NO^+, but not $NO^\bullet$, inhibits respiratory oscillations in ethanol-grown chemostat cultures of *Saccharomyces cerevisiae*

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Abstract A continuous culture of *Saccharomyces cerevisiae* strain IFO 0233 growing aerobically at pH 3.4 shows persistent high-amplitude respiratory oscillations with a period of about 45 min. These robust autonomous cycles are accompanied by changes of product accumulation (acetaldehyde and acetic acid), intracellular pH, and intracellular redox state, as indicated by continuously monitored NADH fluorescence and the glutathione content of cell-free extracts. Perturbation of the oscillation of dissolved O_2 was produced on addition of 100 μM glutathione, >10 nM Na nitroprusside, 8 μM $NaNO_2$, or 10 μM *S*-nitrosoglutathione. NO gas, putative $NO^\bullet$-releasing agents, or an inhibitor of NO synthase were ineffective. We suggest that nitrosation by NO^+ of a component of a redox switch can account for these data, and we emphasise the different modes of action of the different redox forms of nitrogen monoxide.

Key words: Nitric oxide; Nitrosation reaction; Redox switch; Oscillation; *Saccharomyces cerevisiae*

1. Introduction

An understanding of the pervasive diversity of nitric oxide-mediated effects and functions in biological systems [1] demands rigorous distinction between its inter-related redox forms: the nitrosonium cation (NO^+), the nitrogen monoxide radical ($NO^\bullet$), and the nitroxyl anion (NO^-) [2,3]. The iron-nitrosyl complex, Na nitroprusside ($Na_2[Fe(CN)_5NO]$) has a strong NO^+ character, and nitrosation reactions dominate its chemistry [4]. Other nitrosating agents include $NaNO_2$ [5] and *S*-nitrosoglutathione (SNOG) [6]. Here we show that an autonomously oscillating respiratory system that involves NAD(P)H and glutathione cycling in yeast [7,8] is exquisitely sensitive to perturbation in the dark by Na nitroprusside (minimal effective concentration, 10 nM). $NaNO_2$ and SNOG were also active, whereas injections of NO gas, putative $NO^\bullet$-releasing agents, or an inhibitor of NO synthase were without effect. We suggest that nitrosation by NO^+ interferes with a redox-sensing switch [9] responsible for generation of the oscillations of respiration.

2. Methods

2.1. Organism and culture conditions

Saccharomyces cerevisiae strain IFO 0233, an aneuploid wild-type selected for both ethanol and low pH values, was grown in chemostat culture with 340 mM (2%, v/v) ethanol as carbon and energy source. Under these conditions this strain exhibits a robust respiratory oscillation (Fig. 1a). The optimal conditions necessary for the production and maintenance of sustained oscillatory dynamics are [10]: (i) batch culture grown at 30°C through a prolonged stationary phase (12 h) until all the carbon sources were depleted (dissolved O_2 attains air-saturated level) before setting dilution rate at 0.085 h^{-1}, (ii) pH control at 3.4, (iii) O_2 supplied by aeration at 180 ml/l and with impeller rotation at 800 rpm. Experiments in the dark were performed in a fermenter spray painted matt black. The autonomous oscillation could be maintained indefinitely, but elevation of the pH to 4.4, decrease of the growth temperature to 25°C, or either increased or decreased air supply rates, are all conditions leading to attenuation of the oscillations.

2.2. Monitoring of NAD(P)H fluorescence

The probe used was that designed [11] and supplied by Mr Norman Graham, Biomedical Instrumentation Group, Department of Biochemistry and Biophysics, University of Pennsylvania, PA, USA.

2.3. Metabolite assays

Ethanol, acetaldehyde and acetic acid in the growth medium were analysed by HPLC using a Shodex SH101 column (Showa Denko, Japan). Intracellular acetate was determined (Boehringer, Japan) by difference between that in perchloric acid extracts and culture filtrates: intracellular pH was calculated from these data [12]. Glutathione, the major intracellular thiol in yeast, was assayed by reaction with Ellman's reagent after cell disruption by three freeze-thaw cycles [13].

3. Results

Fig. 1a shows high-amplitude oscillations in dissolved O_2, with maxima at 70% air saturation and minima at 40% (corresponding to 165 and 95 μM O_2 respectively). The waveform of the 46.5 min cycle was characteristic; the accelerating phase of O_2 consumption decreased dissolved O_2 to 50% saturation before slowing down to a rate where supply just satisfied demand. Then, further decreased respiratory activity was accompanied by a phase of rapidly increasing liquid phase O_2 content. These cycles of changing O_2 consumption were accompanied by an oscillation in the ethanol content of the culture. Timings of maxima and minima of ethanol concentration occurred 4 min after the onset and cessation of rapid O_2 consumption; ethanol decreased from 130.5 mM to 124.5 mM. Thus the peak-trough amplitude represents a variation of 4.6% of the peak ethanol concentration.

Product concentrations also showed high-amplitude oscillations during this cycle but in antiphase with ethanol. Both acetaldehyde (Fig. 1b) and acetic acid (Fig. 1b) accumulated as ethanol was consumed; maximal extracellular concentrations attained were 920 and 180 μM respectively. At their minima, the concentrations of these excreted metabolites were decreased to 350 μM and 0–10 μM respectively. Intracellular acetate (Fig. 1c) oscillated between 24 and 42 μM with phases of increase 5 min in advance of those of acetate in the growth medium. Intracellular pH values, calculated from

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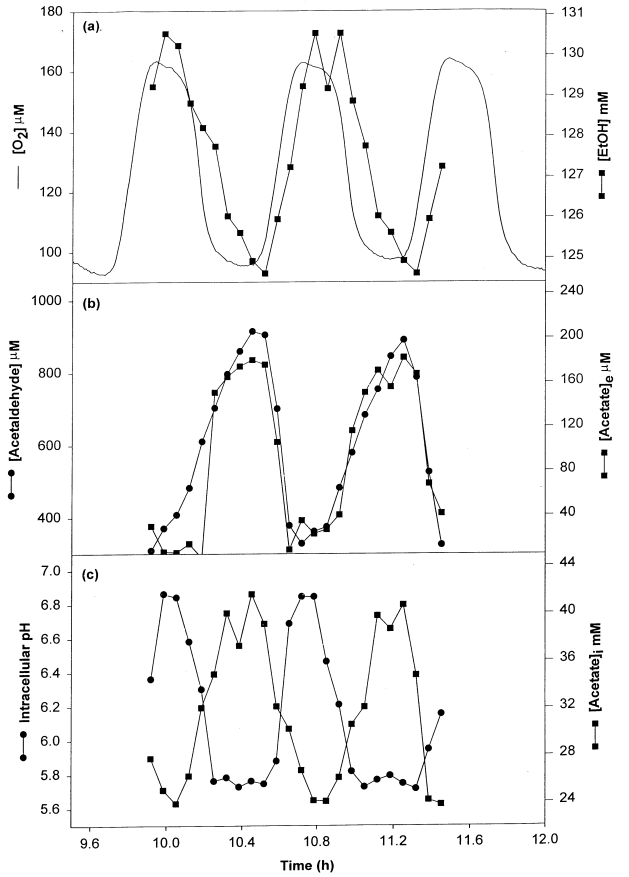

Fig. 1. Respiratory oscillations in an ethanol-grown chemostat culture of *Saccharomyces cerevisiae* IFO 0233. a: Dissolved O_2 measured continuously on-line (Orion polarographic electrode) and ethanol. b: Acetaldehyde and extracellular acetic acid. c: Intracellular pH values and intracellular acetate.

these data on acetate distributions, cycled from pH 6.9 to 5.75, exactly in phase with dissolved O_2 (Fig. 1c).

Two independent indicators of intracellular redox state were assessed during the oscillations (Fig. 2). The redox state of the NAD(P)/NAD(P)H couple was monitored continuously on-line by measurement of fluorescence (365→450 nm). Maximum NAD(P)H fluorescence was observed in each cycle as respiration slowed to its minimum (i.e. as dissolved O_2 reached a maximum). Fluorescence then decreased as respiration increased to its maximum rate. Recovery of NAD(P)H occurred in two stages as respiratory chain activity slowed, so that a secondary maximum of fluorescence was recorded between peaks of dissolved O_2. Maximum glutathione level corresponded with maximum respiration rate. Minimal glutathione level occurred as dissolved O_2 started to increase.

Redox perturbation was achieved by addition of 100 μM glutathione (Fig. 3a). Irrespective of when in the cycle injection was performed, O_2 consumption was immediately inhibited. Measurements of intracellular glutathione indicated that uptake had occurred. Consequently, dissolved O_2 increased to levels never observed in the unamended culture. The duration of respiratory inhibition was dose-dependent, lasting as long as three oscillatory periods: depletion of intracellular glutathione accompanied this phase. Recovery was in stages, with episodic restoration of respiration. Full restoration of the stable oscillation was preceded by cycles characterised by long periods.

Addition of Na nitroprusside in the dark showed an effect similar to that produced by glutathione (Fig. 3b), in that respiration was immediately inhibited and then showed a complex time course of recovery, with long-lasting effects on period and amplitude of the oscillations. Again dose dependence was established. An effect was obtained with as little as 10 nM nitroprusside. Other nitrosating agents (8 μM $NaNO_2$ and 10 μM *S*-nitrosoglutathione, Fig. 3c,d) also gave perturbations.

For $NaNO_2$ respiratory inhibition was delayed, so as to become evident at the next minimum of dissolved O_2 (Fig. 3c). The oscillation was thereby shifted to higher values of minima and maxima dissolved O_2 through four cycles with increased period and decreased amplitude. Recovery occurred with gradually increasing amplitude after loss of the oscillatory state for more than 2 h. Addition of 10 μM *S*-nitrosoglutathione (Fig. 3d) gave yet another different response. In the first cycle, respiratory inhibition, allowing dissolved O_2 to increase above its usual maximal level, was followed by an overshoot that drove the O_2 concentrations below those observed in previous cycles. The oscillatory state was thereafter characterised by a complex waveform which suggested interaction with a lower frequency. Recovery (not shown) to the normal waveshape was not complete until >12 h after addition of the nitrosation agent. These different perturbation effects suggest that the modes of action of the three agents are not the same; possible reaction mechanisms have previously been discussed [5,6]. We stress that for nitroprusside or nitrite these are largely independent of $NO^{\bullet}$ release [14–17]. Under physiological conditions, *S*-nitrosoglutathione can act as NO^-, as well as NO^+ and $NO^{\bullet}$ donor [6,18]. A specific inhibitor of NO synthase (N^G-nitro-L-arginine-methyl ester, L-NAME) [19], NO gas (final concentration 30 nM) or putative

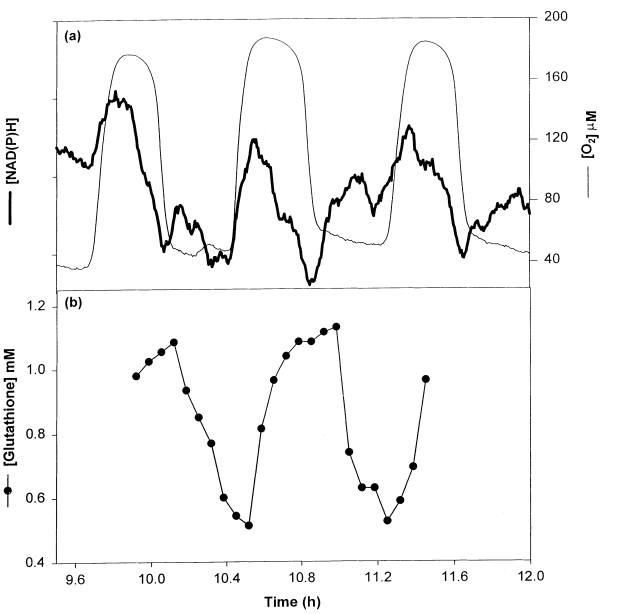

Fig. 2. Respiratory oscillations and the changing intracellular redox states of yeast. a: Dissolved O_2 and NAD(P)H fluorescence (365→450 nm) measured continuously on-line. b: Total intracellular glutathione.

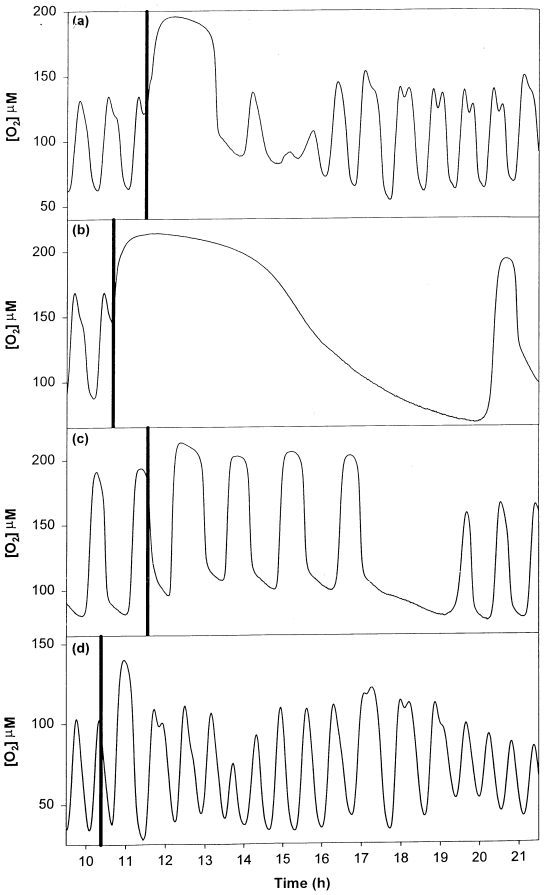

Fig. 3. Perturbation of respiratory oscillations in a yeast culture. Continuous traces of dissolved O_2 are shown; vertical bars indicate time of addition of (a) 100 μM glutathione, (b) 5 μM Na nitroprusside, (c) 8 μM $NaNO_2$, (d) 10 μM *S*-nitrosoglutathione.

NO•-releasing agents [20] (L-arginine, up to 1 mM, NOR1 (up to 8 μM), or 1-OH-2-oxo-3,3-bis-(3-NH_2-ethyl) triazine, NOC 18 (at up to 7.6 μM) were all without effect on the oscillations. 3-Morpholino-sydnomide (SIN-1), a compound successively producing both NO and $O_2^{-\bullet}$, and hence possibly the peroxynitrite anion ($ONOO^-$) [21], was also without effect at concentrations up to 15.5 μM.

4. Discussion

In the dark, and under physiological conditions, Na nitroprusside does not spontaneously release NO• since its stability constant is very high [2]; irradiation with visible light is necessary for NO• release [22]. It is best regarded as a NO^+-metal adduct [6]. In the dark, this compound acts as an excellent nitrosating agent; transfer of NO^+ from the nitrosyl complex to specific targets is its suggested mode of action. The favoured chemistry of this highly reactive nitrosonium cation species is characterised by addition and substitution reactions with nucleophiles (electron-rich bases and aromatics). At pH 7, nitrite is a rather ineffective nitrosylating agent (only 0.022% present as HNO_2); increased protonation (at pH 3.4 in the experiments described here) increase its effectiveness [23]. *S*-Nitrosothiols predominate over other reaction products under physiological conditions [24]. Metalloproteins, such as aconitase, are also important potential targets, especially at acid pH, where nitrosylation of the [4Fe-4S] centre is favoured [25]. That the effects of Na nitroprusside reported here occur in the dark makes it unlikely that NO• inhibition of mitochondrial cytochrome *c* oxidase is the mechanism of perturbation of respiration [26]. Interference with redox signalling at a thiol or metal target is likely. Further investigations will provide new insights into the control of intracellular redox levels [27], redox switching by transcriptional activation [28], and autonomously oscillatory respiratory activity in yeast.

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