FEMS Microbiology Letters 84 (1991) 91–96

Published by Elsevier

FEMSLE 04671

Confirmation by using mutant strains that the membrane-bound H^+-ATPase is the major source of non-linear dielectricity in *Saccharomyces cerevisiae*

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Received 22 July 1991
Revision received 19 August 1991
Accepted 26 August 1991

Key words: Yeast; *Saccharomyces*; H^+-ATPase; Non-linear dielectric spectroscopy

1. SUMMARY

Non-linear dielectric spectroscopy is a novel technique for determining the activity of (predominantly) membranous enzymes as their ability to generate harmonics when excited with a sinusoidal electrical field. In washed suspensions of yeast cells, the ability to generate harmonics is inhibited by low concentrations of sodium vanadate, suggesting that the vanadate-sensitive H^+-ATPase is the major source of the non-linear dielectricity. This conclusion is greatly strengthened by the demonstration herein that the generation of harmonics by a strain containing a vanadate-resistant H^+-ATPase is also highly resistant to sodium metavanadate.

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2. INTRODUCTION

We have recently developed the technique of non-linear dielectric spectroscopy and applied it to the study of biological systems [1–5]. In this approach, one excites the system of interest with a sinusoidally modulated electrical field via a pair of electrodes, and observes the response of the system using a separate pair of electrodes. The response of the system is manifest as its ability to generate harmonics when the exciting field is a sinusoid of a single frequency [1,3–5], whilst 'beat' frequencies or sidebands are observed when the exciting field consists of two sinusoids [2]. The effect may be understood in terms of the ability of (especially) membranous enzymes to couple exogenous electric field energy to their normal catalytic cycles [6–11], and thereby effect energy transduction.

In the case of washed cell suspensions of *Saccharomyces cerevisiae*, we found [1] that substantial, odd-numbered harmonics were generated by

these cells when stimulated by very modest exciting fields (approx. 2 $V \cdot cm^{-1}$, 20 Hz). The generation of these harmonics occurred only in living cells, and in a cell concentration-dependent manner. The ability to generate a third harmonic was observable only within rather narrow voltage and frequency windows, and was strongly inhibited by low concentrations of sodium metavanadate. Since the cell membrane acts strongly to amplify the exogenous field at these low frequencies [12,13], we took this as evidence that the generation of a third harmonic was due largely to the vanadate-sensitive H^+-ATPase present in the plasma membranes of these cells. (When the enzyme was not at static head, and the cells were glycolysing, the 3rd harmonic disappeared and strong second and 4th harmonics were observed; these were also sensitive to low concentrations of vanadate [1].)

Although no other vanadate-sensitive, membrane-located enzymes are known in *S. cerevisiae*, we felt that the above interpretation would be greatly strengthened if we were to compare the non-linear dielectric properties of otherwise isogenic strains whose ATPase was mutated in an appropriate fashion. Since the enzyme is required for growth of the cell [14], it seemed appropriate to use strains in which the enzyme either was or was not sensitive to sodium metavanadate.

Hygromycin B is a positively charged, aminoglycoside antibiotic, whose active uptake is thought to be driven by the membrane potential produced via ATP hydrolysis by the membrane H^+-ATPase; Perlin and colleagues have shown that resistance to hygromycin B can therefore be caused not only by mutations in the genetic loci coding for proteins for which the antibiotic is the target, but also in the PMA1 locus coding for the structural gene of the membrane H^+-ATPase [15]. Such mutants are (somewhat) defective in their ability to generate a membrane potential [16] and in the kinetic and other properties of their membrane H^+-ATPase [17,18]. In particular, the H^+-ATPase of strain pma1-105, whose mutation has been shown to be due to a single base-pair change causing a Ser-368 → Phe substitution, is, in contrast to that of its parental strain Y55, insensitive to inhibition by vanadate [17].

In the present work we have studied the non-linear dielectric properties of strains Y55 and pma1-105. We found that the ability to generate a third harmonic when excited by a field of appropriate frequency and amplitude is similar in the two strains, and that the third harmonic is inhibited strongly by low concentrations of vanadate in strain Y55 but only very weakly in strain pma1-105. This provides powerful evidence that the membrane H^+-ATPase is indeed the major source of non-linear dielectricity in this organism.

3. MATERIALS AND METHODS

3.1. Organism, maintenance and growth

S. cerevisiae strains Y55 and pma1-105 were generous gifts of Dr. David Perlin, and were maintained at 4 °C on plates containing 1% yeast extract (w/v). 1% bactopeptone (w/v) and 5% D-glucose (w/v), solidified with 1.5% (w/v) agar.

Cells were grown in 250-ml shake flasks in aerobic batch culture in 100 ml of a medium consisting of 1% yeast extract (w/v). 1% bactopeptone (w/v) and 5% D-glucose (w/v), harvested in early stationary phase by centrifugation (30 min, $1000 \times g$), washed twice in 1% yeast extract and stored in the same medium prior to initiation of non-linear dielectric measurements.

Non-linear dielectric measurements were performed essentially as described previously [1]. The system is based on an IBM-PC-AT-compatible microcomputer (Viglen III, 80386 main processor, Cyrix 83C87 coprocessor). One of the expansion ports of the computer is furnished with a Data Translation model 2823 16-bit ADC/DAC board. The four electrodes consist of collinear gold pins. Signals were applied to the outer, current electrodes by means of a Krohn-Hite model 4400A ultra-low-noise function generator. The frequency and amplitude of these signals was checked using a Solartron 1200 Signal Processor (Schlumberger Instruments, Farnborough, Hampshire) and a Hameg HM 208 Digital Storage Oscilloscope.

The acquisition of the data and its subsequent processing and display was performed using the ILS software (Signal Technology Inc., Goleta,

CA), running under control files written in-house. Spectra were collected as follows: the system of interest was pipetted into the electrode cell, the test waveform applied to the current electrodes, and the data logged from the voltage electrodes at a sampling frequency (which for the data displayed herein was 500 Hz) and for a time equivalent to 30 blocks, each block consisting of 512 samples. At the end of the time specified, the data were Fourier-transformed as follows. First, the mean of the block was subtracted from the individual samples. Each block was then windowed using a Blackman window [19] and fast-Fourier-transformed using routines within the ILS software to form an ensemble of power spectra. These were then averaged in order to enhance the signal:noise ratio. The spectral data were stored on the computer's hard disk.

A reference spectrum was acquired using the supernatant, whose conductivity had been adjusted to be identical to that of the sample at the frequency of interest. Finally the 'sample' power spectrum so obtained was divided by the 'reference' power spectrum, equivalent to deconvolving the spectrum due to the cells from that due to the experimental apparatus, and also stored on the disk.

3.2. Chemicals and biochemicals

Chemicals and biochemicals were obtained from the Sigma Chemical Company or BDH chemicals, Poole Dorset and were of the highest grade available. Water was singly distilled in an all-glass apparatus.

4. RESULTS AND DISCUSSION

Preliminary experiments (not shown) showed that the optimal conditions for generating harmonics were a field strength of ± 1.33 V·cm^{-1} and a frequency of 10 Hz (strain Y55) or 15 Hz (strain pma1-105). We therefore chose an exciting frequency of 10 Hz.

Figure 1 shows the non-linear dielectric behaviour of a resting, washed-cell suspension of strain Y55, (Fig. 1A), the reference supernatant (Fig. 1B) and their difference (Fig. 1C). It may be observed that a substantial third harmonic is generated by these cells, that is similar to that observed previously with a different strain [1].

Cells from the culture displayed in Fig. 1, and those from a similar culture of strain pma1-105, were taken and their non-linear dielectric behaviour was studied in the presence of increasing concentrations of sodium metavanadate. It was found (Fig. 2) that whilst the ability of strain Y55 to generate a third harmonic was strongly inhibited by sodium metavanadate (with an apparent K_i of some 0.25 mM), the generation of a third harmonic by cells of strain pma1-105 (which in

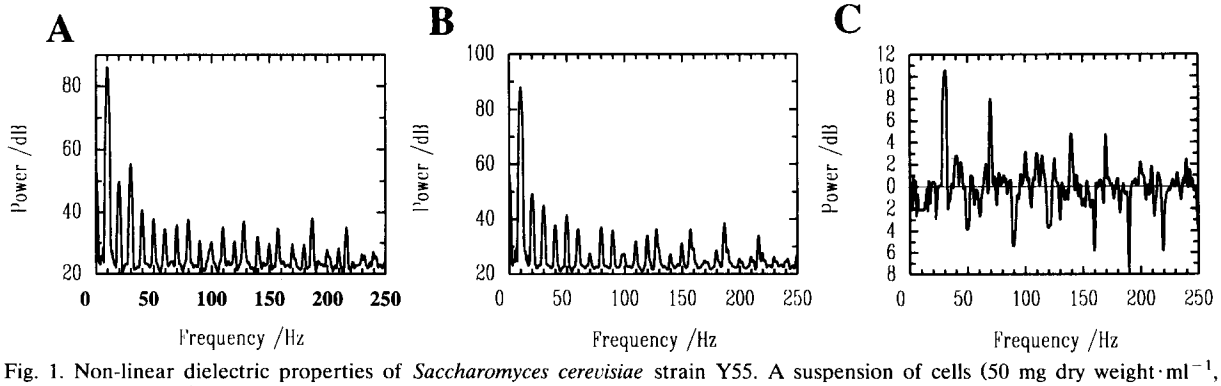

Fig. 1. Non-linear dielectric properties of *Saccharomyces cerevisiae* strain Y55. A suspension of cells (50 mg dry weight·ml^{-1}, prepared as described in MATERIAL AND METHODS, was placed in the test cell, and a sample of its supernatant (with its conductivity at 1 kHz adjusted to match that of the cells) was placed in the reference cell. Spectra were obtained using an exciting voltage of ± 1.0 V zero-to-peak (field strength ± 1.33 V·cm^{-1} as measured between the outer electrodes, ± 165 mV·cm^{-1} as measured between the inner electrodes) at a frequency of 10 Hz, and each spectrum represents the average of 30 blocks. **A.** Test spectrum. **B.** Reference spectrum. **C.** Test spectrum minus reference spectrum.

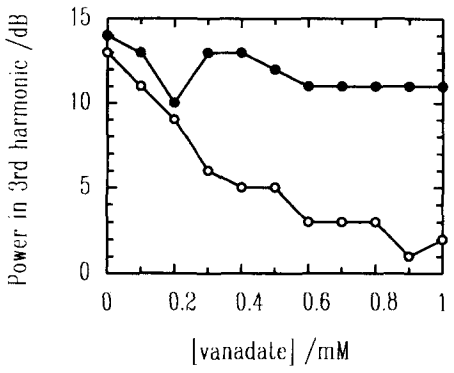

Fig. 2. Effect of sodium metavanadate on the non-linear dielectric properties of *Saccharomyces cerevisiae* strains Y55 and pma1-105. Measurements were performed exactly as described in the legend to Fig. 1C, save that the cells were preincubated for 5 min with sodium metavanadate at the concentrations indicated. Open symbols: strain Y55; closed symbols: strain pma1-105.

the absence of vanadate was similar to that of strain Y55) was virtually insensitive to sodium metavanadate. The conclusion is obvious: cells containing a vanadate-sensitive H^+-ATPase give non-linear dielectric spectra that are sensitive to sodium metavanadate, whilst otherwise isogenic cells containing a vanadate-resistance H^+-ATPase give non-linear dielectric spectra that are resistant to sodium metavanadate. This provides compelling evidence that the H^+-ATPase in the plasma membrane of *S. cerevisiae* is indeed the dominant source of non-linear dielectricity in this organism.

Since strain pma1-105 is known to be defective, relative to strain Y55, in its ability to generate a membrane potential [16], it might be wondered why the non-linear dielectric properties of the two strains in the absence of vanadate were so similar (Fig. 2) (since it might be imagined, for instance, that the mutant strain would be less effective at generating harmonics). However, the fact that the enzyme in resting cells is apparently at static head [1] means that whilst the mutant enzyme might a priori be less effective at transducing the free energy in the exciting field, and thereby generating harmonics, the magnitude of the membrane potential in the absence of the exciting field is also less in the mutant strain than in strain Y55. Given the absence of knowledge of the thermokinetic behaviour (stoichiometry and turnover number as a function of the input and output free energies) of the two enzymes, it is therefore quite reasonable that their non-linear dielectric behaviour in the absence of vanadate should be similar.

Since the non-linear dielectric approach can monitor both the presence and the turnover of the target enzymes in vivo [1–5], and in many cases the yeast H^+-ATPase has a flux-control coefficient (see [20]) for growth of 1 [21], it would appear that non-linear dielectric spectroscopy can provide a novel and useful means of monitoring the vitality of cultures in situ. This approach would act nicely to complement the ability of linear dielectric measurements at radio frequencies to provide a real-time estimation of microbial biomass [22–24].

ACKNOWLEDGEMENTS

We are indebted to the Wolfson Foundation for financial support of this work, and to Dr. David Perlin for his kindness in making available strains Y55 and pma1-105. We thank an anonymous referee for a useful comment.

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