In Vivo Phosphorus-31 Nuclear Magnetic Resonance Saturation Transfer Studies of Adenosinetriphosphatase Kinetics in *Saccharomyces cerevisiae*†

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ABSTRACT: Phosphorus-31 saturation transfer NMR techniques have been employed to measure the unidirectional P_i consumption rate by respiration competent suspensions of the yeast *Saccharomyces cerevisiae* while the levels of ATP, ADP, and P_i are constant. These experiments are performed by saturating the ATP_γ phosphate resonance and observing the changes in the P_i resonance intensity while the yeast are respiring on endogenous substrates. The unidirectional P_i consumption rate is 3.5 ± 1 μmol s^{-1} (g of wet cells)$^{-1}$. The rate is reduced 10-fold upon addition of oligomycin (80 μg/mL), suggesting that at least 90% of the P_i consumption activity is due to the mitochondrial F_1–F_0 ATPase. We have not been able to conclusively assign the remaining 10%. When the yeast are glycolyzing anaerobically, the unidirectional P_i consumption rate was 1.0 ± 0.2 μmol s^{-1} (g of wet cells)$^{-1}$. At most, 80% of this is due to P_i consumption by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase leaving a residual activity of at least 0.2 μmol s^{-1} (g of wet cells)$^{-1}$. Thus the activity in the oligomycin-inhibited cells under respiratory conditions and the nonglycolytic activity in anaerobic cells are equal to within the experimental errors. Furthermore the unidirectional rate of P_i consumption during anaerobic glycolysis is insensitive to oligomycin. These data suggest that the mitochondrial adenosinetriphosphatase is not turning over during anaerobic glycolysis. Possible explanations for this inhibition are discussed.

It is now generally accepted that the oxidative phosphorylation of ADP in mitochondria, chloroplasts, and bacteria is thermodynamically coupled to the transmembrane electrochemical gradient of the proton (Boyer et al., 1977). However, the mechanistic details of this coupling and the enzymology of the various coupling factors remain subjects of intense research. In this research it has been necessary to prepare stable preparations of closed vesicular membranes which support ion gradients and have the coupling factors asymmetrically imbedded in the membrane. The problems involved with such preparations have been avoided with NMR techniques which allow the ATPase[1] kinetics to be measured in the intact cell or the intact organelle. Furthermore, such techniques provide a means of studying the kinetic regulation of the coupling factor activity in its normal physiological state. ^{31}P NMR has been used extensively to measure the concentrations of ATP, ADP, and P_i as well as of other phosphorylated compounds in closed vesicular membranes (Ogawa et al., 1978; Casey et al., 1977; Daniels et al., 1974), whole cells (Ugurbil et al., 1979), and tissue [see Gadian et al. (1979) for review]. In preliminary work we showed that the saturation transfer technique extends the capabilities of ^{31}P NMR to allow kinetic measurements of the bacterial ATPase (Brown et al., 1977). In this paper we describe ^{31}P NMR saturation transfer measurements of ATPase activity in intact yeast cells and develop methods for study of the bioenergetically significant ATPase under in vivo conditions.

While yeasts are complex eukaryotic organisms with many ATPases, the two largest activities are found in the mitochondria and on the plasma membrane (Serrano, 1978). The mitochondrial enzyme is a class I enzyme, similar in structure and function to the proton translocating ATPases of the bacterial plasma membrane, the chloroplast membrane, and the mammalian mitochondrial membrane (Willsky, 1979). Its primary function is to couple ATP production to the membrane's protonmotive force. The major yeast plasma membrane enzyme is a class II proton translocating ATPase which is believed to help regulate cytoplasmic pH and facilitate metabolite transport (Willsky, 1979; Ohsumi & Anraku, 1981; Malpartida & Serrano, 1981; Serrano, 1980). In the present experiments both ATPase's are present, and it is important to establish the relative contribution of each to the saturation transfer signal which measures the sum of all P_i consumption activities. By performing experiments with oligomycin and in the absence of oxygen, we establish that the dominant saturation transfer signal during respiration arises from the mitochondrial ATPase.

Except for a few applications to cellular and vesicular systems (Alger & Prestegard, 1979; Brown et al., 1977, 1978, 1980; Brown, 1980), saturation transfer has been generally used to investigate unidirectional kinetics in equilibrated chemical reaction mixtures (Faller, 1973; Hoffman & Forsén, 1966). Saturation transfer experiments require that there be a steady state in the reactant concentrations during the measurement. Simple chemical reaction mixtures maintain steady state by being at equilibrium whereas living systems are capable of setting up nonequilibrium steady-state concentrations by use of complex interconnecting reaction schemes and by use of compartmentation and transport. During our experiments the cells maintain the concentrations of ATP, ADP, and P_i in a steady state, and we have observed the effects of saturating the ATP_γ resonance upon the P_i signal intensities. In order to provide for the possibility that the ATPase is not in equilibrium during this steady state, we use the following

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[1] Abbreviations: NMR, nuclear magnetic resonance; P_i^{in}, cytosolic and mitochondrial orthophosphate; P_i^{vac}, orthophosphate in polyphosphate vacuoles; SP, sugar phosphates; NTP_γ, γ-phosphate of nucleoside triphosphates; NDP_β, β-phosphate of nucleoside diphosphates; NDP_α and NTP_α, α-phosphates of nucleoside di- and triphosphates; NTP_β, β-phosphate of nucleoside triphosphates; NAD(H), oxidized and reduced form of nicotinamide adenine dinucleotide; PP_1, terminal phosphate of polyphosphate; PP_2 and PP_3, penultimate phosphates of polyphosphate; PP_4, inner phosphates of longer polyphosphates; ATPase, adenosinetriphosphatase; rf, radio frequency; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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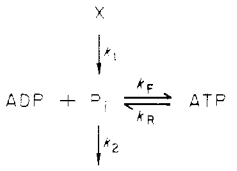
general kinetic scheme for analysis of the saturation transfer.



Note that this scheme completely describes the flows into and out of P_i but does not include other saturation transfer silent reactions involving ATP and ADP.

This scheme has four pertinent rate equations:

$$V_1 = k_1(X) \quad (1)$$

$$V_2 = k_2(P_i) \quad (2)$$

$$V_F = k_F(P_i) \quad (3)$$

$$V_R = k_R(ATP) \quad (4)$$

Although all the steps in this scheme are enzyme-catalyzed reactions and not necessarily described by simple linear rate equations, such equations are convenient in understanding the spin magnetization flows. Thus the rate constants k_1, k_2, k_F, and k_R are termed "apparent rate constants" whose values are not necessarily independent of reactant and product concentrations. (X), (P_i), and (ATP) in eq 1–4 denote the amounts of these materials present under the steady-state conditions. The resolution of the NMR signals defines the extent to which compartmentation can be introduced into the scheme. P_i's in vacuoles and outside the cell are resolved by NMR from internal P_i^{in}. However, mitochondrial P_i has not been resolved from cytoplasmic P_i in yeast, so in our scheme internal P_i^{in} includes contributions from cytosolic and mitochondrial pools. Equation 1 describes the rate of appearance of inorganic phosphate (V_i) from some set of unknown compounds, X. These reactions may include transport of P_i into the cytoplasmic compartment, as well as phosphatase reactions. Equation 2 describes the rate of consumption of P_i by reactions other than ATPase reactions. These reactions include the P_i consumption by glyceraldehyde-3-phosphate dehydrogenase during glycolysis. Equations 3 and 4 describe the forward (V_F) and reverse (V_R) ATPase reactions. We emphasize that V_F and V_R are unidirectional rates, and their difference ($V_F - V_R$) gives the net rate of ATP production.

Saturation transfer is best understood by considering that the NMR intensities (or spin magnetizations) are proportional to the difference in population between the spin states for each nucleus and that these populations can be selectively altered with rf fields (Alger & Prestegard, 1979). The intensity variations are quantitatively described by the modified Bloch equations (McConnell, 1958) for the spin magnetization in inorganic phosphate (M_i) and the ATP_γ phosphate (M_γ) which exchange via the ATPase reaction.

$$\frac{dM_\gamma}{dt} = \frac{1}{T_{1\gamma}}(M^0{}_\gamma - M_\gamma) - k_R M_\gamma + k_F M_i \quad (5)$$

$$\frac{dM_i}{dt} = \frac{1}{T_{1i}}(M^0{}_i - M_i) - k_F M_i - k_2 M_i + V_1 + k_R M_\gamma \quad (6)$$

Here $M^0{}_i$ and $M^0{}_\gamma$ are the fully relaxed intensities and T_{1i} and $T_{1\gamma}$ are the nuclear spin relaxation times in the absence of exchange. This formalism assumes that the nuclear spin does not relax during the time its molecule is undergoing enzyme catalysis. A "label" is introduced at ATP_γ by adding a saturation term to eq 5 which includes a radio frequency field resonant with the ATP_γ transition. This causes the spin state populations in the ATP_γ phosphorus to be equalized so that $M_\gamma = 0$ as long as the rf is on. Under these conditions the label, $\Delta M_i = (M^0{}_i - M_i)$, reaches a steady-state value obtained by solving eq 6.

$$\Delta M_i = \frac{k_F + k_2}{1/T_{1i} + k_F + k_2} M^0{}_i - \frac{V_1}{1/T_{1i} + k_F + k_2} \quad (7)$$

The reduction in P_i signal intensity observed when ATP_γ is saturated depends on the apparent rate constants for P_i disappearance ($k_F + k_2$), the T_1 for P_i, and the rate of P_i appearance by pathways other than the ATPase (V_1). In our experiments V_1 is small because we are not able to observe saturation transfer in any phosphate compounds (except NTP_γ) in the cytosolic or other compartments, in experiments where P_i is saturated (data not shown). Furthermore transport of P_i between external, vacuolar, and cytosolic compartments under several conditions studied occur with time constants of minutes (Gillies et al., 1981; den Hollander et al., 1981). To be saturation transfer sensitive the reactions must have time constants similar to the P_i spin–lattice relaxation time which is about 1 s. Equation 7 can then be used to solve for $k_F + k_2$.

$$k_F + k_2 = \frac{(1/T_{1i})(\Delta M_i/M^0{}_i)}{1 - \Delta M_i/M^0{}_i} \quad (8)$$

An inversion recovery experiment performed while the ATP_γ signal is saturated provides a value of $1/T_{1i} + k_F + k_2$ (Mann, 1977; Campbell et al., 1978; Brown, 1980) so that from eq 7 we have

$$k_2 + k_F = (1/T_{1i} + k_F + k_2)(\Delta M_2/M^0{}_i) \quad (9)$$

Thus the sum of the apparent rate constants for P_i consumption ($k_F + k_2$) is obtained from measurement of the steady-state values of ΔM_i and $M^0{}_i$ and from an inversion recovery measurement of T_1 performed while the ATP_γ is saturated. The unidirectional rate of P_i consumption ($V_F + V_2$) can then be obtained from eq 2 and 3 by using $k_F + k_2$ and the intensity of the unperturbed P_i resonance as a measure of the amount of inorganic phosphate present in the system. During respiration on endogenous carbon sources where exogenous glucose is not present, k_2 is small because 90% of our observed signal is oligomycin sensitive, reflecting that there are no major P_i consuming pathways, other than the ATPase, operating during respiration. Thus our measurement yields primarily V_F, the unidirectional rate of ATP synthesis by ATPase. In the anaerobic glycolysis experiments, we cannot ignore k_2, because our measured glucose consumption rates predict that P_i is consumed rapidly enough by the oxidative phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate to lead to a saturation transfer effect. Our measured rate is the sum of the ATP synthesis rate plus the rate of this glycolytic enzyme and any other rapid P_i consuming paths.

Materials and Methods

The *Saccharomyces cerevisiae* strain NCYC 239 was grown aerobically for 24 h at 30 °C to stationary phase in a liquid medium containing 2% Bactopeptone, 1% yeast extract, and 2% glucose. When grown in this manner, the cells become derepressed, possess many mitochondria, and are respiration competent (Stevens, 1977). The cells were harvested by low-speed centrifugation, after the cultures were cooled to 5 °C, and then washed twice in ice-cold buffer (5 mM KH_2PO_4, 0.86 mM K_2HPO_4, 2 mM $MgSO_4$, 1.7 mM NaCl, 50 mM Mes, pH adjusted to 6.0 by using NaOH). The suspension used for the NMR experiments consisted of equal weights of wet cell pellet and buffer and was kept as long as 12 h at ice temperatures prior to use.

The NMR samples consisted of 15 mL of the cell suspension, in a 20-mm NMR tube into which a multibubbler apparatus was inserted. For the aerobic experiments, the samples were oxygenated by continuously bubbling 95% O_2/5% CO_2 gas through the suspension, at 200 mL/min in two streams below and at 1000 mL/min in two streams above the observation coil. No exogenous carbon source was added. The oxygen level, monitored in a similar apparatus (outside the magnet) which had a Yellow Springs oxygen electrode placed at the height of the NMR detection coil, was at least 60% of saturation under the conditions of the NMR experiment. These bubbling conditions were maintained for at least 10 min prior to beginning the saturation transfer experiments. The anaerobic glycolysis experiments were performed while bubbling 100 mL/min of 95% N_2/5% CO_2 through the two lower bubblers. A 1.67-g sample of glucose was added to the suspension to maintain glycolysis. After a few minutes the glycolytic intermediates came to a steady state which was maintained for about 1 h, during which the NMR experiments were completed. The temperature was regulated to 25 ± 1 °C during the NMR measurements.

A Bruker WH 360 WB NMR spectrometer operating at 145.8 MHz was used for ^{31}P NMR experiments. A frequency synthesizer driving an auxillary amplifier generated the rf field used for saturation. For our instrument and conditions, about 0.5 W was sufficient to keep the ATP resonance saturated. The protocol for the saturation transfer experiments was as follows. After a presaturation period of 2 s, ^{31}P free induction decays were collected by using a pulse interval of 0.6 s and a flip angle of 68°. The saturating rf field was turned off during only the acquisition period of 0.1 s. Blocks of 64 FID's were alternately collected with the saturation frequency at the ATP_γ signal and at a point an equal distance to the low field side of the P_i resonance. In this way the spectrum having ATP_γ saturated (M) and the normal unsaturated spectrum (M^0) were collected "simultaneously". Furthermore the difference spectrum (ΔM) will show no direct saturation effects in the P_i resonance because of the symmetrical relationship of the saturating frequencies about the P_i resonance in the M^0 and M spectra. Even when this compensation is not used we have found the direct saturation of the P_i resonance to introduce insignificant errors.

The analysis of saturation transfer data using the formalism outlined above requires that the M^0 spectra be observed under fully relaxed conditions and that the M spectra be observed only after complete development of the ΔM. The ΔM remains at the steady-state value during data collection because (1) prior to the collection of each data block there is a long presaturation period and (2) the saturation period between FID collections is long with respect to the acquisition time during which saturation is off. So that more efficiency in signal averaging could be obtained, pulse delays shorter than $4T_1$ were used with pulses less than 90°. As a result both the M and M^0 spectra are somewhat saturated. We have found that doubling the delay and using the same flip angle do not change the observed value of $\Delta M/M^0$, so we conclude that the saturation effects in the M and M^0 spectra are identical, leaving the correct value of $\Delta M/M^0$.

Results

In Figure 1 the ^{31}P NMR saturation transfer spectra of an aerobic suspension of yeast cells are presented. Figure 1a, the M^0 spectrum, was taken while the frequency of the saturating rf field was downfield of the P_i resonance. In this spectrum signals from cytoplasmic P_i, the P_i in intracellular compartments which are thought to be polyphosphate vacuoles, and

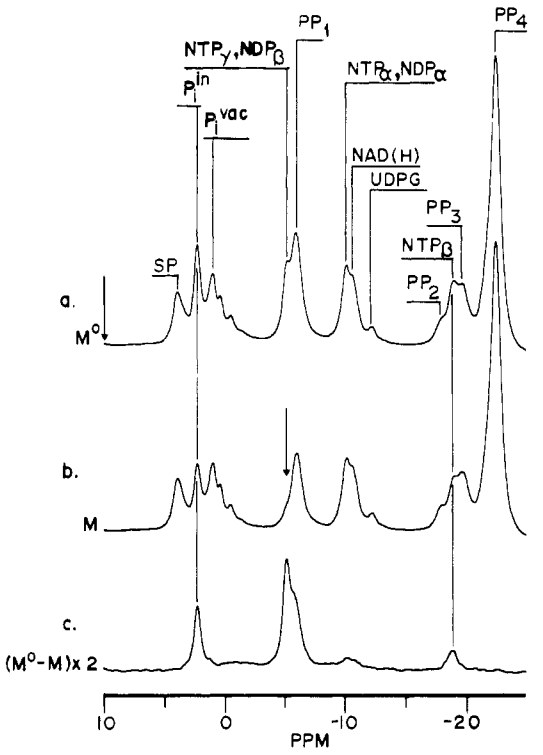


FIGURE 1: Saturation transfer spectra of respiring *S. cerevisiae*. Sample conditions and data acquisition procedures are described under Materials and Methods. The M^0 (a) and M (b) spectra were obtained by saturating at the positions shown by the arrows. (c) The difference (ΔM) spectrum.

from nucleoside phosphates were observed and identified as previously (Salhany et al., 1975; Navon et al., 1979; den Hollander et al., 1981). We have observed that the external P_i is consumed by the cells during the 5–10-min period prior to the time the saturation transfer experiment commences; hence, an external P_i resonance is absent in Figure 1. To obtain Figure 1b, the M spectrum, we saturated the terminal phosphate resonances of the nucleoside phosphates, which includes ATP_γ and ADP_β. The excursions in Figure 1c, the difference spectrum (ΔM), show the intensity changes caused by the saturation of these resonances. There is an excursion in the difference spectrum at the position of only one of the two P_i peaks, the one assigned to intracellular P_i, but not the peak assigned to the polyphosphate vacuoles. There is also a difference signal at the ATP_β position. The broad envelope around 10 ppm is due to direct saturation of the broad signals in that portion of the spectrum. This was confirmed by the lack of a difference signal in an experiment in which the saturation frequency was an equal distance upfield of this region while the M^0 spectrum was obtained. The peak labeled PP_i (terminal phosphate of polyphosphate) also experiences direct saturation, as it is clearly resonant with the saturating radio frequency field.

In seven parallel experiments done with different cell batches we have found $\Delta M_i/M^0_i$ to be 0.29 with a standard deviation from the mean of 0.03. The parameter $(k_F + k_2 + 1/T_{1i})^{-1}$ was determined to be 0.7 s in an inversion recovery experiment (not shown) which was performed while keeping the ATP_γ resonance saturated. Using eq 9, we obtain a value for $k_F + k_2$ of 0.44 s^{-1} with an estimated uncertainty of 20%. Taking the intracellular P_i concentration to be 12 mM (den Hollander et al., 1981) and the internal volume to be two-thirds of the wet pellet (Gancedo & Gancedo, 1973), we obtain a unidirectional P_i consumption rate, $V_F + V_2$, of 3.5 ± 1 μmol s^{-1} (g of wet cells)$^{-1}$.

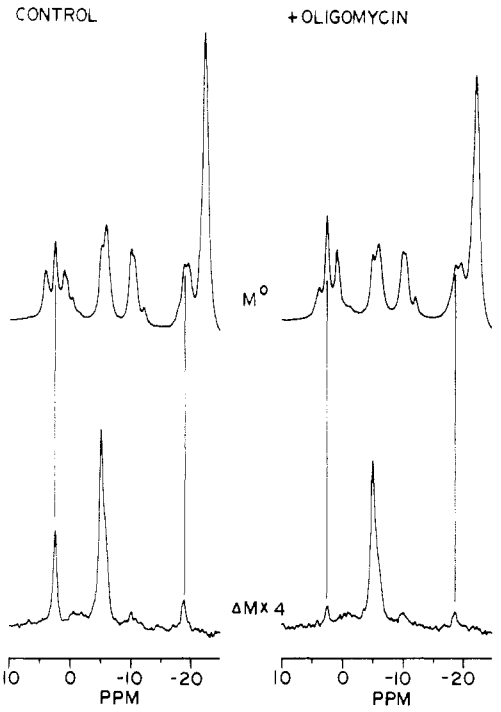


FIGURE 2: Effect of oligomycin upon saturation transfer spectra of respiring *S. cerevisiae*. Only the M^0 and ΔM spectra are shown. Control data, equivalent to Figure 1, are shown in the left panel. The right panel shows the effect of oligomycin at a concentration of 80 μg/mL. Resonance assignments are the same as for Figure 1.

The ATP_γ and ADP_β resonances overlap so that when ATP_γ is saturated, ADP_β is also at least partially saturated. The ΔM_β appearing at −18.8 ppm in Figure 1c is therefore thought to arise from transfer of saturation from the ADP_β resonance. The $\Delta M_\beta/M^0_\beta$ in this case, however, will be related to the rate constant for the ATP hydrolysis reaction, k_R. Quantitative determination of k_R and V_R from ΔM_β is complicated for a number of reasons: The resonance comes from a number of nucleoside triphosphates (Salhany et al., 1975; Navon et al., 1979; den Hollander et al., 1981), each giving an unknown contribution to the total M^0_β and each having a different hydrolysis rate (Serrano, 1978; Pedersen, 1976). The adenylate kinase reaction can also contribute to the observed ΔM_β. The overlap of this resonance with the polyphosphate signals also makes it difficult to obtain an accurate measurement of the T_1. To the extent that these contributions can be evaluated, the single difference spectrum (Figure 1c) simultaneously gives information about both the forward and reverse ATPase reactions.

The relative values of the ΔM_i and ΔM_β can, however, help in determining how close the forward and reverse ATPase rates are to being equal. Analysis similar to that of eq 9 shows that

$$\frac{V_F}{V_R} = \frac{(1/T_{1i})\Delta M_i/(1 - \Delta M_i/M^0_i)}{(1/T_{1\beta})\Delta M_\beta/(1 - \Delta M_\beta/M^0_\beta)}$$

If we assume that both the $\Delta M/M^0$ are small with respect to unity, then

$$\frac{T_{1\beta}\Delta M_i}{T_{1i}\Delta M_\beta}$$

gives the ratio of forward to reverse rates. We measured values of 0.7 s for T_{1i} and 0.2–0.3 s for $T_{1\beta}$ so that if $\Delta M_i/\Delta M_\beta$ = 2.3–3.5, the forward and reverse ATPase rates are equal. In seven independent determinations under respiratory conditions, the values of $\Delta M_i/\Delta M_\beta$ ranged from 2.1 to 3.7, indicating that $V_F \approx V_R$ and therefore that the coupled ATPase reaction was close to equilibrium presumably with the protonmotive force. However, in the presence of oligomycin (Figure 2) or under anaerobic glycolytic conditions (Figure 4), ΔM_i became equal to or smaller than ΔM_β, indicating either a bias in the ATPase toward hydrolysis or the importance of additional pathways. We leave more quantitative evaluation of the ATP hydrolysis rate for the future and concentrate in this paper on the unidirectional ATP synthesis rate.

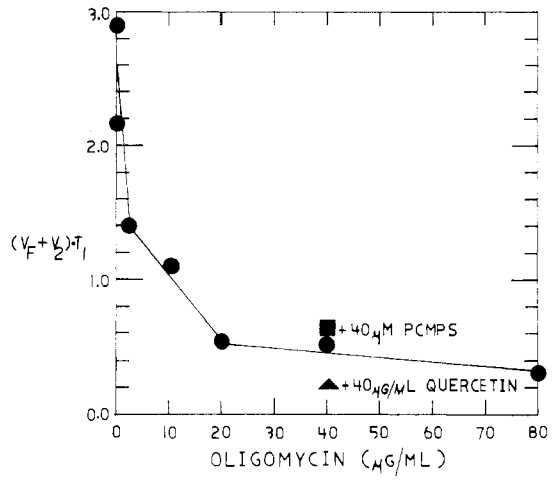


FIGURE 3: Dependence of unidirectional P_i consumption activity in respiring *S. cerevisiae* upon oligomycin. The activities (ordinate values) were obtained from saturation transfer measurements as described in the text. Each data point represents a separate sample. (●) With oligomycin alone; (■) with oligomycin and 40 M *p*-(chloromercuri)phenylsulfonate; (▲) with oligomycin and 40 μg/mL quercetin.

We have measured the rate of O_2 consumption by these yeast under respiratory conditions to be approximately 0.02 μmol s^{-1} (g of wet cells)$^{-1}$. Assuming a P/O ratio of 3, the net rate of ATP synthesis was therefore 0.06 μmol s^{-1} (g of wet cells)$^{-1}$. The fact that this net rate was much smaller than the unidirectional rate measured by saturation transfer [3.5 μmol s^{-1} (g of wet cells)$^{-1}$] provides additional evidence that the coupled ATPase reaction is very close to equilibrium during respiration.

The fraction of the total P_i consumption activity arising from the mitochondrial ATPase was determined by measuring the saturation transfer after various amounts of oligomycin, a specific inhibitor of the mitochondrial enzyme, were added (Serrano, 1978). When present at high concentrations, oligomycin appreciably reduced both ΔM_i and ΔM_β, while it caused only a small increase in M^0_i and a small decrease in M^0_β (Figure 2). Such decreases in these two $\Delta M/M^0$'s indicate that oligomycin is reducing both the forward and reverse rates of the ATPase. Figure 3 shows the effect of increasing concentrations of oligomycin upon the P_i consumption rate. We have avoided the complications of T_1 measurement by plotting $T_1(V_F + V_2)$ as the ordinate in Figure 3. This parameter was determined by measuring $\Delta M_i/M^0_i$ and calculating $(K_F + K_2)T_1$ by using eq 8. Subsequently $(V_F + V_2)T_1$ was calculated from eq 3 by using the relative peak intensities for M^0_i. We expect that T_1 for P_i is independent of oligomycin concentration, so the ordinate in Figure 3 is proportional to the total unidirectional P_i consumption rate. This activity was reduced to 20% by 20 μg/mL oligomycin. Oligomycin at still higher concentrations gave a less than linear reduction, leaving about 10% of the total activity even at 80 μg/mL.

We have attempted to assign the remaining 10% of the P_i consumption activity by using other inhibitors. The residual activity was sensitive to the ATPase inhibitor quercetin (Figure 3), which has a greater effect upon the plasma membrane

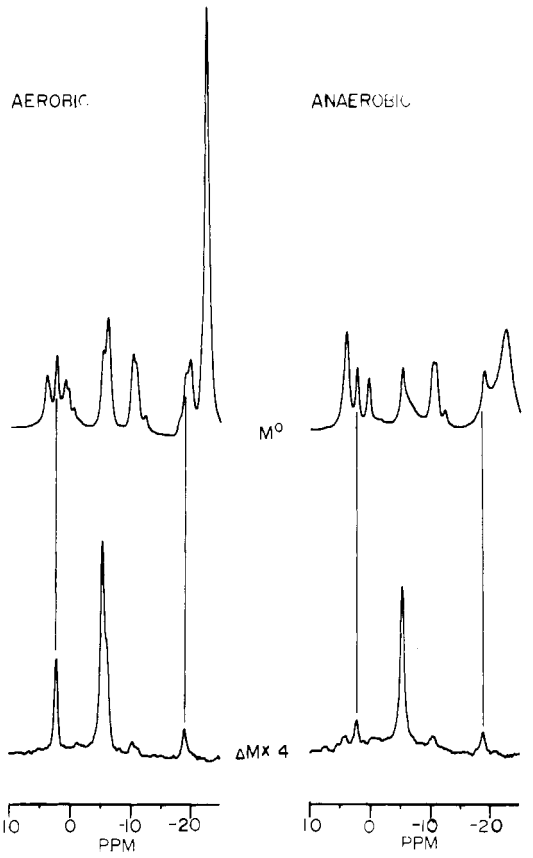


FIGURE 4: Comparison of saturation transfer data collected during respiration on endogenous substrate and during anaerobic glycolysis. Conditions are described under Materials and Methods. Only the M^0 and ΔM spectra are shown for clarity. (Left panel) Conditions equivalent to Figure 1; (right panel) data collected during anaerobic glycolysis.

ATPase. The P_i consumption rate was 2–3-fold lower in the presence of 40 μg/mL quercetin and 40 μg/mL oligomycin than it was in the presence of the oligomycin alone. However, this rate is only slightly lower than that obtained with 80 μg/mL oligomycin, so that this experiment did not identify the origin of the residual 10% activity. The residual oligomycin insensitive activity was not sensitive to 40 μM *p*-(chloromercuri)phenylsulfonate (Figure 3) which is a potent specific inhibitor of the plasma membrane ATPase (Serrano, 1978). The inhibitor's lack of effect could be due to its not having penetrated the cell because of its negative charge or to the plasma membrane ATPase not having been responsible for the 10% residual activity.

The influence of oxygen on the P_i consumption rate is shown in Figure 4, in which the saturation transfer spectrum taken under aerobic conditions is compared with that taken under anaerobic glycolytic conditions. Peak assignments for the anaerobic spectrum are similar to those for the aerobic spectrum (Figure 1a); however, sugar phosphate peaks are larger in the anaerobic spectrum (Salhany et al., 1975; Navon et al., 1979; den Hollander et al., 1981). Furthermore the polyphosphate signals are substantially smaller under anerobic conditions because during the first minutes after glucose addition to these respiration competent cells, the polyphosphate is hydrolyzed to form P_i which is ultimately used to make sugar phosphate.

Under anaerobic glycolytic conditions, the difference spectrum shows that the saturation introduced at ATP_γ and ADP_β appears in the cytoplasmic P_i and ATP_β; however, the value of $\Delta M_i/M^0_i$ was considerably smaller than it was during respiration. In three independent determinations, we found

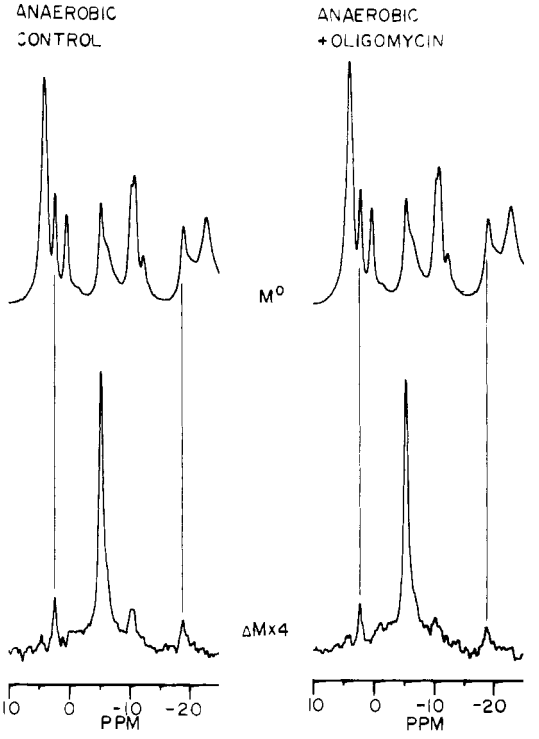


FIGURE 5: Effect of oligomycin on saturation transfer during anaerobic glycolysis. (Left panel) Control spectrum; (right panel) data from a separate sample to which 40 μg/mL oligomycin was added.

that $\Delta M_i/M^0_i$ was 0.10 ± 0.02, and analysis as described above leads to a value of 0.15 s^{-1} for $k_F + k_2$. The peak intensity indicates the cytoplasmic P_i concentration is about 10 mM so we obtain a total P_i consumption rate of 1.0 μmol s^{-1} (g of wet cells)$^{-1}$ with an estimated error of 30%.

The value $\Delta M_i/M^0_i$ for P_i during anaerobic glycolysis was reduced about 5-fold from its value under respiratory conditions. This reduction is similar to that caused by high concentrations of oligomycin under respiratory conditions, particularly in view of the fact that the k_2, from glyceraldehyde-phosphate dehydrogenase, is appreciable in the anaerobic experiment where exogenous glucose is present but not in the aerobic one, where glucose is not present. This prompted us to determine whether oligomycin had any effect on the $\Delta M_i/M^0_i$ during anaerobic glycolysis. The results are shown in Figure 5, where saturation transfer data from anaerobic glycolyzing cells with and without oligomycin (40 μg/mL) are compared. The spectra are quite similar, with the levels of nucleotide phosphates, sugar phosphates, and intercellular P_i being nearly the same in both. The ΔM_i values in the difference spectra are statistically identical. This means that oligomycin, an inhibitor of the mitochondrial ATPase, has a negligible effect on the unidirectional P_i consumption rate during anaerobic glycolysis.

Discussion

The experiments described in this paper were performed on suspensions of respiration-competent yeast cells. These cells possess active mitochondria, and therefore both the mitochondrial ATPase and the plasma membrane ATPase might be expected to contribute to the overall in vivo P_i consumption activity. Studies of isolated plasma and mitochondrial membrane fragments from yeast have shown that oligomycin at concentrations up to 40 μg/mL is a potent inhibitor of mitochondrial ATPase but is ineffective against the plasma membrane ATPase (Serrano, 1978). In order the evaluate the relative contribution of mitochondrial ATPase activity to the

total P_i consumption rate in vivo, we performed a series of experiments with oligomycin. We have shown that about 90% of the initial P_i consumption activity is inhibited by 80 μg/mL oligomycin. This suggests that at least 90% of the overall P_i consumption rate during respiration is due to ATP synthesis by the mitochondrial ATPase. Thus other P_i-consuming pathways including the plasma membrane ATPase contribute less than 10% to the total activity. Furthermore analysis of the ΔM_i and ΔM_β or comparison of the unidirectional ATPase rate with the O_2 consumption rate indicates that the coupled mitochondrial ATPase is operating very close to equilibrium under these conditions. This result is in accordance with those previously obtained by Wilson (Wilson et al., 1974).

Our finding that under conditions of respiration on endogenous substrate a large fraction of the P_i consumption activity is due to the mitochondrial ATPase poses questions about compartmentation. We are uncertain of what fraction of the internal P_i is within the mitochondria. If a large fraction is in fact entrapped within the mitochondria, then V_F is a measure of only the unidirectional ATPase rate. However, if a major fraction of the internal P_i is outside mitochondria, then V_F may include a contribution from the mitochondrial P_i transport system. It is known that the mitochondria in yeast occupy less than 10% of the total cell volume (Stevens, 1977) and that P_i is distributed across the matrix membrane as a weak acid reflecting the pH gradient (Ogawa et al., 1978). Cohen et al. (1978) showed that the mitochondrial P_i could be observed in hepatocytes to which valinomycin had been added to swell the mitochondria and give the maximum ΔpH. The mitochondrial pH was about 7.6 in those experiments. Because the cytoplasmic pH is about 7.2 in the present experiments, the largest pH difference we expect is 0.4, so P_i will be at most 3-fold more concentrated in the matrix. Hence, the mitochondrial P_i peak should be less than 30% of the cytosolic peak and will not be well resolved, which is presumably why it is not observed. In other words the peak assigned as internal P_i may have up to 30% of its intensity from unresolved mitochondrial P_i. Consequently our observed V_F may involve the transport of P_i into the matrix. At this point we are not able to decide unequivocably whether the ATP synthesis or the P_i transport is rate limiting.

We have also studied in vivo P_i consumption activity during anaerobic glycolysis. Here the total unidirectional rate of P_i consumption is about 30% of what it was during respiration. During glycolysis the glyceraldehyde-3-phosphate dehydrogenase step represents a substantial additional pathway for P_i consumption which we have labeled k_2. We have estimated the glycolytic rate to be 0.4 μmol s^{-1} (g of wet cells)$^{-1}$ from a parallel experiment in which the disappearance of the natural abundance ^{13}C NMR signals from the glucose was observed (data not shown). An estimate of the rate for P_i disappearance due to glycolysis (V_2) is therefore 0.8 μmol s^{-1} (g of wet cells)$^{-1}$ assuming that all the glucose flows down the glycolytic pathway. Our saturation transfer experiments show the total rate of P_i consumption ($V_F + V_2$) is 1.0 $\pm$ 0.2 μmol of P_i s^{-1} (g of wet cells)$^{-1}$. Therefore glycolysis is responsible for a major portion of the total rate of P_i disappearance. By difference we obtain a value of 0.2 μmol of P_i s^{-1} (g of wet cells)$^{-1}$ for nonglycolytic P_i consumption which is the same within experimental error as that determined in aerobic experiments in the presence of oligomycin (80 μg/mL). Furthermore this anaerobic activity is virtually insensitive to oligomycin (Figure 5). These data are summarized in Table I. We also conclude from these fluxes that to keep P_i at steady state during glycolysis the unidirectional rate of ATP hydrolysis by some ATPase, V_R, could be as much as 5 times the rate of ATP synthesis by ATPase, V_F, since most of the ATP is being synthesized by substrate level phosphorylation. This is probably the reason the ΔM values for P_i and ATP_β are closer to being equal under glycolytic conditions.

Table I: Unidirectional P_i Consumption Rates in *S. cerevisiae*

conditions	rate[a]
aerobic endogenous respiration[b]	3.5
aerobic endogenous respiration + oligomycin	0.3
anaerobic glycolysis[c]	1.0
anaerobic glycolysis + oligomycin	1.0

[a] Units are μmol s^{-1} (g of wet cells)$^{-1}$. [b] O_2 consumption rate is about 0.02 μmol s^{-1} (g of wet cells)$^{-1}$. [c] Glucose consumption rate is about 0.4 μmol s^{-1} (g of wet cells)$^{-1}$.

It is conceivable that during anaerobic glycolysis, ATP formed by substrate level phosphorylation could be transported into the mitochondria and be hydrolyzed by the ATPase to form a protonmotive force. Eventually the protonmotive force would come to equilibrium with the phosphate potential. Our aerobic data show that the coupled mitochondrial ATPase reaction comes to an equilibrium with the cytosolic ATP, ADP, and P_i levels within a few seconds (the rate constant turnover of the cytoplasmic P_i is 0.44 s^{-1}). Thus by the time the anaerobic saturation transfer experiment commences, we might expect the mitochondrial ATPase to be "turning over" the cytoplasmic P_i with a unidirectional rate which is similar to that observed in the aerobic experiment. We emphasize that this would be the case in this model even though O_2 is not present and the mitochondria are not capable of producing net ATP by oxidative phosphorylation. Since our experiments measure unidirectional flux and not net ATP synthesis, we would, if this model held, expect to measure a $\Delta M_i/M^0_i$ of appreciable value corresponding to turnover through the mitochondrial ATPase. We do not observe an appreciable $\Delta M/M^0$ during anaerobic glycolysis, so we conclude that the model does not hold because the transport systems and/or the ATPase is kinetically inhibited during anaerobic glycolysis.

There are several mechanisms by which this inhibition might occur. Inhibition of the mitochondrial ATPase activity during anaerobic glycolysis may be the result of the presence of the ATPase inhibitor protein (Ernster et al., 1979; Ebner & Maier, 1977; Satre et al., 1979; Landry & Goffeau, 1975; Satre et al., 1975). The inhibitor binding to the F_1 ATPase of mitochondrial preparations (Galante et al., 1981; Klein et al., 1981) and its influence on ATP-dependent energy conversion reactions, ATP hydrolysis, and oxidative phosphorylation of ADP have been studied (Asumi et al., 1970; Horstman & Racker, 1970; van de Stadt et al., 1973; Ferguson et al., 1977; Gomez-Puyou et al., 1979; Harris et al., 1979). Initially the inhibitor was thought to inhibit only ATP hydrolysis and ATP-dependent energy conversion reactions, but more recently it has been shown to inhibit oxidative phosphorylation as well (Harris et al., 1979; Gomez-Puyou et al., 1979). In the absence of a protonmotive force the inhibitor protein binds to and inactivates the ATPase (Harris et al., 1979). Under anaerobic conditions the inhibition may prevent the formation of a protonmotive force from ATP hydrolysis, thereby keeping the inhibitor attached. Recently Schwerzmann & Pedersen (1981) have proposed this role for the mitochondrial inhibitor protein. An alternative model based on in vitro experiments (Boyer, 1975; Boyer et al., 1977) suggests that the ATPase activity is inhibited in the absence of a protonmotive force by virtue of a conformational change in the ATPase itself. The low ATPase activity may prevent the formation of a proton-

motive force from ATP hydrolysis, thereby keeping the enzyme in an inactive form. Still another model based on in vitro experiments of Klingenberg (1980) suggests that the ATP/ADP exchanger may not be capable of transferring ATP into the mitochondria because the exchanger is electrogenic and any protonmotive force (negative inside) created by ATP hydrolysis would resist further inward movement of negatively charged ATP. Whichever of these models is responsible, the present experiments have provided physiological connections to these previous in vitro experiments. In any case, our results demonstrate that the cell is capable of inhibiting the mitochondrial energy conversion during anaerobic periods.

To conclude, we have shown that the ^{31}P NMR saturation transfer technique can measure the unidirectional rate of P_i consumption in intact yeast cells by the ATPase. The mitochondrial ATPase gives the larger contribution to the total rate of P_i consumption under respiratory conditions, but during glycolysis it is not turning over. We intend to study the effect of various compartmental metabolic perturbations on the rate of this ATPase to obtain a more detailed understanding of its control and its role in cellular energetics.

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References

Alger, J. R., & Prestegard, J. H. (1979) *Biophys. J. 28*, 1–14.
Asumi, K., Juntti, K., & Ernster, L. (1970) *Biochim. Biophys. Acta 205*, 307–311.
Boyer, P. D. (1975) *FEBS Lett. 58*, 1–6.
Boyer, P. D., Chance, B., Ernster, L., Mitchell, P., Racker, E., & Slater, E. C. (1977) *Annu. Rev. Biochem. 46*, 955–1026.
Brown, T. R. (1980) *Philos. Trans. R. Soc. London, Ser. B B289*, 441–444.
Brown, T. R., Ugurbil, K., & Shulman, R. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5551–5553.
Brown, T. R., Gadian, D. G., Garlick, P. B., Radda, G. K., Seeley, P. J., & Styles, P. (1978) *Frontiers in Biological Energetics II*, pp 1341–1349, Academic Press, New York.
Brown, T., Chance, E. M., Dawson, M. J., Gadian, D. G., Radda, G. K., Wilkie, D. R. (1980) *J. Physiol. (London) 305*, 84P.
Campbell, I. D., Dobson, C. M., Ratcliffe, R. G., & Williams, R. J. P. (1978) *J. Magn. Reson. 29*, 397–417.
Casey, R. P., Njus, D., Radda, G. K., & Sehr, P. A. (1977) *Biochemistry 16*, 972–977.
Cohen, S. M., Ogawa, S., Rottenberg, H., Glynn, P., Yamane, T., Brown, T. R., Shulman, R. G., & Williamson, J. R. (1978) *Nature (London) 273*, 554–556.
Daniels, A., Korda, A., Tanswell, P., Williams, A., & Williams, R. J. P. (1974) *Proc. R. Soc. London, Ser. B 187*, 353–361.
den Hollander, J. A., Ugurbil, K., Brown, T. R., & Shulman, R. G. (1981) *Biochemistry 20*, 5871–5880.
Ebner, E., & Maier, K. (1977) *J. Biol. Chem. 252*, 671–676.
Ernster, L., Carlsson, C., Hundal, T., & Nordenbrand, K. (1979) *Methods Enzymol. 55*, 399–407.
Faller, J. W. (1973) in *Determination of Organic Structures by Physical Methods* (Nachod, F. G., & Zukerman, J. J., Eds.) pp 75–97, Academic Press, New York.
Ferguson, S. J., Harris, D. A., & Radda, G. K. (1977) *Biochem. J. 162*, 351–357.
Gadian, D. G., Radda, G. K., Richards, R. E., & Seeley, P. J. (1979) in *Biological Application of Magnetic Resonance* (Shulman, R. G., Ed.) pp 463–529, Academic Press, New York.
Galante, Y. M., Wong, S.-Y., & Itateti, Y. (1981) *Biochemistry 20*, 2671–2678.
Gancedo, J. M., & Gancedo, C. (1973) *Biochimie 55*, 205–211.
Gillies, R. J., Ugurbil, K., den Hollander, J. A., & Shulman, R. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2125–2129.
Gomez-Puyou, A., Tuena de Gomez-Puyou, M., & Ernster, L. (1979) *Biochim. Biophys. Acta 547*, 252–257.
Harris, D. A., von Tscharner, V., & Radda, G. K. (1979) *Biochim. Biophys. Acta 548*, 72–84.
Hoffman, R. A., & Forsēn, S. (1966) *Prog. Nucl. Magn. Reson. Spectrosc. 1*, 15.
Horstman, L. L., & Racker, E. (1970) *J. Biol. Chem. 245*, 1336–1344.
Klein, G., Satre, M., Dianoux, A.-C., Vignais, P. V. (1981) *Biochemistry 20*, 1339–1344.
Klingenberg, M. (1980) *J. Membr. Biol. 56*, 97–105.
Landry, Y., & Goffeau, A. (1975) *Biochim. Biophys. Acta 376*, 470–484.
Malpartida, F., & Serrano, R. (1981) *J. Biol. Chem. 256*, 4175–4177.
Mann, B. E. (1977) *J. Magn. Reson. 25*, 91–94.
McConnell, H. M. (1958) *J. Chem. Phys. 28*, 430–431.
Navon, G., Shulman, R. G., Yamane, T., Eccleshall, T. R., Lam, K.-B., Baronofsky, J. J., & Marmur, J. (1979) *Biochemistry 18*, 4487–4499.
Ogawa, S., Rottenberg, H., Brown, T. R., Shulman, R. G., Castillo, C. L., & Glynn, P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1796–1800.
Ohsumi, Y., & Anraku, Y. (1981) *J. Biol. Chem. 256*, 2079–2082.
Pedersen, P. L. (1976) *J. Biol. Chem. 251*, 934–940.
Salhany, J. M., Yamane, T., Shulman, R. G., & Ogawa, S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4966–4970.
Satre, M., deJerphanion, M.-B., Huet, J., & Vignais, P. V. (1975) *Biochim. Biophys. Acta 387*, 241–255.
Satre, M., Klein, G., & Vignais, P. V. (1979) *Methods Enzymol. 55*, 421–426.
Schwerzmann, K., & Pedersen, P. L. (1981) *Biochemistry 20*, 6305–6311.
Serrano, R. (1978) *Mol. Cell. Biochem. 22*, 51–63.
Serrano, R. (1980) *Eur. J. Biochem. 105*, 419–424.
Stevens, B. J. (1977) *Biol. Cell. 28*, 37–55.
Ugurbil, K., Shulman, R. G., & Brown, T. R. (1979) in *Biological Applications of Magnetic Resonance* (Shulman, R. G., Ed.) pp 537–589, Academic Press, New York.
van de Stadt, R. J., de Boer, B. L., & van Dam, K. (1973) *Biochim. Biophys. Acta 292*, 338–349.
Willsky, G. R. (1979) *J. Biol. Chem. 254*, 3326–3332.
Wilson, D. I., Stubbs, M., Veach, R. L., Erecinska, M., & Krebs, H. (1974) *Biochem. J. 140*, 57–64.