Triton X-100: $F = 20$, df = 32 and 30, $P < 0.001$. Data corresponds to the experiments illustrated in Figs. 1–3.

Fraction	K_m (μM)		V_{max} (μmol P_i /mg per min)	
	K_{m1}	K_{m2}	V_{max1}	V_{max2}
Crude membrane	35 ± 18	476 ± 143	0.035 ± 0.013	0.262 ± 0.021
Plasma membrane	37 ± 26	583 ± 85	0.062 ± 0.029	0.389 ± 0.025
Triton X-100 residual	7 ± 4	488 ± 133	0.030 ± 0.006	0.670 ± 0.110
	58 ± 8[a]	1959 ± 152[a]	0.646 ± 0.040[a]	4.035 ± 0.191[a]
Zwittergent soluble	60 ± 11	1206 ± 152	0.413 ± 0.036	1.710 ± 0.102
Triton X-100 Zwittergent soluble	24 ± 6	939 ± 80	0.501 ± 0.093	5.82 ± 0.175

[a] With 300 μg/ml of albumin and 400 μg/ml soybean phospholipids added to the incubation media.

below 100 μM appear above the fitting curve representing a single hyperbola. A rigorous statistical analysis using the Snedecor's F test applied to the differences between experimental and theoretical points systematically favored the two-Michaelian model. This indicates that the best fit was not due just to the larger flexibility introduced by adding more parameters in the two-Michaelian approach. The mean and standard error of the fitting parameters for the two-sites model for all conditions represented in the figures are summarized in Table II while the values of the Snedecor's F test are given in the legend to the figure. The dose–response experiments were repeated at least three times, and in each case the results obtained were similar to those described in the table. The values of K_m and V_{max} for the low ATP affinity component in Table II are similar to those already reported in the literature, even for cases where a single Michaelian kinetics was observed [1–3]. On the other hand, K_m and V_{max} values for the high ATP affinity component were not given before except for higher plants [14]. It is possible that in several instances the high ATP affinity component was missed because the investigated ATP concentrations were not low enough. In addition, in *Neurospora*, micromolar vanadate concentrations transform the [ATP]–activity relationship from sigmoid into hyperbolic [23]; the fact that for several years vanadate containing ATP was routinely used could also explain why this high affinity component was not detected [3].

In summary, our results show that regardless of the degree of purification, the [ATP]–activity relationship of the H^+-ATPase from *S. cerevisiae* is complex and can be adequately described by the sum of two Michaelians. The behavior is similar to that described for the animal Na^+/K^+-ATPase [24], Ca^{2+}-ATPase [25] and H^+/K^+-ATPase [26]. In these enzymes the low-affinity site is related to a regulatory non-phosphorylating role of ATP, accelerating the E_1–E_2 transition. The functional significance of the two components for the ATP stimulation of the H^+-ATPase described here and before [14] is still uncertain. Possible alternatives are: (i) A regulatory role of the substrate. (ii) Two active substrate sites in a monomeric molecule; this is unlikely because the primary structure of this enzyme shows only one phosphorylating site [27]. (iii) A heterogeneous preparation with two enzyme species. Although yeasts have at least two isoforms, this can be ruled out because, under normal conditions, only one isoform is expressed [28,29]. However, posttranslational modifications cannot be ruled out. (iv) A dimeric enzyme with two catalytic subunits operating in a kind of flip-flop mode. (v) Negative cooperativity. Whatever the mechanism, there is an important kinetics consideration to make: in order to obtain a substrate activation curve given by the sum of two hyperbolae it is necessary that the enzyme can hydrolyze ATP even when only one site is occupied. If in order to function the system requires that both sites are occupied, the relationship will be sigmoid but not biphasic [30].

Acknowledgements

This work was supported by Grants from CONICET (BID-PID 1053), CONICOR (2733) and Fundación Pérez Companc. G.B. and L.B. are established investigators from CONICET.G.H. holds a R. Helman fellowship from FUNINAR. We wish to thank Myriam Siravegna for her skillful technical assistance.

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