Differential Sensitivity of the Cellular Compartments of *Saccharomyces cerevisiae* to Protonophoric Uncoupler under Fermentative and Respiratory Energy Supply†

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ABSTRACT: The effect of a protonophoric uncoupler (CCCP) on the different cellular compartments was investigated in yeast grown aerobically on lactate. These cells were incubated in a resting cell medium under three conditions; in aerobiosis with lactate or glucose or in anaerobiosis with glucose as energetic substrate. For each condition, in vivo ^{31}P NMR was used to measure pH gradients across vacuolar and plasma membrane and phosphorylated compound levels. Respiratory rate (aerobic conditions) and TPP^+ uptake were measured independently. Concerning the polyphosphate metabolism, spontaneous NMR-detected polyphosphate breakdown occurred, in anaerobiosis and in the absence of CCCP. In contrast, in aerobiosis, polyphosphate hydrolysis was induced by addition of either CCCP or a vacuolar membrane ATPase-specific inhibitor, bafilomycin A1. Moreover, polyphosphates were totally absent in a null vacuolar ATPase activity mutant. The vacuolar polyphosphate content depended on two factors: vacuolar pH value, strictly linked to the vacuolar H^+-ATPase activity, and inorganic phosphate concentration. CCCP was more efficient in dissipating the proton electrochemical gradient across vacuolar and mitochondrial membranes than across the plasma membrane. This discrepancy can be essentially explained by a difference of stimulability of each proton pump involved. As long as the energetic state (measured by NDP + NTP content) remains high, the plasma membrane proton ATPase is able to compensate the proton leak. Moreover, this ATPase contributes only partially to the generation of ΔpH. The maintenance of the ΔpH across the plasma membrane, that of the energetic state, and the cellular TPP^+ uptake depend on the nature of the ATP-producing process. As expected, these parameters are more sensitive to CCCP when the ATP-producing process is only oxidative phosphorylation (lactate condition) than when in the aerobic glucose condition, thus suggesting the role of the glycolytic pathway in the resistance to this compound. However, the resistance of cells to CCCP is not effective when the ATP-producing process is only fermentation (anaerobic glucose condition), although under these conditions glycolysis is optimal. The role of mitochondrial uncoupled respiration in maintaining the cellular ATP level in the presence of glucose is discussed.

It is well documented that in yeast cytosolic pH is rather independent from the carbon source and remains quite constant near neutrality, since the external pH is modulated between 3 and 7.2 (den Hollander et al., 1981; De la Pena et al., 1982). Thus, this homeostasis of the intracellular proton concentration must be carefully controlled by several systems which also participate in the maintenance of the transmembrane difference in proton electrochemical potential, $\Delta\tilde{\mu}H^+$, which has two components: electrical potential ($\Delta\Psi$) and pH gradient (ΔpH). In such processes, the plasma membrane proton ATPase plays a key role (Goffeau & Slayman, 1981; De la Pena et al., 1982; Serrano, 1988).

However, two other intracellular membranes, vacuolar and inner mitochondrial membranes, contain proton pumping systems involved in the establishment of proton electrochemical gradients. The vacuole is the most acidic compartment of the cell, maintaining its low pH by an ATP-dependent proton pump embedded in the membrane (Kakinuma et al., 1981; Forgac, 1989; Klionsky et al., 1990). It has been reported that $\Delta\tilde{\mu}H^+$ is used for active transport of some metabolites like basic amino acids and mineral cations like Ca^{2+} [for review see Anraku et al. (1989)]. Moreover, this compartment contains numerous enzymes concerned with the degradation and storage of cellular components like polyphosphates [poly(P)],[1] suggesting a major role in cellular homeostasis for this organelle (Gillies et al., 1981; Lichko et al., 1982; Bostian et al., 1983; Wood & Clark, 1988; Schuddemat et al., 1989; Klionsky et al., 1990). A change in the vacuolar pH has three consequences: (i) since enzymes are generally strongly pH-dependent, a change in vacuolar pH has significant effects on the degradative activity of phosphatases (Greenfield et al., 1987); (ii) a vacuolar pH change should modify the metabolic content of this compartment via the proton substrate antiporters (Anraku et al., 1989); and (iii) vacuolar biogenesis and protein sorting should be defective under growth conditions [Banta et al., 1988; for review see Klionsky et al. (1990)].

When yeasts are grown aerobically on a neoglucogenic substrate like lactate, the mitochondria are well differentiated with regard to oxidative phosphorylation [for review see De Vries and Marres (1987)]. From the chemiosmotic viewpoint, this energy transduction is accomplished by the respiratory chain and ATP synthase proton pumps coupled through the common intermediate, $\Delta\tilde{\mu}H^+$ (Mitchell, 1961).

So plasma, vacuolar and inner mitochondrial membranes contain different proton pumps able to maintain various proton electrochemical gradients. Some lipophilic weak acids, like

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; α- and β-NDP, α- and β-phosphate of nucleoside diphosphates; NMR, nuclear magnetic resonance; α-, β-, and γ-NTP, α-, β-, and γ-phosphate of nucleoside triphosphates; poly(P), polyphosphates; T1, spin-lattice relaxation time; TPP^+, tetraphenyl phosphonium.

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phenol derivatives, are now named protonophores since they increase the proton permeability of both artificial and natural membranes (Mac Laughlin & Dilger, 1980). Consequently, in mitochondria, these agents uncouple energy-yielding processes (i.e., oxidative activity) from energy-consuming processes (i.e., ATP synthesis and active transport) by dissipating transmembrane $\Delta\tilde{\mu}H^+$ (Mitchell, 1961). Although these drugs have been largely studied on yeast, either on isolated organelles like mitochondria (Rigoulet et al., 1987) or on purified membrane fractions, for example, vacuolar membrane vesicles (Kakinuma et al., 1981), little is known about their differential effect in vivo on each compartment of the eukaryotic cells.

It has been previously reported that the concentration of CCCP required to inhibit yeast cell growth on a fermentative carbon source is higher than that needed to inhibit the growth on a nonfermentative carbon source (Dupont et al., 1984). The purpose of this work is to analyze the effect of a protonophoric uncoupler, CCCP, on the energetic state of the yeast cells and the capacity of the various energy-transducing membranes to maintain proton gradients in vivo. Using the ^{31}P NMR technique, it is possible to investigate the metabolism of phosphorylated compounds and to determine cytosolic and vacuolar pH (Salhany et al., 1975; Navon et al., 1979; den Hollander et al., 1981; Nicolay et al., 1982). In the present study, by using this technique and other classical methods, we have found that CCCP is more efficient in dissipating the proton gradient across vacuolar and mitochondrial membranes than across the plasma membrane.

A detailed study of poly(P) pool evolution was also performed under conditions where vacuolar pH was modulated by addition of either CCCP or a vacuolar membrane ATPase-specific inhibitor, bafilomycin A1. Moreover, poly(P) was totally absent in a null vacuolar ATPase activity mutant.

During aerobic glucose consumption, the pH gradient and electrical potential across the plasma membrane and energetic state were maintained in the presence of 20 μM CCCP, whereas the mitochondria were fully uncoupled. By switching from aerobic to anaerobic conditions, these energetic parameters became sensitive to CCCP, thus raising the question of the role of uncoupled mitochondria in maintaining the cytosolic energetic state.

Materials and Methods

Yeast Strain, Media, and Growth. The diploid yeast strain used was the wild strain *Saccharomyces cerevisiae* (yeast foam). Cells were grown aerobically at 28 °C on a YE medium at pH 4.5, containing 1% yeast extract, 0.1% potassium phosphate, and 0.12% ammonium sulfate and supplemented with 2% lactate as carbon source. Respiratory deficient mutant (Rho^0) was obtained from the previous wild strain and was grown on the YE medium except that the carbon source was 2% galactose.

The Vacuolar Membrane ATPase mutant of *S. cerevisiae*, ΔVMA4-5A (MATα, *vma4*::URA3, *ade2*, *leu2*, *his3*, *trp1*, *rho*$^+$) was a gift of Dr. Françoise Foury, Unité de Biochimie Physiologique, Université de Louvain, Louvain-la-Neuve, Belgium. This strain was disrupted for the VMA4 gene encoding the vacuolar ATPase 26.6-kDa polypeptide (Foury, 1990). The corresponding wild-type strain was W303-1B (MATa, *ura3*, *ade2*, *leu2*, *his3*, *trp1*, *rho*$^+$). Since null *vma4* mutant exhibits poor growth on neoglucogenic substrate (Foury, 1990), cells were grown aerobically at 28 °C in a YE medium at pH 4.5, supplemented with 2% glucose as the carbon source.

Cells were harvested in the logarithmic growth phase and washed twice with cold water.

NMR Spectroscopy. In vivo ^{31}P NMR spectra were recorded at 161.9 MHz using a Bruker AM400 spectrometer. Spectra were obtained at 25 °C by using a 20-mm NMR probe. Acquisition parameters were as follows: 60° pulse angle, 0.135-s acquisition time, 0.065-s delay, 750 scans, and 4K memory size. An exponential line broadening of 15 Hz was applied.

The cells (500 mg dry wt) were suspended in a buffer containing 2 mM $MgSO_4$, 1.7 mM NaCl, 2 mM potassium phosphate, and 50 mM Mes, pH 6.0, in a final volume of 10 mL (Brindle & Krikler, 1985). A total of 50 μL of antifoam was added to the cell suspension prior to each experiment, and aerobic conditions were obtained by bubbling 95% O_2/5% CO_2 through the suspension. Substrates (220 mM lactate or 250 mM glucose), CCCP, or bafilomycin A1 was injected directly into the NMR tube (20-mm diameter).

In perifusion experiments, cells (400 mg dry wt) were immobilized in low-gelling agarose as described previously (Brindle & Krikler, 1985). Perifusion was carried out on the same oxygenated buffer supplemented with 40 mM glucose, at a flow rate of 30 mL/min. This technique was used to make a long time average determination of longitudinal relaxation time (T1) for the α-phosphate of di- and trinucleotides, poly(P) and P_i pools. T1 values were determined by using the progressive saturation method, and saturation factors were calculated from

$$S = \sin\theta(1 - e^{-t/T1})/(1 - \cos\theta(e^{-t/T1}))$$

where θ is the pulse angle and t is the interpulse delay.

Analysis of ^{31}P Spectra. Assignments of resonances were made by comparing with spectra reported in previous papers (Navon et al., 1979; den Hollander et al., 1981). Chemical shifts were given relative to an internal reference of methylenediphosphonic acid at 18.45 ppm (sealed capillary). The pH value of the different compartments was determined by using the chemical shifts of inorganic phosphate within each compartment and by comparison with a P_i calibration curve drawn from a sample containing 30 mM NH_4Cl, 200 mM KCl, 20 mM $MgCl_2$, and 10 mM potassium phosphate (Gancedo & Gancedo, 1973; den Hollander et al., 1981). Moreover, the external medium pH evolution was followed by pH electrode measurements. To better characterize P_i pools, resolution enhancement was applied consisting in the difference spectrum of data treated with two exponential line broadenings (15 and 80 Hz). Peak areas were determined by using a software integration routine. Poly(P), nucleoside di- and triphosphate, and P_i contents were calculated by taking into account their respective saturation factors and by comparing the metabolite area with that of the methylenediphosphonate standard (2 μmol of P_i equivalents). External P_i was also measured colorimetrically according to Sumner (1944).

Oxygen Consumption Measurements. Oxygen consumption rates were measured at 25 °C with a Clark oxygen electrode (Gilson) in a 1.5-mL thermostatically controlled chamber. Measurements were performed on the bench under conditions simulating NMR experiments. Aliquots were taken and diluted to final concentration of 1 mg dry wt/mL in oxygenated buffer. Rates were determined from the slope of a plot of O_2 concentration versus time.

Determination of CCCP Concentration in the Extracellular Medium. CCCP showed a maximum absorbance at 355 nm, pH 4.5 (Dupont et al., 1984). The measurements were performed on the bench under conditions simulating NMR experiments, except that Mes buffer was replaced by sodium phthalate, pH 4.5. The amount of CCCP in the extracellular

medium was obtained by measuring at 355 nm the remaining CCCP concentration of the supernatant after centrifugation.

Determination of TPP^+ Uptake (De la Pena et al., 1982). Measurements were performed on the bench under conditions simulating NMR experiments. Radioactive TPP^+ uptake was determined as follows: cells were incubated in NMR buffer supplemented with 0.5 μM [^{3}H]TPP^+ (0.1 μCi/mL). It was verified that the equilibrium distribution was reached in about 5 min. Routinely, aliquots were taken and yeast cells separated from the medium by rapid centrifugation through a silicone oil layer (silicone AR200 fluid). Both the supernatant and pellet were counted, and TPP^+ uptake was calculated assuming an internal volume of 2 μL/mg dry wt (Gancedo & Gancedo, 1973).

Results

Quantitation of Phosphorylated Metabolites and Intracellular pH Determination. A typical ^{31}P NMR spectrum of a suspension of well-oxygenated yeast cells in the presence of 220 mM lactate is illustrated in Figure 1A. The resonances were assigned according to previous papers (Salhany et al., 1975; Navon et al., 1979; den Hollander et al., 1981; Beauvoit et al., 1989). The main resonances were from intracellular polyphosphates [PP_4, inner phosphate of long-chain poly(P) at −22.5 ppm, PP_2–PP_3, penultimate phosphates centered at −21.7 ppm, and finally, PP_1, terminal phosphate around −7 ppm] and nucleoside di- and triphosphates (β-NTP at −18.3 ppm, α-NTP + α-NDP at −10 ppm, and γ-NTP + β-NDP at −5 ppm). The region between 0 and 3 ppm contained the inorganic phosphate resonances that were better characterized after resolution enhancement (inset of Figure 1A) and were assigned to external (1 ppm), vacuolar (1.2 ppm), and cytosolic (2.1 ppm) P_i. Other signals belonged to various phosphodiesters (−1 ppm) and phosphomonoesters (4 ppm). Usually the area of β-NTP resonance gave a good estimate of the NTP level within the cell, while either the α-phosphate or the terminal phosphate of di- and trinucleotides could be used to measure NTP + NDP content. Unfortunately, in yeast cells, the β-NTP resonance was obscured by the presence of resonances from poly(P) (PP_2–PP_3). However, when the poly(P) pool did not vary much, this resonance gave an estimation of the NTP pool. In contrast, under large variations in poly(P) chain length, such estimations of the NTP pool are not valid. Since γ-NTP + β-NDP overlaps with poly(P) resonances (PP_1), only α-phosphate peak areas (which overlap with fairly unvariable NAD + NADH resonances) can be used to give a good estimate of the energetic state of cells (den Hollander et al., 1981; Brindle et al., 1990).

The repetition rate of the flip angle was selected to optimize the signal to noise ratio with respect to NTP signals (Becker et al., 1979). Under these conditions the resonances were saturated and a saturation parameter was computed using T1 values, as described in the Materials and Methods section. Saturation parameters were 0.29 ± 0.01 for cytosolic P_i, 0.54 ± 0.08 for α-NDP + α-NTP, and 0.66 ± 0.05 for PP_4. Using these parameters, the absolute contents of these intracellular phosphorylated metabolites could be calculated.

Using the phosphorus NMR technique, it has been shown that the pH of the cytosol and the vacuole can be determined separately in intact yeast cells (Nicolay et al., 1982, 1983; Greenfield et al., 1987). This is possible because distinct resonances from cytosolic, vacuolar, and external P_i can be recorded by ^{31}P NMR (Figure 1A), allowing the pH of the compartments to be determined by comparing their chemical shift values to a standard calibration curve (see Materials and Methods section). However, under a wide variety of experimental conditions, the vacuolar P_i is not well resolved. Nevertheless, other phosphate compounds like poly(P) can be used as pH probes (Lundberg et al., 1989). Indeed, NMR-detected poly(P) are mainly localized in vacuoles [Urech et al., 1978; Wood & Clark, 1988; see also Beauvoit et al. (1989)], and the vacuolar pH can be further determined from the titration behavior of the phosphate resonance of these compounds. Both PP_1 and PP_2 peaks, respectively terminal and penultimate phosphates from poly(P) chains, have been shown to be sensitive to changes in pH, whereas the PP_4 peak, the inner phosphate resonance from these polymers, is relatively insensitive (Mac Donald & Mazurek, 1987; Lundberg et al., 1989). Consequently, a plot of the difference in chemical shifts between PP_4 and PP_1 in relation to vacuolar pH, obtained when vacuolar P_i was visible, was drawn as a calibration curve. Figure 1B represents the sum of four different types of experiments including (i) respiratory-deficient mutant (Rho^0-type) yeast cell suspension fermenting galactose, (ii) wild-type

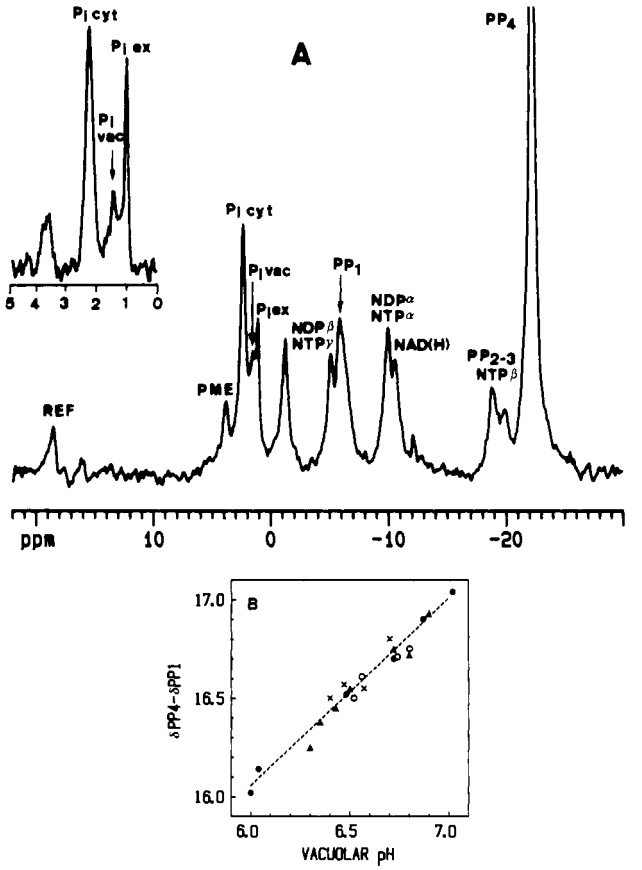

FIGURE 1: Titration behavior of phosphate resonances of polyphosphate in relation to vacuolar pH. (A) ^{31}P NMR spectrum of aerobic suspension of yeast cells in presence of 220 mM lactate. The spectrum was recorded as described in the Materials and Methods section: 42 mg dry wt/mL, 750 scans, 2.5-min accumulation. Abbreviations: PME, phosphomonoesters; $P_i$cyt, $P_i$ext, and $P_i$vac, cytosolic, external, and vacuolar inorganic orthophosphate, respectively; α- and β-NDP, α- and β-phosphate of nucleoside diphosphates; α-, β-, and γ-NTP, α-, β-, and γ-phosphate of nucleoside triphosphates; PP_n, phosphate residue at the nth position in polyphosphate chains; REF, reference signal of methylenediphosphonic acid (capillary). The inset shows the expanded region of the spectrum, between 4 and 0 ppm, treated with resolution enhancement as described in the Materials and Methods section. (B) Plot of the chemical shift difference between PP_4 and PP_1 resonances versus vacuolar pH. Vacuolar pH was determined from the corresponding P_i chemical shift and a standard pH calibration curve. Experimental conditions: (×) Rho^0-type yeast cell suspension with 250 mM galactose; (○) wild-type yeast cell perifusion with 40 mM glucose; (●, ▲) wild-type yeast cell suspension with 250 mM glucose and 220 mM lactate, respectively, and different CCCP additions.

yeast cells utilizing glucose in perifusion experiments, and wild-type yeast cell suspensions utilizing aerobically either (iii) glucose or (iv) lactate, in the presence of different concentrations of CCCP. All these experimental conditions exhibited a unique relationship between PP_4 and PP_i chemical shift differences versus vacuolar pH determined by the position of P_i resonance. The chemical shift variation in the range of pH 6–7 was linear, as already found by Mac Donald and Mazurek (1987). This pH calibration obtained in vivo could now be used to estimate the vacuolar pH even in the absence of direct detectability of the vacuolar P_i pool.

Metabolic Steady States in Yeast Cells after either Glucose or Lactate Feeding. ^{31}P NMR spectra were sequentially recorded after addition of 250 mM glucose on a respiratory competent yeast cell, and the results are presented in Figure 2A,B. Glucose addition induced a transient acidification of cytosolic pH as already reported with glucose-repressed yeast cells (den Hollander et al., 1981). In contrast, the vacuolar pH value remained nearly constant at 6.2. Under our particular conditions (low external P_i concentration), we observed that (i) the external medium pH decreased slowly from 6.0 to 5.5 (Figure 2A); (ii) poly(P) breakdown, calculated from PP_4 resonance, occurred at a slow constant rate of 0.06 μmol of P_i equiv per minute per gram dry wt (Figure 2B); (iii) the internal P_i content decreased and remained constant and could be estimated at around 1.8 mM using an internal volume value of 2 μL/mg dry wt (Gancedo & Gancedo, 1973); (iv) the sugar phosphate pool increased rapidly after glucose addition. It should be noted that the reestablishment of a high ΔpH across the plasma membrane allowed the recovery of the initial NTP level and was due essentially to the increase in cytosolic pH. Initial poly(P) breakdown and the decrease in the cytosolic P_i pool should be interpreted as a direct consequence of P_i mobilization during the start of glycolysis. When the cells were perifused with glucose and 2 mM P_i, poly(P) breakdown did not occur and intracellular P_i was maintained near 4.1 mM (data not shown), thus illustrating the role of poly(P) as an intracellular P_i pool generator. According to the data shown in Figure 2A,B, a metabolic steady state [except for poly(P)] was reached 10 min after the onset of glucose feeding, and a typical ^{31}P NMR spectrum is shown in Figure 2C.

Similar experiments were performed in the presence of 220 mM lactate as substrate, and the spectra recorded from aerobic suspension of cells indicate that the steady state was obtained at the end of the first spectrum (2.5 min). Such a spectrum is presented in Figure 1A. The cytosolic and vacuolar pH are respectively 7.1 and 6.4, and external pH remains around 6.0. Compared to glucose, the lactate metabolism was characterized by the maintenance of poly(P) and P_i pools, strengthening the idea of an equilibrium between poly(P) and cytosolic P_i pools (Gillies et al., 1981). It was also observed that pH gradients across various compartments were slightly lower in the lactate-containing medium.

Role of Vacuolar Membrane ATPase Activity in Vacuolar pH Homeostasis and Polyphosphate Storage. Bafilomycin A1 is a well-known specific inhibitor of vacuolar membrane H^+-ATPase (Bowman et al., 1988). The addition of 320 μM bafilomycin A1 to a yeast cell suspension in the presence of 250 mM glucose induced both a dissipation of the pH gradient across vacuolar membrane and a large decrease of poly(P) content. The ^{31}P spectrum obtained after 30 min is shown in Figure 3A. Vacuolar and cytosolic pH values were identical and equal to 7.15. It should be noticed that vacuolar ATPase inhibition does not significantly change the cytosolic pH and

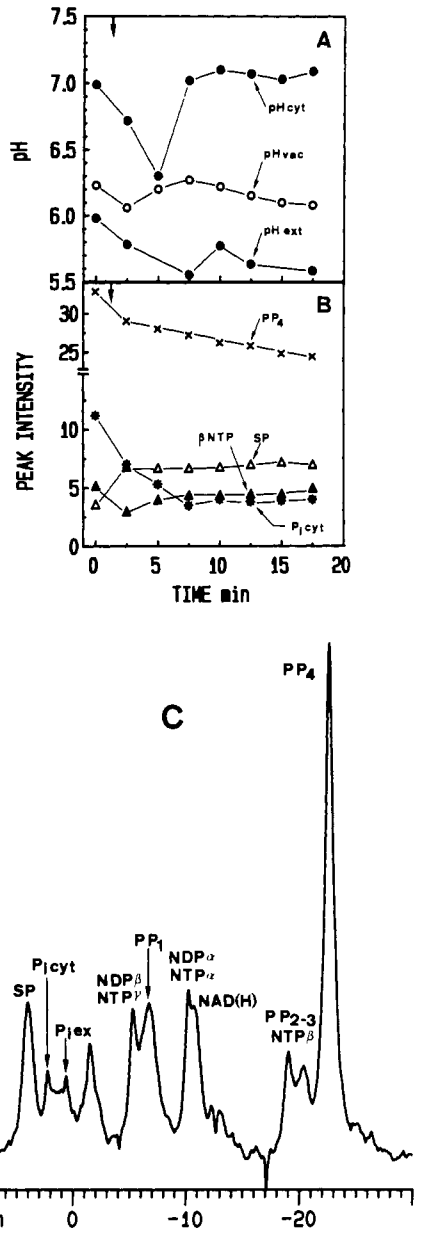

FIGURE 2: Evolution of energetic metabolism during aerobic glucose consumption. ^{31}P NMR spectra were recorded as described in the Materials and Methods section: 58 mg dry wt/mL, 750 scans, 2.5-min accumulation. At $t = 0$, 250 mM glucose was added to the suspension. (A) Time courses of the external (⊕), cytosolic (●), and vacuolar pH (○) determined as described in the text. (B) Time courses of the relative areas of the cytosolic Pi (*), sugar phosphates, SP (△), inner phosphates of polyphosphate, PP_4 (×), and β-phosphate of nucleoside triphosphate resonances (▲). (C) Typical spectrum at metabolic steady state of glucose consumption (10 min). Abbreviations are the same as in Figure 1A, except SP is sugar phosphates.

α-NDP + α-NTP content (compare Figures 3A and 2C). The poly(P) hydrolysis generated shorter poly(P) chains and cellular P_i accumulation (compare Figures 3A and 2C). Poly(P) breakdown was completely achieved after 45 min (data not shown).

VMA4 is a gene encoding a polypeptide required for the catalytic activity of the yeast vacuolar ATPase (Foury, 1990). Null *vma4* haploid mutant (ΔVMA4-5A), obtained by disruption, is viable but exhibits poor growth on lactate. Consequently, this strain and the corresponding wild-type strain (W303-1B) were grown on glucose as the carbon source. As shown in Figure 3B, aerobic suspension of wild-type yeast cells, in the presence of glucose, exhibited a large amount of poly(P). In contrast, the ^{31}P spectrum of a null *vma4* mutant suspension

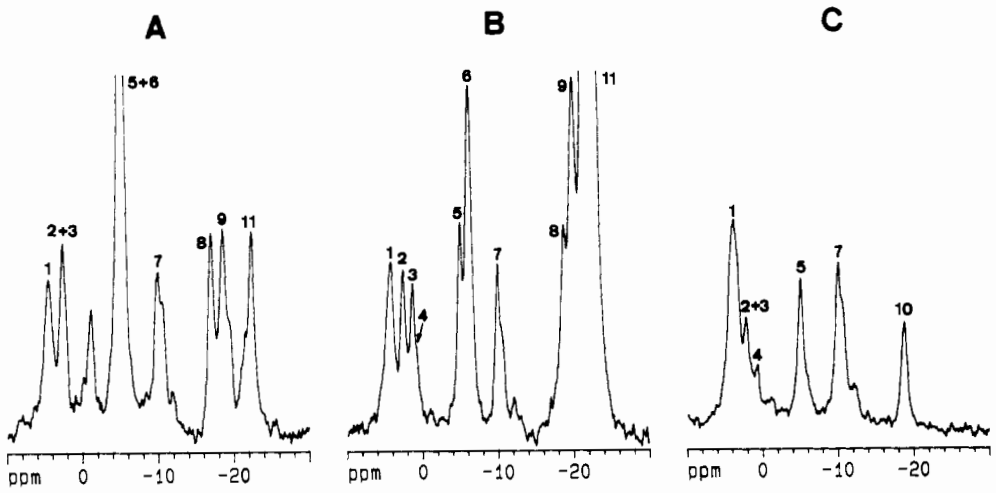

FIGURE 3: Effect of vacuolar membrane ATPase activity deficiency on vacuolar pH and polyphosphate content. ^{31}P NMR spectra were recorded as described in the Materials and Methods section: 50 mg dry wt/mL, 750 scans, 2.5-min accumulation. Experimental conditions were as follows. (A) Aerobic yeast cell suspension (yeast foam strain), with 250 mM glucose and 320 μM bafilomycin A1 for 30 min. A corresponding control spectrum (without inhibitor) is shown in Figure 2C. (B) Aerobic yeast cell suspension (W303-1B strain), with 250 mM glucose for 15 min. (C) Aerobic suspension of Vacuolar Membrane ATPase mutant cells (ΔVMA4-5A strain), with 250 mM glucose for 15 min. The resonances assignments are as follows: 1, sugar phosphates; 2, cytosolic P_i; 3, vacuolar P_i; 4, external P_i; 5, γ-phosphate of nucleoside triphosphates and β-phosphate of nucleoside diphosphates; 6, terminal phosphate of polyphosphate (PP_1); 7, α-phosphate of nucleoside di- and triphosphates; 8 and 9, penultimate phosphate of polyphosphate (PP_2, PP_3) including β-phosphate of nucleoside triphosphates; 10, β-phosphate of nucleoside triphosphates; 11, inner phosphate of polyphosphate (PP_4).

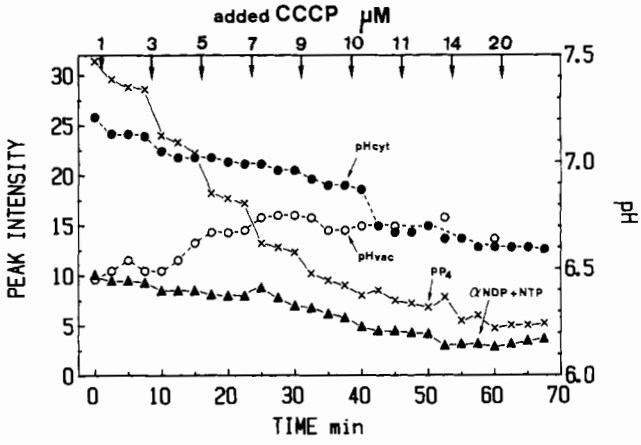

FIGURE 4: Time-dependent changes in pH compartments and phosphorylated compound levels upon CCCP additions during aerobic metabolism of lactate. ^{31}P NMR spectra were recorded as described in the Materials and Methods section: 52 mg dry wt/mL, 750 scans, 2.5-min accumulation. pH compartments were determined as described in the Materials and Methods section, and peak intensities are expressed in relative areas. CCCP was added directly to the suspension as indicated by arrows. Symbols: (●) cytosolic pH, (○) vacuolar pH, (▲) α-phosphate of di and tri-nucleotides, and (×) inner phosphate of polyphosphate (PP_4).

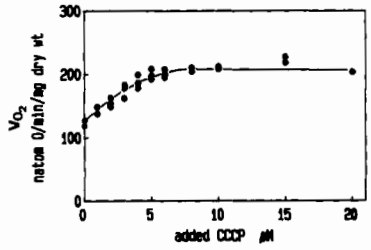

FIGURE 5: Effect of CCCP addition on oxygen consumption rate of yeast cell suspension in presence of lactate. Respiratory rate was measured as indicated in the Materials and Methods section, under the conditions that simulated the experiment of Figure 4 (50 mg dry wt/mL).

did not present any resonance assigned to poly(P) (Figure 3C). Moreover, only one small intracellular P_i pool was detected, indicating the absence of a pH gradient across the vacuolar membrane (compare parts C and B of Figure 3).

Effect of CCCP Titration on pH Gradients and Phosphorylated Metabolite Contents of Yeast Cells under Aerobic Conditions. The pH variations within various compartments and the evolution of poly(P) (PP_4) and nucleotide contents (α-NDP + α-NTP) under aerobic lactate utilization in the presence of CCCP are given in Figure 4.

Under CCCP titration, the external pH remained nearly constant at a value of 6.1 (data not shown); in contrast, the cytosolic pH slowly decreased with CCCP concentration to 10 μM, whereas the vacuolar pH increased to 6.7. For 10 μM CCCP, the pH gradient between vacuolar and cytosolic compartments was partially maintained at a value of 0.25 pH unit.

The existence of this residual gradient correlated to a relative stability in the nucleotide level. To better document the origin of the relative stability of nucleotide pool, we measured the rate of oxygen consumption in relation to CCCP concentration under similar experimental conditions. VO_2 reached a maximum value for 8–10 μM CCCP (Figure 5), indicating that the nucleotide levels were maintained as long as the respiratory rate was stimulable. At higher CCCP concentrations (up to 10 μM), the collapse of the residual pH gradient across the vacuolar membrane, which was due to a drop in the cytosolic pH, correlated to a decrease in the nucleotide level (Figure 4) and a maximum oxygen consumption rate. A further increase in CCCP concentration did not modify the cytosolic pH (Figure 4), and even for 20 μM CCCP, the ΔpH across the plasma membrane was maintained at a residual value of 0.5. In the presence of lactate and bubbling N_2 to collapse the nucleotide level, an identical residual pH gradient between cytosol and external medium could be measured (data not shown), demonstrating that only a part of the internal pH is maintained by energetic processes, including ATP-dependent proton pumping.

Each CCCP addition induced a drop of poly(P) content as indicated by the decrease of the PP_4 signal area (Figure 4). Quantitative analysis of the NMR data indicated that there was a good recovery of the hydrolyzed poly(P) in the cellular P_i. During the entire titration protocol, the internal P_i evolved from 13 μmol/g dry wt to 30 μmol/g dry wt. Surprisingly,

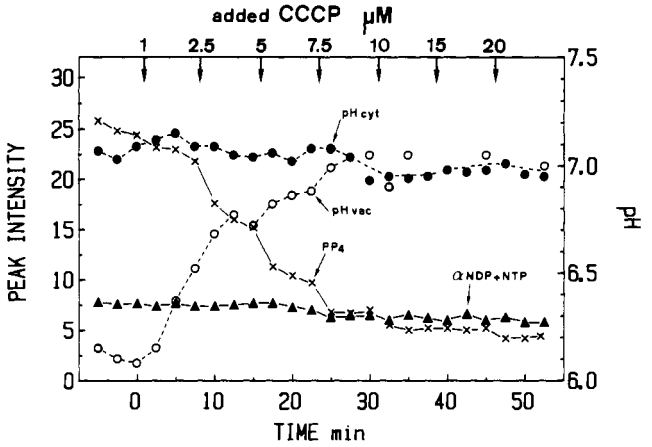

FIGURE 6: Time-dependent changes in pH compartments and phosphorylated compound levels upon CCCP additions during aerobic metabolism of glucose. ^{31}P NMR spectra were recorded as described in the Materials and Methods section: 52 mg dry wt/mL, 750 scans, 2.5-min accumulation. pH compartments were determined as described in the Materials and Methods section, and peak intensities are expressed in relative areas. CCCP was added directly to the suspension as indicated by arrows. In order to avoid substrate lack, 250 mM glucose was added every 30 min. Symbols are the same as in Figure 4.

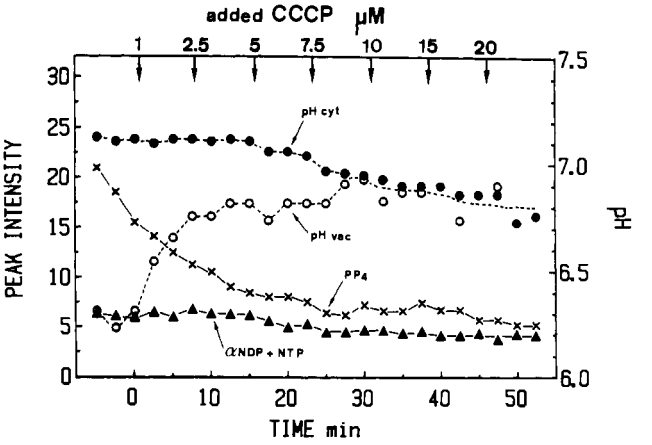

FIGURE 7: Time-dependent changes in pH compartments and phosphorylated compound levels upon CCCP additions during anaerobic metabolism of glucose. ^{31}P NMR spectra were recorded as described in the Materials and Methods section: 52 mg dry wt/mL, 750 scans, 2.5-min accumulation. Strictly anaerobic conditions were obtained by replacing bubbling O_2 by N_2 and by the addition of 0.25 mg/mL antimycin A in the presence of 250 mM glucose. pH compartments were determined as described in the Materials and Methods section, and peak intensities are expressed in relative areas. CCCP was added directly to the suspension as indicated by arrows. In order to avoid substrate lack, 250 mM glucose was added every 30 min. Symbols are the same as in Figure 4.

the external P_i concentration remained constant at 1.7 mM as determined by quantitative NMR and chemical analysis. Also it can be observed that poly(P) hydrolysis correlated with vacuolar pH change.

The effect of the substrate on the CCCP response of yeast cells was investigated by replacing lactate by 250 mM glucose. Results are shown in Figure 6. The external pH is lower than that in the presence of lactate, and the pH gradient between vacuole and cytosol is higher (ΔpH = 1.0 pH unit) due to the acidification of the vacuolar compartment (pHvac = 6.1). As observed with lactate, CCCP collapsed this pH gradient and induced a large hydrolysis of the poly(P) pool. Unlike the result in lactate, within the range of CCCP used (1–20 μM), the nucleotide levels and cytosolic pH remained quite constant at their basal value. It is worth noting that on cell suspensions supplemented with lactate and in the presence of 20 μM CCCP, 250 mM glucose addition reestablished both basal ΔpH across the plasma membrane and the basal nucleotide level (data not shown).

CCCP Titration in the Presence of Glucose under Anaerobic Conditions. In order to further document the role of glucose fermentation in maintaining a high level of nucleotide and pH gradients, cells were supplemented with 250 mM glucose and antimycin to block the respiratory chain, and the bubbling O_2 was replaced by N_2. Figure 7 shows the results of CCCP titration on cytosolic and vacuolar pH and phosphorylated metabolite pools. Cell behavior was similar to that observed during lactate feeding, i.e., loss of nucleotide pools, complete or partial collapse of the pH gradients across the vacuolar and plasma membranes, respectively. However, unlike with lactate or aerobic glucose feeding conditions, a large spontaneous poly(P) hydrolysis was observed. Very surprisingly, fermentation alone could not explain the behavior of the cells under aerobic glucose consumption, which strongly suggests the participation of uncoupled mitochondria in the energetic metabolism under these conditions.

Determination of External CCCP Concentration. It has been shown that CCCP uptake by the cells is very high, and free internal CCCP represents only about 1% of the total intracellular CCCP (Dupont et al., 1984). Under the experimental condition described in Figure 6, the CCCP concentration in the extracellular medium was measured at each steady state (data not shown). As expected, the extracellular CCCP concentration was low compared to the amounts of CCCP added, reflecting a high CCCP uptake. For 5 μM CCCP added, the amount of internal CCCP may be calculated to be equal to 47 μM (assuming that CCCP is not bound). For this amount of added CCCP, the respiratory rate reaches a maximum value (see Figure 5). However, it is well known that isolated mitochondria are fully uncoupled by 1 μM CCCP (Mazat et al., 1986). It is therefore clear that internal free CCCP represents only 2% of the total amount of CCCP contained by the cell, the greater part being associated with cellular membrane and/or proteins.

Measurement of TPP^+ Uptake. The proton gradient across the various membranes is a function of two components, the pH gradient across the membranes (ΔpH) and the electrical potential ($\Delta\Psi$). NMR is a useful technique for determining ΔpH but is not able to give any information on electrical potential. Estimation of this parameter in isolated mitochondria by the equilibrium distribution of a lipophilic cation as TPP^+ is now a well-established technique (Rottenberg, 1979). More recently, this method has been used to measure either both the cell membrane potential and mitochondrial membrane potential in intact mammalian cells (Berry et al., 1988) or only the plasma membrane potential in yeast (Borst-Pauwels, 1981; De la Pena et al., 1982; van de Mortel et al. 1988). In this latter case, the authors consider that this method gives the value of the plasma membrane potential ($\Delta\Psi_p$), assuming that the mitochondrial electrical potential ($\Delta\Psi_m$) is negligible. This assumption could be ruled out when yeast cells were grown on lactate which possessed well-differentiated mitochondria. We then measured TPP^+ uptake by yeast cells incubated in the presence of glucose under aerobic or anaerobic conditions. As shown in Figure 8, at equilibrium distribution of the probe, the cellular TPP^+ content in aerobic conditions was 1.55 μM, while the external TPP^+ concentration was only 0.5 μM. In anaerobiosis, the intracellular TPP^+ content was only 1.29 μM. These results suggest that mitochondrial $\Delta\Psi$ ($\Delta\Psi_m$) participates significantly in total TPP^+ uptake (see Figure 8).

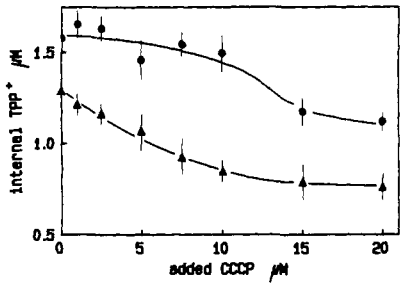

FIGURE 8: Changes in TPP^+ uptake upon CCCP additions during both aerobic and anaerobic metabolism of glucose. Radioactive internal TPP^+ concentration was determined in the presence of 0.5 μM external [^{3}H]TPP^+ as described in the Materials and Methods section. Cells (50 mg dry wt/mL) were incubated with 250 mM glucose either aerobically (●) or anaerobically in the presence of 0.25 mg/mL antimycin A (▲).

In oxygenated cells, CCCP titration induced a decrease in internal TPP^+ (from 1.55 to 1.15 μM). The fact that this value for a CCCP concentration up to 10 μM corresponds to the anaerobic control value (in the absence of CCCP) is in good agreement with the assumption that the decrease in internal TPP^+ is essentially due to mitochondrial uncoupling. The level of TPP^+ uptake for an external CCCP concentration up to 10 μM in aerobic conditions illustrates the relative maintenance of an electrical potential across the plasma membrane. In contrast, when the mitochondrial energetic metabolism was blocked, CCCP titration induced a TPP^+ efflux, leading to a concentration of 0.65 μM at 20 μM CCCP added, thus indicating an efficient dissipation of $\Delta\Psi_p$.

Discussion

Polyphosphate Metabolism. In *S. cerevisiae*, the poly(P) account for about 37% of the total phosphate content (Kulaev, 1979). Their subcellular localization has been described by several authors, and it has been shown that the most acid-soluble poly(P) detected by ^{31}P NMR are localized in the vacuoles (Urech et al., 1978; Wood & Clark, 1988). The observation that deficiency of vacuolar ATPase activity, either by bafilomycin A1 addition or by genetic modification, induced a total poly(P) hydrolysis is in agreement with the vacuolar NMR-detected poly(P) localization. Since both PP_1 and PP_2 resonances are found to be sensitive to pH change whereas PP_4 resonance is relatively insensitive (Mac Donald & Mazurek, 1987), the chemical shift difference has been proposed as a probe for vacuolar pH measurement (Lundberg et al., 1989). As other factors may modify the chemical shift of this polymer (for instance Mg^{2+} concentration), it is of interest to check the in vivo relationship between the chemical shift difference of poly(P) resonances and the vacuolar pH estimated by using the chemical shift of vacuolar P_i when visible. Indeed, using different experimental conditions including the modulation of vacuolar pH by adding various amounts of CCCP, this pH calibration curve proved to be valid, at least, between pH 6.0 and 7.1.

An interesting problem is the role of poly(P) in the energetic metabolism of the yeast cell. We report above that content of this polymer depends on many different factors, i.e., vacuolar ATPase activity, vacuolar pH, cell P_i concentration, and the energetic state of the cell. Our NMR experiments confirm that vacuolar acidification is directly controlled by the vacuolar H^+-ATPase activity [see also Banta et al. (1988), Umemoto et al. (1990), and Yamashiro et al. (1990)]. It has been reported that alkalinization of the vacuolar compartment induces an activation of phosphatases (Bostian et al., 1983; Greenfield et al., 1987) catalyzing the hydrolysis of poly(P). Whatever the mean by which vacuolar pH was modulated, we observed a good correlation between poly(P) hydrolysis and intracellular P_i accumulation, whereas the external P_i concentration remained nearly constant (data not shown). It must be noticed that, in contrast to bafilomycin, a CCCP addition sufficient to collapse vacuolar pH gradient does not induce a total poly(P) breakdown.

The poly(P) hydrolysis does not seem only controlled by the value of vacuolar pH. For instance, glucose addition induced a transient poly(P) hydrolysis, under aerobic conditions, corresponding to an increase in the phosphomonoester pool. This observation can be interpreted as a hydrolysis of the poly(P) pool, which is localized in the vacuolar compartment, partly in response to a fall in the P_i cytosolic content. The signal transduction between the compartments could be mediated by P_i transport through the vacuolar membrane. This interpretation is strengthened by the observation that poly(P) hydrolysis occurred only when the extracellular P_i pool was not maintained at a high level. It should be stressed that under these conditions, poly(P) hydrolysis occurred without a significant vacuolar pH change. Under aerobic conditions and with lactate or glucose, CCCP addition induced a significant poly(P) hydrolysis, with vacuolar pH reaching 6.5. An interesting observation is the transient hydrolysis of poly(P) upon CCCP addition. Time-dependent poly(P) hydrolysis is such that it is higher in the pre steady state than in the new steady state defined by the vacuolar pH and the P_i concentration. An increase in the vacuolar P_i concentration could negatively control poly(P) hydrolysis. Such an interpretation supposes that the efflux of P_i from the vacuole to the cytosol is rather low. In agreement with this, a relative independence of P_i distribution across the vacuolar membrane from the pH gradient has already been proposed in *Candida utilis* (Nicolay et al., 1982, 1983). It should be noted that, in our experiments, CCCP addition seemed to induce an increase in vacuolar P_i rather than cytosolic P_i (data not shown). In contrast with observations made under aerobic conditions, hydrolysis of poly(P) in yeast incubated with glucose in the absence of mitochondrial activity is spontaneous and does not necessitate CCCP addition (Figure 7), as already shown (den Hollander et al., 1981; Campbell-Burk et al., 1987). This hydrolysis is not correlated to vacuolar pH. The only difference that can be noticed in comparing the different situations is the low content of the NDP + NTP pool under these conditions.

Effect of CCCP on the Different Cell Compartments. It is well-known that CCCP, like other lipophilic weak acids, acts as a protonophore by increasing the proton conductance of biological membranes [for review, see Mac Laughlin et al. (1980)]. In *S. cerevisiae*, aerobic cell growth is completely inhibited for 16 μM CCCP when carbon sources are ethanol plus glycerol, whereas 70 μM CCCP is required to stop cell multiplication on glucose (Dupont et al., 1984). Such a difference may indicate that CCCP acts essentially as an uncoupler of oxidative phosphorylation. We report above that sensitivity of CCCP varies from one compartment to another. Our results indicate that the sensitivity of intracellular membranes (vacuolar and mitochondrial) is essentially higher than that of the plasma membrane. When glucose was used aerobically as carbon source, vacuolar ΔpH was suppressed for 7.5 μM CCCP (Figure 6); at this protonophore concentration, the internal TPP^+ concentration was still maximum (Figure 8), indicating that mitochondria were still energized. Mitochondrial uncoupling occurred between 10 and 15 μM CCCP, as shown by the internal TPP^+ decrease on glucose and also by the nucleotide drop under lactate conditions. In contrast,

the electrochemical potentials of both plasma membrane and nucleotide pools were unaffected by this CCCP concentration range.

The effect of CCCP depends on many factors: the pK of the chemical group from which the proton is dissociable (Wilson et al., 1971); the pH of the different aqueous phases (Wilson et al., 1971); the solubilities of both the protonated and the anionic forms in membranes (Wilson et al., 1971); the free concentration of the protonophore; and finally the size of the electrochemical proton gradient across the membrane. In addition, the uncoupling effect, i.e., the capacity of these molecules to dissipate the $\Delta\tilde{\mu}H^+$, depends also on the ability of the different processes generating this force, i.e., the capacity of proton pumps and their substrate supply. Indeed $\Delta\tilde{\mu}H^+$ depression is a direct function of the kinetic competition between an increase in proton conductance and the response of the proton pump involved. In this respect, the great sensitivity of the vacuolar compartment to CCCP under conditions in which the nucleotide pools are not modified suggests a poor stimulability of the proton ATPase of this membrane in response to an increase in proton leak.

On the other hand, in the presence of lactate and in the range of CCCP where the respiratory activity is stimulable (below 10 μM), protonophore addition modifies neither the trans-plasma membrane ΔpH nor the NDP + NTP content of the cell. In contrast, above this threshold value, an increase in proton conductance is not compensated by an increase in redox proton pump activities, so the energetic parameters fall. As previously described in mammals, as long as the respiratory chain is stimulable, the nucleotide level is maintained; this reflects a great sensitivity of the respiratory chain toward a change in the forces that it generates (Hassinen et al., 1990; Kingsley-Hickman et al., 1990). Therefore, the high stimulability of the mitochondrial redox proton pumps could explain the higher resistance of this organelle to CCCP than that of the vacuolar compartment.

The plasma membrane clearly appears to be the more resistant, since under aerobic conditions with glucose 20 μM CCCP did not modify the external cytosolic proton gradient. This observation is consistent with a high efficiency of the plasma membrane proton ATPase. However, when the energetic state, measured by the NTP + NDP content, drops after CCCP addition (lactate or glucose in anaerobiosis), the cytosolic pH does not fall below 6.7. Therefore, the trans-plasma membrane ΔpH depends on the external pH. The presence of such a residual ΔpH was already reported by Campbell-Burk et al. when yeast cells were incubated either in the presence of ethanol and antimycin or in that of glucose and iodoacetate (Campbell-Burk et al., 1987). Taking these results together, it can be deduced that the plasma membrane H^+-ATPase contributes only partially to the generation of ΔpH. It can be hypothesized that the residual ΔpH measured at low phosphate potential is due to the high buffering capacity of cytosol and to the acido-basic metabolic scalar reactions.

It must be pointed out that the evolutions of the three parameters measured, i.e., trans-plasma membrane ΔpH, TPP^+ accumulation, and nucleotide content, are strictly linked. As CCCP is a protonophoric uncoupler, it is widely accepted that oxidative phosphorylation is very sensitive to this compound. Indeed, cell growth on neoglucogenic substrates like lactate is more sensitive than growth on glucose (Dupont et al., 1984). This fact has been generally interpreted as an indication of a link between resistance to CCCP and the glycolytic pathway. The main observation reported in the present work is the same high sensitivity of cells to CCCP when the ATP-producing process is either only oxidative phosphorylation (lactate condition) or only fermentation (anaerobic glucose condition). However, the mechanism causing this CCCP sensitivity could be different. In effect, CCCP does not inhibit ATP production in anaerobic conditions, in contrast to aerobic conditions. However, this high sensitivity may be due to a low adaptability of the fermentation ATP-producing system in response to the protonophore-induced ATP hydrolysis increase. Of importance is the low apparent sensitivity to CCCP of the plasma membrane when cells are incubated aerobically with glucose, thus raising the question of the role of mitochondrial uncoupled respiration in maintaining the cellular ATP level in the presence of glucose. It is well-known that the glucose consumption rate is stimulated by a transition of yeast cells from aerobiosis to anaerobiosis [Gancedo & Serrano (1989), for a review]. We also observed this stimulation under our conditions, and this can be related to the level of the adenylic nucleotide pool (cf. Figures 6 and 7). However, CCCP at a concentration that completely uncouples the oxidative phosphorylation neither decreases the adenylic pool (Figure 6) nor stimulates the glucose consumption rate (data not shown). As expected, the size of the adenylic pool correlates with the glucose consumption rate. In contrast to other reports (Stickland, 1956), the Pasteur effect, defined by an inhibition of sugar consumption by respiration, appears to be more related to respiration per se than to oxidative phosphorylation. It is possible that uncoupled mitochondria are able to sustain an appreciable ATP synthesis rate by the substrate level phosphorylation pathway. It has been shown in isolated yeast mitochondria that substrate level phosphorylations are able to maintain a high internal phosphate potential (Rigoulet et al., 1985), provided that respiratory rate and transport processes are not limiting. Such a process could be partly responsible for the maintenance of the nucleotide pool and glycolysis control in the presence of glucose. Another possibility is that in anaerobiosis the cellular energetic efficiency is particularly low due to the presence of futile cycles. This is a priori possible with derepressed cells (this work) which contain a high level of neoglucogenic enzymes, if the inhibition of these cycles by certain effectors [e.g., fructose 2,6-bis(phosphate)] is not total under these conditions. It will therefore be of interest to measure the effect of uncoupled respiration on the level of metabolites likely to be involved in futile cycles. It is worth noting that the glucose consumption rate may be controlled not only by the rate of ATP synthesis (in the presence of absence of oxidative phosphorylation) but also by the energy-dissipating pathways which probably differ from one steady state to another.

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Registry No. CCCP, 555-60-2.

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