

In vivo ^{31}P nuclear magnetic resonance saturation transfer measurements of phosphate exchange reactions in the yeast *Saccharomyces cerevisiae*

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^{31}P saturation transfer techniques have been used to measure phosphate kinetics in the yeast *Saccharomyces cerevisiae*. The phosphate consumption rate observed in acetate grown mid-log cells was combined with measurements of O_2 consumption to yield P/O ratios of 2.2 and 2.9, for cells respiring on glucose and ethanol, respectively. However, no phosphate consumption activity was observed in saturation transfer experiments on anaerobic glucose fed cells. The phosphate consumption rates measured by saturation transfer in cells respiring on glucose and ethanol was attributed to the unidirectional rates of mitochondrial ATP synthesis.

ATPase Kinetics Saturation transfer ^{31}P -NMR (Saccharomyces cerevisiae) Metabolism

1. INTRODUCTION

^{31}P NMR saturation transfer has been performed in a variety of in vivo systems to investigate kinetics in a non-invasive manner [1,2]. In previous work [3], ^{31}P NMR saturation transfer studies were conducted in the yeast *Saccharomyces cerevisiae* to study phosphate kinetics in cells respiring on endogenous substrates. The phosphate consumption activity was observed in these studies by saturating the $\text{NTP}\gamma$ peak and observing the reduction in the intracellular phosphate (P_i^{in}) peak intensity. The calculated P_i -consumption rate was assigned to the mitochondrial ATPase since 90% of the P_i -consumption activity was sensitive to oligomycin. The large P/O ratio of 87 determined

in these experiments suggested rapid equilibrium of the ATPase. However, our recent investigation of this system indicates that oligomycin did not enter the yeast cell and a different explanation of the oligomycin effect has been proposed (in preparation). Furthermore, the observation of P_i -consumption activity in anaerobic glucose fed cells, indicated that contributions from other P_i -consuming pathways such as glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase (GAP/PGK) may be important.

These previous kinetic studies had been performed with glucose-grown stationary phase cells. To restrict the P_i -consumption activity as much as possible to mitochondrial ATPase kinetics, the present saturation transfer studies were conducted in cells grown to mid-log phase with acetate as the carbon source. They differ from glucose-grown stationary phase cells in that they are actively dividing and thus require more energy for biosynthesis. Moreover, these cells are respiratory competent and contain lower levels of glycolytic enzymes than cells grown in the presence of glucose [4-7]. This work was performed on acetate-grown cells supplied with glucose under both aerobic and

Abbreviations: rf, radiofrequency; $\text{NTP}\gamma$, γ -phosphate of nucleoside triphosphates; $\text{NDP}\beta$, β -phosphate of nucleoside diphosphates; $\text{NDP}\alpha$ and $\text{NTP}\alpha$, α -phosphates of nucleoside di- and triphosphates; $\text{NTP}\beta$, β -phosphate of nucleoside triphosphates; NADP(H) , oxidized and reduced form of nicotinamide adenine dinucleotide phosphate; PP_1 , terminal phosphate of polyphosphate; PP_2 and PP_3 , penultimate phosphates of polyphosphate

anaerobic conditions and with cells fed ethanol aerobically.

2. EXPERIMENTAL

The *S. cerevisiae* strain NCYC 239 was grown to mid-log phase at 30°C on a liquid medium as described [8]. The cells were harvested according to [3], and resuspended in the harvesting media to a density of 40% wet wt.

Glucose (2 g) was added to 15 ml of the 40% cell suspension in a 20 mm NMR tube and bubbled with either oxygen or nitrogen. For aerobic experiments using exogenously added ethanol, 15 ml cell suspension was pelleted and resuspended in the buffer containing 3.3% ethanol. The aerobic experiments were bubbled with 95% O₂/5% CO₂ at rates which maintained the level of O₂ in the suspension well above air-saturated levels.

³¹P NMR spectra were obtained at 145.78 MHz using a Bruker WH 360 NMR spectrometer at 25°C. Saturation transfer experiments were conducted according to Alger et al. [3]. The rf field was positioned alternately between the NTP_γ resonance (*M*) and control (*M*⁰) region downfield from P_iⁱⁿ every 24 scans and spectra collected in files of 144 scans using a 90° pulse and a 2.7 s relaxation delay to obtain a fully relaxed P_iⁱⁿ peak. A capillary containing methyl phosphonate was used as an external concentration standard to calculate the P_iⁱⁿ concentration.

Oxygen-consumption rates were monitored with a Yellow Spring oxygen electrode contained in a model 5301 bath stirrer assembly unit at 25°C.

3. RESULTS

The saturation transfer control (*M*⁰) and the difference spectrum (*M*⁰−*M*) of aerobic cells supplied with ethanol is shown in fig.1a and b, respectively. The *M*⁰ spectrum was obtained with the rf field positioned downfield from the intracellular phosphate (P_iⁱⁿ) as illustrated by the arrow. Resonance assignments for the *M*⁰ spectrum were obtained from previously assigned ³¹P spectra of acetate-grown yeast respiring on acetate, glucose, and from anaerobic glucose-fed cells [8]. The peak labelled P_i^{other} is predominately composed of phosphate from the external medium (since the buffer contains 7 mM phosphate) and vacuolar

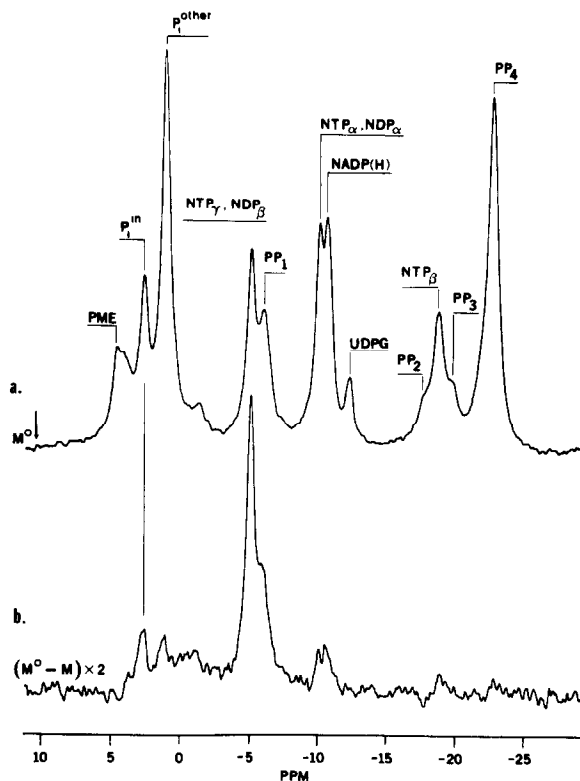


Fig.1. Saturation transfer spectra of aerobic cells supplied with ethanol. Only the *M*⁰ and *M*⁰−*M* spectra are shown. Spectra were accumulated for 1.5 h after waiting 15 min for sample equilibration. NMR pulsing and sample conditions are described in the text.

phosphate. This resonance also contains glycerophosphorylcholine. The *M* spectrum (not shown) was taken during steady-state saturation of the NTP_γ resonance, and was subtracted from the *M*⁰ spectrum to obtain the difference spectrum (*M*⁰−*M*) shown in fig.1b. The P_iⁱⁿ resonance is affected by saturation of the NTP_γ peak as shown by the excursion at 2.8 ppm. In addition, the difference peaks at −5 and −10 ppm corresponding to NTP_γ, NTP_α and NDP_α, respectively, are due to direct saturation effects from rf irradiation of NTP_γ peak during acquisition of the *M* spectrum. A difference signal is also observed at ~0 ppm and may result from either direct saturation effects (due to its proximity to the NTP_γ resonance) or transfer via exchange. However, this cannot be tested by placing a control equidistant from NTP_γ on the downfield side due to interfering phosphomonoester (PME) and P_iⁱⁿ peaks.

We have used the model and the modified Bloch equations described by Alger et al. [3] to analyze the saturation transfer data where the following equation was used to determine the flux:

$$k_2 + k_f = (1/T_{1_i} + k_2 + k_f) \Delta M_i / M_i^0 \quad (1)$$

In this expression, k_f corresponds to the unidirectional apparent rate constant for mitochondrial ATP synthesis while the apparent rate constant k_2 denotes P_i consumption by reactions other than the ATPase such as the GAP/PGK-coupled reaction in glycolysis. An apparent relaxation rate $(T_{1_i})_{\text{apparent}}^{-1} = (k_f + k_2 + 1/T_{1_i}) = 2.50 (\pm 0.38) \text{ s}^{-1}$ was obtained from an inversion recovery experiment performed during steady-state saturation of the NTP_γ peak. The error was estimated using a least-squares linear regression program. Using eqn 1, and $\Delta M / M^0 = 0.21$ with a standard deviation from the mean of 0.04 obtained from 4 independent saturation transfer experiments, a $k_f + k_2$ value $= 0.52 (\pm 0.13) \text{ s}^{-1}$ was obtained. The uncertainty ($\Delta k / k$) was estimated using the following expression [1],

$$\Delta k / k = (2N_{\text{RMS}} / M^0) (\Delta M / M^0)^{-1} + \Delta R / R \quad (2)$$

where $\Delta R / R$ is the uncertainty in the apparent relaxation rate. A P_i consumption rate of $1.3 (\pm 0.4) \mu\text{mol/min per g wet cells}$ was then evaluated by multiplying the $k_f + k_2$ value by the intracellular phosphate concentration (table 1).

Measurements of oxygen consumption were combined with the saturation transfer measured phosphate-consumption rate, and a P/O ratio of 2.9 was determined. The oxygen-consumption rate of $0.22 (\pm 0.02) \mu\text{mol/min per g wet cells}$ ($n=6$) for acetate mid-log cells respiring on ethanol is approx. 11-fold higher than the rate measured in glucose-grown derepressed cells respiring on endogenous substrates. A P/O ratio of 2.2 was determined from experiments performed on acetate-grown cells supplied with glucose aerobically with the data listed in table 1 (not shown).

In fig.2, saturation transfer spectra taken during anaerobic glycolysis are presented. Changes in intracellular pH, phosphomonoester, nucleotides, intracellular P_i , and polyphosphates in acetate-grown cells under the conditions studied here have been described previously [8]. No P_i^{in} difference signal arising from saturation transfer was observed in these anaerobic glucose-fed cells, as shown in fig.2b, in contrast to the observations on aerobic cells (see table 1).

4. DISCUSSION

Phosphate-consumption activity was observed in saturation transfer experiments during aerobic metabolism on glucose and ethanol. The net rates of ATP synthesis were estimated from oxygen-consumption measurements [9] by assuming that 1 mol O_2 consumed corresponds to 4–6 mol ATP produced (i.e. a P/O ratio of 2–3), and these

Table 1

Condition	$[P_i]^a$ (mM)	$\Delta M / M^0$	T_{1_i} (apparent) (s)	$k_f + k_2$ (s^{-1})	P_i consumption	O_2 consumption	P/O
Anaerobic glucose	4.0	0	—	—	—	—	—
Aerobic glucose	2.6	0.26	0.35	0.74	1.2 ± 0.5^b	0.26	2.2 ± 0.2^c
Aerobic ethanol	4.1	0.21	0.40	0.52	1.3 ± 0.4	0.22	2.9 ± 0.4

^a Intracellular phosphate concentrations were determined assuming 1.67 g wet cells contains 1 ml intracellular volume

^b Estimated error – the calculation is described in the text

^c SD

P_i and O_2 consumption values expressed as $\mu\text{mol/min per g wet cells}$

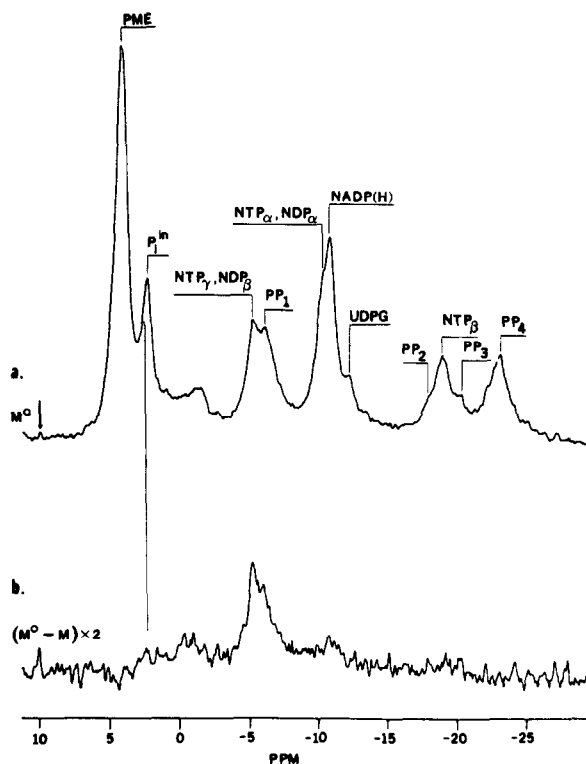


Fig.2. Saturation transfer spectra of anaerobic glucose-fed cells. Conditions equivalent to fig.1.

values agreed with the P_i -consumption rates measured by saturation transfer (table 1). The rates determined by saturation transfer techniques are unidirectional, and since these P_i -consumption rates are approximately equal to net ATP synthesis rates, the F_1F_0 -ATPase appears not to be in equilibrium and to be operating in the direction of ATP synthesis.

Previously, P_i -consumption activity ($\Delta M/M^0 = 0.10$) was observed in saturation transfer experiments performed during anaerobic glucose metabolism in glucose-grown derepressed cells [3]. These investigators suggested that GAP may be responsible for the saturation transfer since this rate was similar to the anaerobic glycolytic flux. However, P_i -consumption activity was not observed in our anaerobic glucose fed cells ($\Delta M/M^0 = 0$). In our present experiments on acetate cells, contributions from glycolytic pathways do not appear to contribute to the total P_i -consumption activity observed by saturation transfer experiments.

This is consistent since acetate-grown cells contain lower levels of glycolytic enzymes than glucose-grown cells, and thus are not as dependent on this pathway. Furthermore, we do not know what fraction of glucose is catabolized via the glycolytic pathway. The flux may be too low to be detected by saturation transfer techniques.

In yeast mitochondrial P_i is not resolved from P_i in the cytosol. The P_i -consumption rates were calculated using the total intracellular phosphate concentration thereby assuming that the ATPase is not limited by P_i transport across the mitochondrial membrane (a 1 pool model), and these rates are consistent with the estimated yeast F_1F_0 ATP synthesis rates. Moreover, studies performed in rat liver mitochondria [10] attest that P_i transport is not a rate-determining step in oxidative phosphorylation.

The role of pathways such as respiration and fermentation in yeast metabolism is largely dependent on growth conditions, (i.e. growth medium, carbon source, and culture age) since there is a correlation to the enzyme content and distribution in the cells. Our results in acetate-grown cells suggest that both GAP/PGK and the mitochondrial ATPase were responsible for the P_i -consumption activity observed in saturation transfer experiments, previously performed in glucose-grown cells. A similar implication has also been made by Brindle and Krikler (in press) in results obtained from their yeast saturation transfer experiments. We have recently completed a study which has confirmed that both of these P_i -consuming pathways contribute to P_i -consumption activity measured by saturation transfer in glucose-grown stationary phase cells, by conducting saturation transfer studies on cells treated with either a glycolytic inhibitor (iodoacetate) or antimycin A, an inhibitor of respiration (in preparation).

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