Respiratory oscillations in yeast: clock-driven mitochondrial cycles of energization

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Abstract Respiratory oscillations in continuous yeast cultures can be accounted for by cyclic energization of mitochondria, dictated by the demands of a temperature-compensated ultradian clock with a period of 50 min. Inner mitochondrial membranes show both ultrastructural modifications and electrochemical potential changes. Electron transport components (NADH and cytochromes *c* and *c* oxidase) show redox state changes as the organisms cycle between their energized and de-energized phases. These regular cycles are transiently perturbed by uncouplers of energy conservation, with amplitudes more affected than period; that the characteristic period is restored after only one prolonged cycle, indicates that mitochondrial energy generation is not part of the clock mechanism itself, but is responding to energetic requirement.

Key words: Mitochondria; Redox regulation; NADH; Cytochrome; Ultradian clock; Yeast

1. Introduction

The time-frame for intracellular processes is provided by biological clocks that act on appropriate time-scales [1]; elucidation of mechanisms that keep circadian time [2] is further developed than that for shorter-period clocks [3–5]. Continuous cultures often show oscillatory dynamics, even when constant conditions are rigorously maintained [5,6]. The most conveniently monitored on-line observable output is usually the oxygen dissolved in the culture medium. Continuous cultures of *Saccharomyces cerevisiae* show autonomous oscillations in respiration that can be monitored continuously over extended experiments (months) by direct measurement of dissolved O_2 [7]. At 30°C, the period of this ultradian rhythm is 50 min, and it hardly changes over a temperature range from 27 to 34°C ($Q_{10} = 1.07$)[8]. This is one output of many, driven by an ultradian clock; others include intracellular pH [7], sulfate uptake [9], ethanol production [10], and intracellular redox state as indicated by assays of glutathione [11]. It has been proposed that in the system described here, synchronization of metabolic events, but not the processes of the cell division cycle, within the population is mediated by periodic release of H_2S [12], or other volatile messenger substances, e.g. acetaldehyde [13]. The respiratory oscillations are not related to a glycolytic oscillatory state, as they also occur in ethanol-grown cultures [10]. The aim of the present work was to investigate mitochondrial function during the oscillatory behavior.

2. Materials and methods

2.1. Organisms and culture

S. cerevisiae strain IFO 0233 was used. The medium consisted of glucose monohydrate (22 g dm^{-3}), $(NH_4)_2SO_4$ (5 g dm^{-3}), KH_2PO_4 (2 g dm^{-3}), $MgSO_4{\cdot}7H_2O$ (0.5 g dm^{-3}), $CaCl_2{\cdot}2H_2O$ (0.1 g dm^{-3}), $FeSO_4{\cdot}7H_2O$ (0.02 g dm^{-3}), $ZnSO_4{\cdot}7H_2O$ (0.01 g dm^{-3}), $CuSO_4{\cdot}5H_2O$ (0.005 g dm^{-3}), $MnCl_2{\cdot}4H_2O$ (0.001 g dm^{-3}), yeast extract (Difco; 1 g dm^{-3}), and 70% (v/v) H_2SO_4 (1 cm^3 added dm^{-3} of medium). Sigma Antifoam A was used at 1 cm^3 dm^{-3}. Continuous culture was carried out as described previously [7]. The fermenter (LH500, Adaptive Biosystems) was operated with a stirrer rate of 900 rpm, a working volume of 0.8 dm^3, an air flow rate of 180 cm^3 min^{-1} and a dilution rate of 0.085 h^{-1}. The temperature was 30°C and the pH was controlled at 3.4 by the addition of 2.5 M NaOH. Dissolved O_2 was monitored continuously in the culture using an Ingold O_2 electrode. Under these conditions the supply of O_2 did not limit growth, as it could be shown that increased O_2 supply did not increase the cell numbers. Furthermore, the cytochrome *c* oxidase was shown to be partially oxidized as shown by determinations of its redox state in sample cells taken directly from the culture and examined by difference spectrometry at 77 K. Experiments were conducted in continuous light. Uncouplers, *m*-chlorocarbonylcyanide phenylhydrazone (CCCP) and 5-chloro-*t*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13), were added as solutions in ethanol.

2.2. Cytochrome spectra and nicotinamide nucleotide redox state

Samples at high and low dissolved oxygen tension (DOT; 3.8 ml culture) were centrifuged at $1000 \times g$ for 2 min in a MSE Minor centrifuge using haematocrit tubes. Resuspension was to a volume of 0.2 ml in 1.0 M mannitol. Reduced-oxidized cytochrome spectra were obtained at 77 K in an SP1800 spectrophotometer, using Perspex cuvettes with a light path of 5 or 2 mm. The oxidized sample was aerated and the reduced sample was kept anaerobic immediately before freezing. Samples were thawed and re-equilibrated to 77 K. Spectra were obtained at a band-width of 2.0 nm and with a scan rate of 2 nm s^{-1} over a spectral range of 500–620 nm.

The redox state of the cellular NAD(P)H/$NAD(P)^+$ couple was monitored continuously in the culture [14] using a fluorimeter fitted with a Corning filter for excitation (366 ± 20 nm) and a Wratten 450 ± 30 nm filter for emission and using a 100 W short-arc Hg lamp.

3. Results

Fig. 1a shows a typical section of the continuously monitored output from the O_2 electrode immersed in the culture. A stable high-amplitude waveform in this case accounted for the observed cyclic variation between 80 and 170 μM O_2. Direct fluorescence monitoring of the culture [14], for intracellular

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Abbreviations: DOT, dissolved oxygen tension

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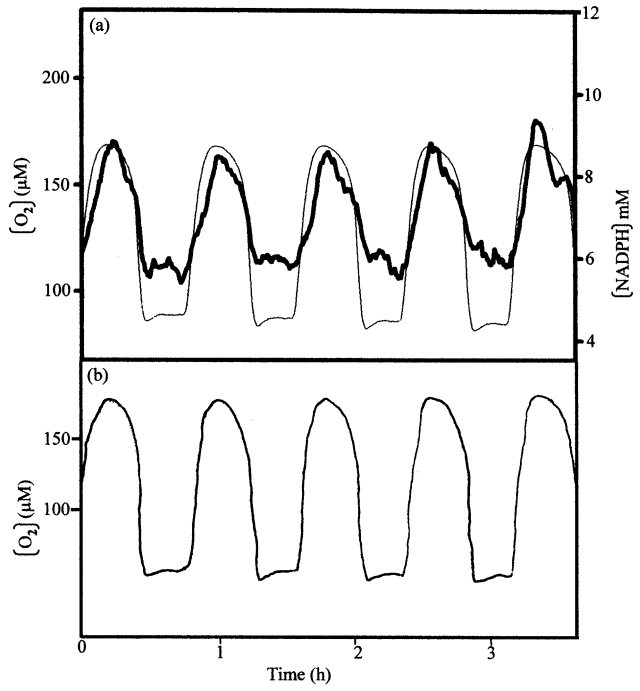

Fig. 1. Dissolved O_2 and reduced nicotinamide nucleotide fluorescence in a continuous culture of *S. cerevisiae*. a: Simultaneous O_2 electrode and fluorimetric monitoring (366 → 450 nm) in the same culture: fluorescence emission was calibrated by nicotinamide nucleotide extraction from the culture. b: Control culture (no UV irradiation).

pools of reduced nicotinamide nucleotides (excitation at 366 nm, emission at 450 nm) indicated that the reduction state of this key indicator of intracellular redox balance also showed oscillations with a period identical with those of respiratory activity measured simultaneously in the same culture. In the control culture (UV irradiation switched off; Fig. 1b) the amplitude and period of the respiratory oscillations were identical to those in a culture during NAD(P)H monitoring. Maximum NAD(P)H reduction occurred just after the times when respiratory rate was minimal (i.e. when dissolved O_2 reached its maxima). Changes in cellular ultrastructure that accompany the changing demands for O_2 were seen in electron micrographs of thin sections of organisms, rapidly fixed at high and at low dissolved O_2 (not shown). At high dissolved O_2, when yeast respiration is at a low rate, mitochondrial sectional areas account for a small proportion of total cell areas and the cristae of the inner mitochondrial membranes are indistinct. At low dissolved O_2, when the respiration is high, mitochondria appeared larger, the cristae are numerous and clearly defined. These extreme conformational states correspond to those originally described for liver mitochondria by Hackenbrock [15] as 'orthodox' and 'condensed' respectively. In the orthodox state there was a relatively large matrix volume with the inner-boundary membrane closely apposed to the outer membrane, with only a very small space between them. In the condensed form, the inner membrane was pulled away from the outer membrane and the cristae became more clearly defined.

Further evidence for changes in mitochondrial function are provided by a study of the cytochrome components of the respiratory chain. Fig. 2a shows the difference spectrum at 77 K (Na dithionite reduced *minus* ammonium persulfate oxidized) of yeasts rapidly harvested when dissolved O_2 was at a maximum. Mitochondrial cytochromes *c*, c_1, *b*, and *c* oxidase are indicated by their α-absorption maxima at 548.5, 554, 559 and 601 nm respectively. Organisms obtained from cultures when dissolved O_2 was at a minimum (Fig. 2b) showed an identical difference spectrum. This shows that the total mitochondrial cytochrome contents are unchanged during the respiratory oscillation.

The effects of addition of an uncoupler of energy conservation on the respiratory oscillations in the continuous culture

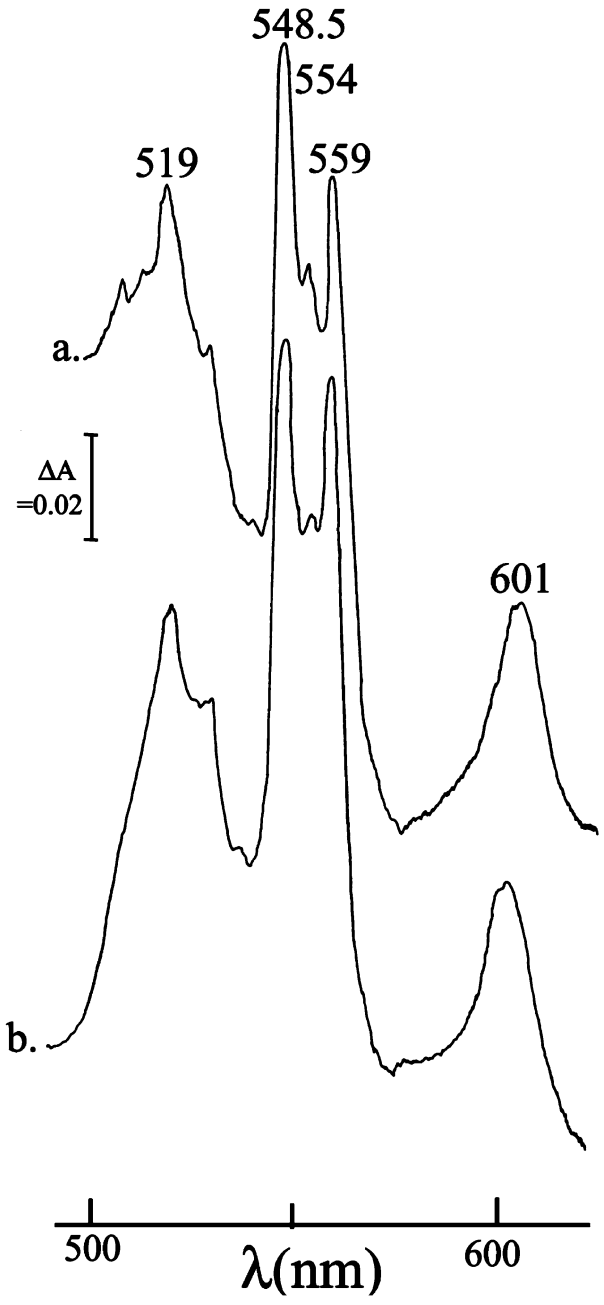

Fig. 2. Absorption difference spectra of samples at 77 K. a: Na dithionite reduced *minus* ammonium persulfate oxidized difference spectrum of *S. cerevisiae* harvested at a maximum of dissolved O_2, and (b) sample taken at a minimum of dissolved O_2

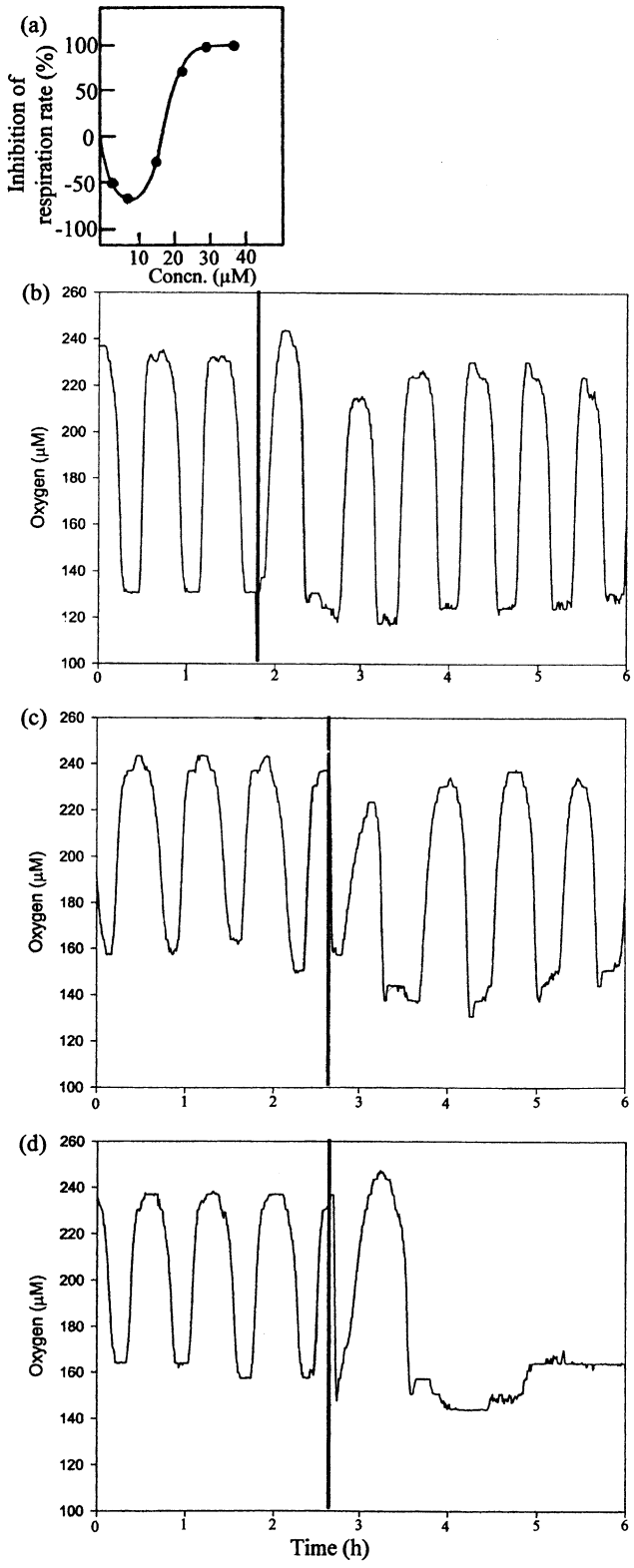

Fig. 3. Effects of CCCP, an uncoupler of energy conservation, on continuous cultures of *S. cerevisiae* showing respiratory oscillations. a: Concentration dependence. Final concentrations (b,c) 5 μM, (d) 10 μM: additions were made at the solid lines.

are shown in Fig. 3; at sub-optimal uncoupling concentrations (Fig. 3a) CCCP (5 μM), added after respiration reached a maximum, gave an immediate effect on the amplitude of the changes in dissolved O_2. This was to increase the rates of respiration both at low and high dissolved O_2 (Fig. 3b). However, at this low concentration, the period was only transiently affected, with a lengthening of the first cycle after uncoupler addition. Addition at a respiratory minimum produces similar effects (Fig. 3c). Recovery of the system thus occurred in two stages; more than five cycles elapsed before the levels of O_2 in the culture returned to normal, although the period length was restored to the normal value much more rapidly. At a higher concentration (10 μM) this uncoupler produced much more evident and clear uncoupling with DOT remaining low (i.e. the respiration was greatly accelerated) as well as other pronounced perturbative effects (Fig. 3d). In this case the period of the next cycle was greatly increased (to 60 min), and the respiration of the culture remained at high levels for more than 5 h during which no oscillation was observed. Recovery to normal amplitudes (not shown) took more than 20 h, although oscillations were again restored before this, albeit with greatly diminished amplitude. Similar results (not shown) were also obtained when another uncoupler of energy conservation, S-13, was employed.

4. Discussion

These data indicate that the mechanism of respiratory oscillation in yeast growing in continuous culture involves cycles of energization and de-energization of mitochondria. Temperature compensation of the period [8,17,18] as well as sensitivity to Li^+ [19,20] strongly suggests, as in circadian timekeepers, connection to a clock output. This is analogous to that situation described previously in cell-division-synchronized cultures of the soil amoeba *Acanthamoeba castellanii*, where in vivo mitochondrial respiratory control was observed [21,22] (i.e. control of respiratory rate by the availability of ADP [16]). As in that case [23], where the period of the oscillations was almost unchanged over a 10°C temperature range, observation of the ultradian clock output driving the respiratory oscillation in yeast requires synchrony between organisms for its detection in the population. However, here, synchronization of the population is metabolically rather than cell cycle mediated. That the oscillatory dynamics are slow by comparison with many of the characteristic energy transformations in mitochondria indicates that the cycles observed are dictated by the slower time-scales of the energy-requiring processes of biosynthesis, so that what we observe are ultradian clock-driven cycles of energy demand.

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