Two-dimensional gel electrophoresis and FTIR spectroscopy reveal both forms of yeast plasma membrane H^+-ATPase in activated and basal-level enzyme preparations

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Received 26 June 2001; revised 27 July 2001; accepted 31 July 2001

First published online 24 August 2001

Edited by Richard Cogdell

Abstract Plasma membrane H^+-ATPase of the yeast *Saccharomyces cerevisiae* was isolated and purified in its two forms, the activated A-ATPase from glucose-metabolizing cells, and the basal-level B-ATPase from cells with endogenous metabolism only. Using two-dimensional gel electrophoretic analysis, we showed that both enzyme preparations are actually mixtures of the non-active, i.e. non-phosphorylated, and the active, i.e. phosphorylated, forms of the enzyme. Previous deliberations suggesting that the B-ATPase displays some activity which is lower than that of A-ATPase were apparently wrong. It seems that, molecularly speaking, the B-form is actually not active at all, and what activity we measure in our preparation is due to an admixture of the true active form (A-form). Fourier transform infrared spectroscopic study of the secondary structure and particularly thermal denaturation data suggest the possibility that the two enzyme forms interact to form complexes less stable than the single forms. On the whole then, there apparently is a different ratio of the active and inactive forms and/or complexes between the two forms present in all enzyme preparations.

Key words: H^+-ATPase; Plasma membrane; Secondary structure; Two-dimensional gel electrophoresis; Fourier transform infrared spectroscopy; *Saccharomyces cerevisiae*

1. Introduction

The yeast plasma membrane H^+-ATPase belongs to the P-type ATPases. This electrogenic proton pump is essential not only for intracellular pH regulation, but also for preservation of basic life functions of yeast cells [1]. It occurs as a single polypeptide chain. The extremely hydrophilic N-terminus is followed by four well-defined hydrophobic segments that span the membrane. The central one-third of the molecule (the H_4–H_5 loop) is relatively hydrophilic and contains residues involved in ATP binding and phosphorylation. The enzyme has six additional hydrophobic stretches at its C-terminus. Both the N- and C-termini are exposed at the cytoplasmic side of the membrane [2]. The C-terminus is an inhibitory/regulatory domain interacting with the ATP-binding site and is involved in the modulation of ATPase activity by glucose metabolism [3,4]. Glucose regulation takes place at two levels. At the transcriptional level, glucose increases the ATPase gene (*pma1*) expression and at the posttranslational level, it induces ATPase activation.

It was suggested [5] that the carboxyl terminus would interact, directly or indirectly, with transmembrane helices 1, 2, and 4 [6] limiting the access of protons to their transport site [7,8], while interaction with the ATP-binding domain would limit ATP access. Glucose would trigger a modification of the ATPase, mediated by phosphorylation, which would release this interaction. The H^+-ATPase has been shown to be activated apparently by protein kinase C (PKC)-catalyzed phosphorylation of some C-terminal amino acid residues. Thr-912 together with Arg-909, and Ser-899 together with Glu-901 could define putative phosphorylation sites [2,4,5,9,10]. The activation, taking place after addition of D-glucose or a similar sugar (cf. [11]), apparently involves the phosphatidyl inositol signaling pathway [12–14], which is triggered through activation of *plc1*-encoded phospholipase C [15]. The involvement of PKC1-MPK1 mitogen-activated protein kinase signaling pathway in glucose activation of the yeast plasma membrane H^+-ATPase was also suggested [16].

After activation, the ATPase retains an altered structure throughout its purification so that it can be isolated from yeast cells in two different states (cf. [17–19]). Here we use two-dimensional (2D) gel electrophoresis and Fourier-transform infrared (FTIR) spectroscopy to analyze both the basal-level (B-ATPase) and the activated H^+-ATPase (A-ATPase) preparation.

2. Materials and methods

2.1. Reagents

Deuterium oxide (99.9% ^2H_2O), ^2HCl and NaO^2H were purchased from Aldrich. All chemicals of special interest for the isolation of the enzyme and all inhibitors were obtained from Sigma-Aldrich, and sodium 5-mercapto-7,8-dicarbanonaborate (MDB) was prepared by

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Abbreviations: A-ATPase, activated H^+-ATPase; B-ATPase, basal-level H^+-ATPase; A-form, active form of H^+-ATPase; B-form, non-active form of H^+-ATPase; MDB, sodium 5-mercapto-7,8-dicarbanonaborate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FTIR, Fourier transform infrared

0014-5793/01/$20.00
PII: S0014-5793(01)02793-4

Dr. J. Plešek and co-workers [20]. All the common chemicals were commercial samples of the highest quality.

2.2. Yeast strain and its cultivation

Saccharomyces cerevisiae K (CCY-21-4-60) was grown for 17 h at 30°C in a medium containing 2% (w/v) yeast extract (Difco) and 5.8% (w/v) glucose, adjusted to pH 4.6 with 1 M HCl. After harvesting, the yeast was washed twice with distilled water. Half of the cells were incubated for 10 min at 30°C with 0.1 M glucose, and the other half with 0.1 M sorbitol. Incubation with glucose resulted in an 'activation' of the plasma membrane H^+-ATPase [17].

2.3. Isolation and purification of H^+-ATPase

The yeast cells were disrupted in a Gaulin laboratory homogenizer Model 15M-8TA (Hilversum, The Netherlands) at 4°C using a mixture of 1 mM $MgCl_2$, 50 mM Tris buffer (pH 7.5) and either 0.25 M glucose for glucose-preincubated cells or 0.25 M sorbitol for the sorbitol-preincubated ones. Phenylmethylsulfonyl fluoride (1 mM) was added to prevent protein degradation. The method of Goffeau and Dufour [21] was used for the purification of plasma membranes, while that of Serrano [8] was preferred for purifying the ATPase.

The activity of H^+-ATPase was determined basically according to [21,22], with the addition of 10 mM KNO_3 to block any trace of vacuolar ATPase that may be present in the plasma membrane preparation (see also [8]). The protein content was determined by Lowry's method. The specific activity of the ATPase was found to be, on average, 4.20 μmol phosphate hydrolyzed from ATP per min per mg protein for the activated enzyme and, on average, 0.59 μmol phosphate for the basal-level enzyme, the ratio of activities being identical with that found with purified plasma membranes, before and after activation [16,23].

2.4. 2D gel electrophoresis

This was done by using isoelectric focussing in the first direction in an Oxford Glycosystem Investigator setup for 15.5 h, with 2500 V applied during the last 30 min. In the second direction, the tube with the gel was electrophoresed for 6 h as in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). The plates with separated proteins were stained with Coomassie blue. The lane corresponding to a molar mass of 100 kDa was then evaluated densitometrically.

2.5. Infrared (IR) measurements

Samples for IR measurements were prepared as previously reported [19]. In particular, 1–1.5 mg of protein were suspended in 300 μl 60 mM MES, 10 mM $MgSO_4$ buffer prepared in D_2O at pH 6.1. The protein suspension was then centrifuged at $500\,000 \times g$ for 20 min and the pellet resuspended in 300 μl of buffer and centrifuged again. This procedure, repeated four times, made it possible to exchange H_2O for D_2O. For the experiments with inhibitors, the pellet was treated in the last washing with 300 μl of buffer containing 0.3 mM of the desired inhibitor. The inhibitors were in contact with the H^+-ATPase for 12 h at 4°C and all samples were in contact with D_2O medium for 24 h before IR analysis.

After the last centrifugation, the pellet, together with some drops of buffer used in the last washing, was collected and placed between two CaF_2 windows, separated by a 25 μm teflon spacer. The windows were fitted in a 20500 cell (Graseby-Specac, Orpington, Kent, UK) and the sample was analyzed using a Perkin-Elmer 1760-x FTIR spectrometer equipped with a deuterated triglycine sulfate detector. At least 24 h before, and during data acquisition, the spectrometer was continuously purged with dry air at a dewpoint of −40°C. Spectra of buffers and samples were acquired at 2 cm^{-1} resolution under the same scanning and temperature conditions. Typically, 256 scans were averaged for each spectrum obtained at 20°C, while 16 scans were averaged for spectra obtained at higher temperatures. In the thermal denaturation experiments, the temperature was raised in 5°C steps from 20°C to 95°C. The sample temperature was controlled by an external bath circulator (Haake F3). Before spectrum acquisition, samples were maintained at the desired temperature for the time necessary for the stabilization of temperature inside the cell (6 min). Spectra were collected and processed using the SPECTRUM software from Perkin-Elmer. An interactive difference routine was used to subtract the spectrum of the buffer from the spectrum of the sample. Proper subtraction of H_2O was judged to yield an approximately flat baseline at 1900–1400 cm^{-1}. Subtraction of D_2O was adjusted to the removal of the D_2O, bending absorption close to 1220 cm^{-1} [24]. The deconvoluted parameters for the amide I band were set with a γ value of 2.5 and a smoothing length of 60. Second-derivative spectra were calculated over a nine-data-point range (9 cm^{-1}).

3. Results and discussion

In an earlier paper [19], we described the structure of A-ATPase and B-ATPase in the absence and in the presence of β,γ-imidoadenosine 5′-triphosphate or of diethylstilbestrol. In that paper we demonstrated that the secondary structures of A- and B-ATPases were the same and that two populations of α-helices, namely more and less exposed to the solvent, were present in both enzyme forms. Thermal denaturation

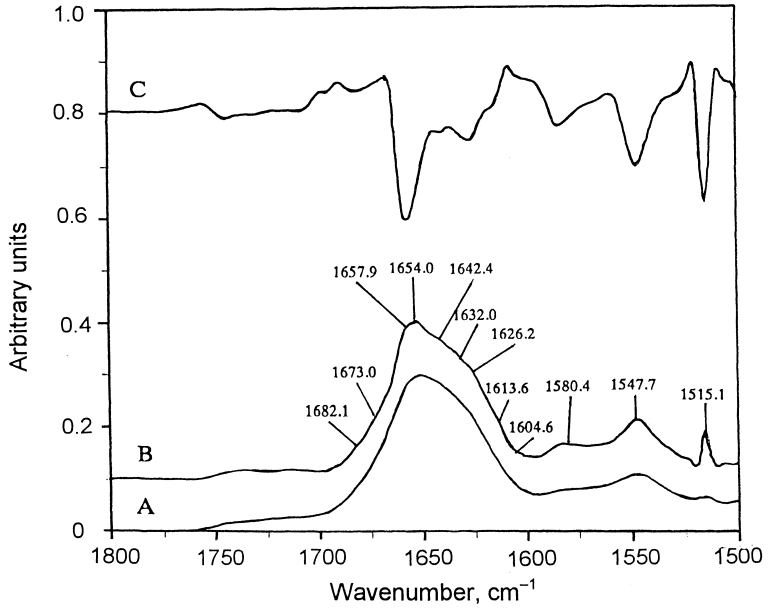

Fig. 1. Absorbance (A), deconvoluted (B) and second-derivative (C) spectra of B-ATPase. The spectra were obtained at 20°C. The spectra of A-ATPase were practically superimposable on those of B-ATPase (not shown).

studies showed that B-ATPase has a more stable structure than A-ATPase, and the lack of significant difference in the H/D exchangeability of the two enzyme forms.

Fig. 1 shows the absorbance, deconvoluted and second-derivative spectra of a new preparation of A- and B-ATPases. The spectrum of this new preparation is very similar to that previously published. The amide I′ component bands are the same and located in similar positions as observed previously, and their assignment was done as follows. The 1657.9 and 1654 cm^{-1} bands were assigned to α-helices and, in particular, to the less and the more solvent-exposed, respectively [19]. The 1632 and 1626.2 cm^{-1} bands were assigned to β-sheets, the 1673 and 1642.4 cm^{-1} bands to turns and unordered structures and/or loops, respectively. The 1682.1 cm^{-1} band was assigned to β-sheets and/or turns. The other bands are due to amino acid side-chain absorption [25], except the band at 1547.4 cm^{-1}, which is due to residual amide II absorption [26]. Although similar, the spectrum of B-ATPase of this new preparation is not exactly the same as that reported in our previous paper [19]. In particular, the intensity of the 1657.9 cm^{-1} band is lower, while the intensity of the 1654 cm^{-1} band is higher. This finding indicates that in the new B-ATPase preparation, the α-helices are more exposed to the solvent than in the old B-ATPase preparation. This effect, namely the exposure of α-helices to the solvent, was induced by diethylstilbestrol in our previous study [19].

While it is possible, by using IR spectroscopy, to obtain information on conformational changes involving the entire secondary structure, in some cases one can detect different populations of a particular secondary structure more or less exposed to the solvent (e.g. α-helices, β-strands), and thus, observe changes in these populations [19,27,28]. However, it is not possible to specify which part of the polypeptide chain underwent a particular conformational change. For instance, different segments of the polypeptide chain may be arranged in α-helices or in β-strands more or less exposed to the solvent – we can detect the different populations, but not the specific segment that is exposed to the solvent under particular conditions.

From these spectra, one can say that the preparation of B-ATPase led to pure samples having the same secondary structure composition, but with small differences in the exposition of α-helices to the solvent. However, a marked difference was found in the thermal denaturation curve of B-ATPase when compared with the published one [19]. Fig. 2 shows that the new B-ATPase preparation displays a thermal denaturation curve with two distinct transitions, one located at the same temperature as found previously, and a new one at about 55°C. In the old preparation of B-ATPase, we observed a slight decrease of amide I′ band-width in correspondence with the shoulder we found in this new preparation of the enzyme. The same is obtained with A-ATPase (Fig. 2; cf. [19]). This finding was surprising because the old and new IR spectra of B-ATPase were very similar and did not show any unusual bands that could indicate contamination. On the other hand, SDS–PAGE revealed a single major band at M_r 100 000, supporting the purity of the preparation.

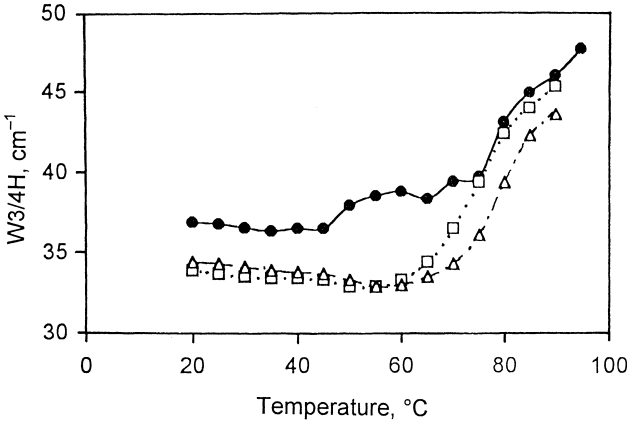

Fig. 2. Thermal denaturation of A- and B-ATPase. Full circles and open triangles refer to the new and old preparations of B-ATPase, respectively. Open squares refer to the new preparation of A-ATPase. The thermal denaturation profiles were obtained by monitoring the amide I′ band-width, calculated at three-quarters of amide I′ band-height (W3/4H), as a function of the temperature [19].

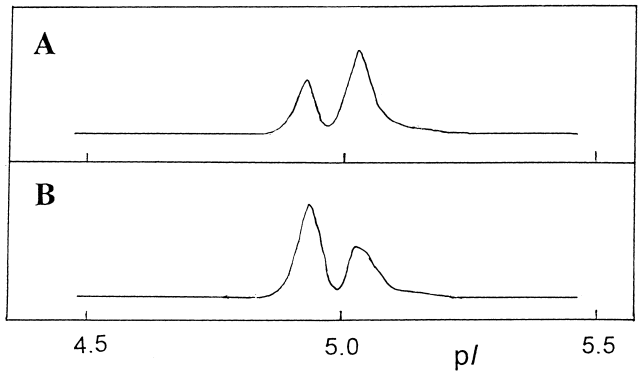

Fig. 3. Densitometric record of a 2D gel electrophoreogram at the level of 100 kDa. Peak 1 corresponds to the phosphorylated form, peak 2 to the non-phosphorylated form, the p*I* representing the negative logarithm of the isoionic point. Panel A: B-ATPase; panel B: A-ATPase.

New evidence comes from 2D gel electrophoresis where two spots of different size, corresponding to the phosphorylated and non-phosphorylated forms, were found consistently in both types of enzyme preparation, their proportions being different – the rest of the field was virtually free of spots, except some minor 'debris' at very low M_r values (Fig. 3).

This finding indicates that both enzyme preparations in fact contain a certain amount of the 'active', i.e. phosphorylated, form (A-form) and of the 'inactive' basal form (B-form) of H^+-ATPase with no activity. What activity is determined in the B-ATPase thus appears to be due to an admixture of the A-form [27]. In fact, both preparations are obtained after growth of yeast cells on glucose and only subsequently they were differentiated by incubation with glucose or sorbitol. Hence, at different activities of PKC and with, presumably, the same activity of phosphatase which hydrolyzes the phosphorylated form of H^+-ATPase, there will be a different proportion of the A- and B-forms.

Although the A- and B-ATPases are a mixture of both enzyme forms, the resolution-enhanced spectra shown in Fig. 1 are the same and very similar to that published previously [19]. In that study, we demonstrated that A- and B-ATPases have the same secondary structure, and thus, the same amide I′ band contour.

We analyzed the secondary structure of both preparations of yeast plasma membrane H^+-ATPase in the presence of various non-specific inhibitors, such as suloctidil, orthovana-

date, erythrosin B and MDB, and no significant changes in this structure were observed (data not shown). In the presence of MDB, we observed that the position of the α-helix band and the intensity of the residual amide II band absorption were lower than those of the control, suggesting that the inhibitor allowed a greater exposure of the α-helices to the solvent (data not shown) [26].

This structural analysis confirmed our previous study [29] where we showed that the effects of the above inhibitors on the activity were very similar on plasma membrane-localized and -purified ATPases of both forms, suggesting that both preparations contain the two ATPase forms, the glucose preincubated one being richer in the A-form, while the sorbitol-preincubated one contains less of it.

It is difficult to find an explanation for the presence of the transition at about 45–55°C in the thermal denaturation curve of B-ATPase. We know that both preparations do not contain any other protein impurities (SDS–PAGE), but they are mixtures of A- and B-ATPases (2D gel electrophoresis). So, the explanation should be searched in this context. One possibility is that the two forms of H^+-ATPase interact. Interaction between a phosphate group of A-ATPase (phosphorylated form) and a positive charge of B-ATPase could lead to a complex less stable than the pure forms. This interaction could destabilize the native conformation of the enzyme by removing specific original salt bridges (ion pairs), showing a transition at about 55°C in the thermal denaturation curve.

In conclusion, 2D gel electrophoresis and FTIR spectroscopy reveal both forms of yeast plasma membrane H^+-ATPase in the activated and basal-level enzyme preparation. There apparently is a different ratio of the A- and B-forms and/or complexes between the two forms present. The ratios of these two forms and/or of the complexes are possibly quite different depending on uncontrolable factors during the cultivation as well as preincubation with glucose or sorbitol. If the phosphorylation and activation of H^+-ATPase is due to PKC, one should accept the fact that there is a certain equilibrium between the non-phosphorylated and the phosphorylated forms under all conditions, the position of the equilibrium being shifted in dependence on factors besides the PKC. The idea tallies with the postulated molecular disposition of the enzyme where the B-form has its ATP-binding site blocked by the C-terminus (e.g. [9,19]), and hence, displays no ATP-hydrolyzing activity.

Acknowledgements: This work was supported by grants from Ancona University (E.B. and F.T.) and by Grant No. 204/98/0474 of the Grant Agency of the Czech Republic (A.K. and G.L.). Our thanks are due to Ms Silvia Bezoušková of the Institute of Microbiology, CzAcadSci, for carrying out the 2D gel electrophoresis.

References

[1] Serrano, R. (1988) Biochim. Biophys. Acta 947, 1–28.
[2] Serrano, R. and Portillo, F. (1990) Biochim. Biophys. Acta 1018, 195–199.
[3] Portillo, F., de Larrinoa, I.F. and Serrano, R. (1989) FEBS Lett. 247, 381–385.
[4] Portillo, F., Eraso, P. and Serrano, R. (1991) FEBS Lett. 287, 71–74.
[5] Eraso, P. and Portillo, F. (1994) J. Biol. Chem. 269, 10393–10399.
[6] Na, S., Perlin, D.S., Seto-Young, D., Wang, G. and Haber, J.E. (1993) J. Biol. Chem. 268, 11792–11797.
[7] Clarke, D.M., Loo, T.W. and MacLennan, D.H. (1990) J. Biol. Chem. 265, 6262–6267.
[8] Serrano, R. (1988) Methods Enzymol. 157, 533–544.
[9] Portillo, F. (2000) Biochim. Biophys. Acta 1469, 31–42.
[10] Goossens, A., de la Fuente, N., Forment, J., Serrano, R. and Portillo, F. (2000) Mol. Cell Biol. 20, 7654–7661.
[11] Kotyk, A. and Georghiou, G. (1994) Cell Biol. Int. 18, 813–817.
[12] Becher dos Passos, J., Vanhalewyn, M., Brandão, R.L., Castro, I.M., Nicoli, J.R. and Thevelein, J.M. (1992) Biochim. Biophys. Acta 1136, 57–67.
[13] Brandão, R.L., de Magalhaes-Rocha, N.M., Alijo, R., Ramos, J. and Thevelein, J.M. (1994) Biochim. Biophys. Acta 1223, 117–124.
[14] Kotyk, A. and Georghiou, G. (1994) Bull. Mol. Biol. Med. 19, 127–134.
[15] Coccetti, P., Tisi, R., Martegani, E., Teixeira, L.S., Brandão, R.L., Castro, I.M. and Thevelein, J.M. (1998) Biochim. Biophys. Acta 1405, 147–154.
[16] de la Fuente, N. and Portillo, F. (2000) Biochim. Biophys. Acta 1509, 189–194.
[17] Serrano, F. (1983) FEBS Lett. 156, 11–14.
[18] Lapathitis, G., Tanfani, F. and Kotyk, A. (1997) Folia Microbiol. 42, 233–234.
[19] Tanfani, F., Lapathitis, G., Bertoli, E. and Kotyk, A. (1998) Biochim. Biophys. Acta 1369, 109–118.
[20] Plešek, J., Jelínek, T., Drdáková, E., Heřmánek, S. and Štíbr, B. (1984) Coll. Czech. Chem. Commun. 49, 1559.
[21] Goffeau, A. and Dufour, J.P. (1988) Methods Enzymol. 157, 528–533.
[22] Dufour, J.-P., Amory, A. and Goffeau, A. (1988) Methods Enzymol. 157, 513–528.
[23] Sychrová, H. and Kotyk, A. (1985) FEBS Lett. 183, 21–24.
[24] Tanfani, F., Galeazzi, T., Curatola, G., Bertoli, E. and Ferretti, G. (1997) Biochem. J. 322, 765–769.
[25] Chirgadze, Y.N., Fedorow, O.W. and Trushina, N.P. (1975) Biopolymers 14, 679–694.
[26] Osborne, H.B. and Nabedryk-Viala, E. (1982) Methods Enzymol. 88, 676–680.
[27] Banecki, B., Zylicz, M., Bertoli, E. and Tanfani, F. (1992) J. Biol. Chem. 267, 25051–25058.
[28] Jackson, M. and Mantsch, H.H. (1992) Biochim. Biophys. Acta 1118, 139–143.
[29] Lapathitis, G. and Kotyk, A. (2000) Folia Microbiol. 45, 221–223.