Involvement of glutathione in the regulation of respiratory oscillation during a continuous culture of *Saccharomyces cerevisiae*

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Respiratory oscillation occurred during aerobic continuous culture of *Saccharomyces cerevisiae*. During oscillation, phase-related changes in NAD(P)H and GSH levels occur. Perturbation of oscillation and inhibition of respiration occurred when GSH or GSSG was injected; however, there was a phase delay in perturbation in the case of an injection during high respiration. The perturbation phase delay was not apparent when a combination of DL-buthionine-(S,R)-sulphoximine, GSH and 5-nitro-2-furaldehyde was injected. Perturbation by GSH injection caused the intracellular GSH concentration to increase, the GSSG concentration to decrease and the cessation of ethanol uptake. NAD(P)H during perturbation was inversely related to dissolved oxygen. Perturbation by calcium pantothenate and pyridoxal-HCl caused a period of enhanced respiration before oscillation returned. These results suggest that the NAD⁺/NADH redox is not directly involved in oscillation control and regulation involves glutathione metabolism. Possible regulation points include alcohol dehydrogenase inhibition and/or respiratory-chain inhibition.

Keywords: Oscillation, redox regulation, glutathione, NADH, yeast

INTRODUCTION

Biological oscillations are intrinsic to all living systems, and have been studied over a wide temporal range (Hess & Boiteux, 1971; Lloyd & Rossi, 1992). Oscillations provide useful systems for the elucidation of biological control mechanisms *in vivo* (Friesen *et al.*, 1993; Lloyd, 1998). We have found a robust autonomous respiratory oscillation ($\tau = 30\text{--}120$ min) in *Saccharomyces cerevisiae* grown under continuous aerobic culture conditions (Satroutdinov *et al.*, 1992). The oscillation occurs independently of glycolysis and the cell cycle (Keulers *et al.*, 1996a), and no difference in oscillation was seen in light or darkness (Murray *et al.*, 1998). The oscillation is dependent on pH (Satroutdinov *et al.*, 1992), aeration (Keulers *et al.*, 1996b) and carbon dioxide (Keulers *et al.*, 1996b). Oscillation occurs when glucose, ethanol or acetaldehyde is used as a carbon source (Keulers & Kuriyama, 1998). In our experience, for the oscillation to occur ethanol has to be present. It has been suggested that a stage of ethanol metabolism may be involved in

both population synchrony and intracellular regulation giving oscillatory dynamics (Keulers *et al.*, 1996a).

During oscillation, calculated metabolic fluxes through the tricarboxylic acid cycle and oxygen uptake are closely coupled (Satroutdinov *et al.*, 1992). However, studies using the effectors of our system, i.e. ethanol (Keulers *et al.*, 1996a), acetaldehyde (Keulers & Kuriyama, 1998), O₂ and CO₂ (Keulers *et al.*, 1996b) did not reveal either a mechanism for synchronisation or regulation. Individually, the effectors did not meet the requirements of a synchronisation agent (Richard *et al.*, 1996), i.e. the substance must be emitted, sensed and dissipated. It should also oscillate and produce phase shifts when introduced at above or below its normal oscillating concentration (Winfree, 1990). It was suggested that the intracellular redox state plays an important role in the regulation of oscillation and it was shown that NAD(P)H fluorescence oscillates in phase with dissolved oxygen (Murray *et al.*, 1998).

Respiratory oscillation can be characterized on the basis of many metabolic variables. When cultures were grown on ethanol media, GSH oscillated about 20° in advance of dissolved oxygen and in phase with ethanol (Murray

Abbreviations: BSO, DL-buthionine-(S,R)-sulphoximine; NF, 5-nitro-2-furaldehyde.

et al., 1998). Acetaldehyde and acetate oscillated 180° out of phase with dissolved oxygen. As ethanol and GSH accumulate, acetaldehyde and acetate are quickly utilized, and as ethanol and glutathione start to be consumed, acetaldehyde and acetate increase. These observations of phase relationships provided evidence of a close link between glutathione metabolism and ethanol metabolism during oscillation.

Recently, it was found that pulse addition of nanomolar concentrations of sodium nitroprusside, a source of NO⁺, perturbed oscillation by inhibiting respiration, whereas the pulse addition of reagents that generate NO[•] did not (Murray *et al.*, 1998). A similar perturbation of oscillation occurred when GSH was injected, leading to the suggestion that redox sensing (Rouault & Klausner, 1996) might be involved. GSH is synthesized from glutamate, cysteine and glycine. In yeast, the regulation of cytosolic and intramitochondrial redox balances are achieved by alteration of the ratios of GSH to its oxidized dimer GSSG (Gilbert, 1995; Penninckx & Elskens, 1993). This redox balance is vital for correct protein folding (Raina & Missiakas, 1997), transcriptional regulation (Demple, 1998) and protection against reactive oxygen species (Meister, 1995a). These mechanisms of thiol:dithiol switching are highly conserved in nature.

In this paper we report an investigation of the role of redox cycling in the regulation of the respiratory oscillation. It is shown that glutathione redox balance rather than the redox state of the NAD⁺/NADH couple is involved in oscillation regulation.

METHODS

Strain and culture conditions. The *S. cerevisiae* strain used in this study was the polyploid strain IFO 0233 (IFO, Institute of Fermentation, Osaka, Japan). Fermentation was carried out as previously described (Keulers *et al.*, 1996a, b). All fermentations used ethanol as the carbon source (Keulers *et al.*, 1996a).

Sample preparation, instrumentation and analytical techniques. Acid-stable metabolites, e.g. NAD⁺, GSH and GSSG, were extracted from cell samples (1 ml) using perchloric acid (0.25 ml; 3 M) followed by three freeze (−70 °C)/thaw (4 °C) cycles. Samples were centrifuged (15000 g; 5 min) and the supernatant was neutralized to pH 7.8 with tri-potassium phosphate (2 M). The samples were centrifuged (15000 g; 5 min) and analysed.

Alkaline-stable metabolites, e.g. NADH, were extracted from cell samples (1 ml) using potassium hydroxide (2 M)/methanol (70%, v/v) mix (4 °C; 0.5 ml) followed by three freeze (−70 °C)/thaw (4 °C) cycles. Samples were centrifuged (15000 g; 5 min) and before analysis, the supernatant was brought to pH 6.6 with triethanolamine (2 M).

The same instrumentation and analytical techniques were used as previously described (Keulers *et al.*, 1994, 1996a; Satroutdinov *et al.*, 1992). All enzyme assays were modified for a microtitre-plate reader and carried out in triplicate (Ashour *et al.*, 1987).

NAD⁺, NADH, NADPH and NAD[•] were determined enzymically (Bergmeyer & Braßl, 1984). GSSG and GSH were determined enzymically (Baker *et al.*, 1990). The intracellular

glutathione redox potential was calculated from levels of GSH and GSSG using the Nernst equation (Hwang *et al.*, 1995):

$$E = E_0 + 2.303 \frac{kT}{nF} \log \frac{[\text{GSH}]^2}{[\text{GSSG}]}$$

where E_0 is the standard potential of glutathione (−0.24 V), k is Boltzmann's constant (8.31 J mol^{−1} K^{−1}), T is the absolute temperature (303 K), n is the number of electrons transferred (2) and F is the Faraday constant (96406 J V^{−1}). Intracellular concentrations of metabolites from extracted cells, were calculated assuming that cytosolic volume was 1.6 cm³ (g dry cell wt)^{−1} (Pampulha & Loureiro-Dias, 1989).

NAD(P)H fluorescence. NAD(P)H fluorescence was measured using a fibre-optic probe supplied by the Biomedical Instrumentation Group of the Department of Biochemistry and Biophysics, University of Pennsylvania (Chance *et al.*, 1975). Fluorescence and reflectance data were normalized then smoothed exponentially (factor 0.90) using Microsoft Excel 97. NAD(P)H fluorescence was calculated by subtraction of normalized reflectance from fluorescence data.

RESULTS

Respiratory oscillation and nicotinamide-nucleotide redox state

The redox state of the NAD⁺/NADH couple could be a possible regulator of oscillation. NAD(P)H fluorescence monitored continuously during oscillation gave a complex output (Fig. 1), compared to dissolved oxygen oscillation. At the beginning of the phase of diminished respiration, a transient rapid increase in NAD(P)H fluorescence was observed, which reached a maximum just before the dissolved oxygen maximum. The NAD(P)H fluorescence then decreased, reaching a minimum just before the dissolved oxygen minimum. When respiration approached a maximum, NAD(P)H fluorescence increased and then oscillated at a higher frequency ($\tau \approx 5$ min). NADPH was below the level of detection [$< 1.75 \mu\text{mol}$ (g dry cell weight)^{−1}], so fluorescence changes were due almost entirely to NADH. Enzymic determination of NAD⁺ and NADH from cell-free extracts obtained every 4 min gave values for

Fig. 1. Dissolved oxygen oscillation (thick line) and NAD(P)H fluorescence (fine line) (365–450 nm) during continuous aerobic yeast culture.

Fig. 2. Perturbation of dissolved oxygen oscillation during continuous aerobic yeast culture, with GSH (50 μM) (a), GSSG (25 μM) (b) and NF (50 μM) (c). The thick lines represent injection at high dissolved oxygen, the fine lines represent injection at low dissolved oxygen and the vertical bars represent the time of addition.

intracellular concentrations ranging from 0.6 to 0.9 mM for NADH and 2.5 to 4 mM for NAD⁺. However, characterization of the rapid dynamics of nicotinamide pools requires more frequent measurements.

Perturbation by addition of glutathione

Injection of GSH (50 μM) at maximal dissolved oxygen (Fig. 2a) gave inhibition of respiration and a transient cessation of oscillation. An injection at low dissolved oxygen produced similar effects; however, these were delayed until the dissolved oxygen was at a maximum,

Fig. 3. Perturbation of dissolved oxygen oscillation during continuous aerobic yeast culture with BSO (50 μM), GSH (50 μM) and NF (50 μM). The thick line represents injection at high dissolved oxygen, the thin line represents injection at low dissolved oxygen and the vertical bars represent the time of addition.

suggesting that oscillation was only sensitive to increased GSH levels during periods of lowered respiration. There was a secondary peak of dissolved oxygen 20 min after injection, suggesting that there may be at least two stages that are sensitive to addition of GSH. Injection of GSSG (25 μM) at high or low dissolved oxygen (Fig. 2b) also gave inhibition of respiration for the first cycle and increased periods of the subsequent three cycles. During injection of GSSG at low dissolved oxygen, there was a similar delay in perturbation as with GSH, confirming the redox sensitivity of the yeast at high dissolved oxygen. Relative phase shifting depending on the timing of perturbation was much more evident for GSSG than for GSH additions.

In *S. cerevisiae*, glutathione reductase is the major enzyme involved in the interconversion of GSH and GSSG (Casalone *et al.*, 1988). When the specific inhibitor of glutathione reductase, NF (5-nitro-2-furaldehyde), was injected at high or at low dissolved oxygen (Fig. 2c) no immediate effect was evident; then after one cycle the oscillation became damped for four cycles. Adding BSO [DL-buthionine-(S,R)-sulphoximine] (200 μM), an inhibitor of GSH synthesis, had no immediate effect; however, two or three cycles later the period of oscillation started increasing from 50 to 70 min. The oscillation period recovered to 50 min after 24 h. The amplitude of oscillation remained unaffected throughout the experiment.

The combined addition of BSO (50 μM), GSH (50 μM) and NF (50 μM) at high dissolved oxygen (Fig. 3) produced an immediate cessation of oscillation and inhibited respiration. The duration of perturbation was twice as long as those produced by separate additions of individual components. When the BSO, GSH and NF cocktail was injected at low dissolved oxygen there was also an immediate inhibition of respiration.

Fig. 4. Perturbation of dissolved oxygen oscillation during continuous aerobic yeast culture with GSH (100 μM). (a) Dissolved oxygen (thick line), acetaldehyde (▲) and ethanol (○). (b) Dissolved oxygen (thick line), GSH (▲) and GSSG (○). (c) Dissolved oxygen (thick line), NAD(P)H fluorescence (fine line) (365–450 nm) and intracellular redox potential (○).

The results suggest that GSH and GSSG play important roles in the regulation of oscillation. Thus, altering the concentrations and interconversion rates of GSH and GSSG leads to oscillation amplitude and period changes; the population is more sensitive during the low-respiration state, implying that a rapid conversion of GSH to GSSG does not occur at this time. Further evidence for this comes from the immediate cessation of respiration during a combined injection of BSO, GSH and NF at high respiratory activity. Combined GSH (50 μM) and NF (50 μM) injections had a similar effect as the BSO, GSH and NF cocktail, with respect to immediately perturbing the oscillation; however, the duration of perturbation was reduced.

Glutathione perturbation caused a reduction in ethanol uptake and respiration

Perturbation of the oscillation by addition of GSH (100 μM) inhibited ethanol uptake and acetaldehyde production (Fig. 4a). Extracellular GSH was not de-

tected 1 h after addition, indicating either GSH uptake or modification by the yeast (D. B. Murray, unpublished results). The duration of oscillation perturbation by GSH was concentration based, i.e. injections of 50 μM and 100 μM gave perturbation times of 2.8 h (Fig. 2) and 6.5 h (Fig. 4), respectively. Intracellular GSH increased from 10 to 25 mM and intracellular GSSG decreased from 1.5 to <0.1 mM (Fig. 4b), indicating that the yeast takes up GSH. Intracellular GSH fluctuated markedly during the first 2 h of perturbation. Biomass concentration in the fermenter declined from 6.45 to 5.84 g l⁻¹ during the period of respiratory inhibition due to wash out of yeast. Biomass then started to increase during the phase of increased respiration before levelling out once oscillation recovered (21.5 h). NAD(P)H fluorescence decreased after injection of GSH, reaching a minimum 20 min after injection, and ethanol uptake was immediately inhibited (Fig. 4c). Then NAD(P)H fluorescence increased during the reduced-respiration period. When the oscillation was perturbed with GSH, almost all of the GSSG is metabolized, resulting in a decrease in

Fig. 5. Perturbation of dissolved oxygen oscillation during continuous aerobic yeast culture by monosodium glutamate (1 mM) (a) and cysteine (500 μM) (b). The vertical bars represent the time of addition.

intracellular glutathione redox from -0.3 to -0.35 mV. Injection of glutamate (Fig. 5a) and cysteine (Fig. 5b) at millimolar concentrations, inhibited respiration and perturbed oscillation: both caused immediate respiratory inhibition. Glycine had no influence probably because of the low uptake of glycine by yeast (Rose & Harrison, 1989). However, to have a similar oscillation perturbation duration as GSH, 50- or 5-fold increases in the concentration of glutamate or cysteine, respectively, had to be used.

Perturbation of oscillation by cofactors

When calcium pantothenate (0.45 μM), required for the synthesis of CoASH, was added, respiration was enhanced and no oscillation was evident for the next 5 h (Fig. 6a). Addition of pyridoxal-HCl (0.98 μM), a cofactor in amino acid synthesis, also gave enhanced respiration with no oscillation over the first 5 h (Fig. 6b). Oscillation resumption involved slowly increasing amplitudes of dissolved oxygen. The enhancement of respiration suggests that ethanol catabolism was promoted during perturbation by either cofactors. Acetyl CoA is an intermediate in ethanol catabolism and is formed by the reaction of CoASH with acetate.

Fig. 6. Perturbation of dissolved oxygen oscillation during continuous aerobic yeast culture by calcium pantothenate (0.45 μM) (a) and pyridoxal-HCl (0.98 μM) (b). The vertical bars represent the time of addition.

DISCUSSION

Previous studies on the respiratory oscillation in continuous cultures of yeast have not identified the mechanism of intracellular regulation. Evidence suggested that metabolic switching occurs during ethanol catabolism, which involves control of the $NAD^+/NADH$ redox couple (Murray *et al.*, 1998). Using continuous readout of fluorescence, we observed a $NAD(P)H$ fluorescence oscillation with the major period similar to that of dissolved oxygen oscillation, but with a superimposed high-frequency, low-amplitude component. The interconversion of organisms' nicotinamide nucleotides between intracellular compartments in the oscillating state is too rapid to be analysed using enzymic techniques.

Investigations on the mechanism of glutathione-induced perturbation of respiratory oscillation indicated that factors increasing GSH concentration, e.g. glutamate, cysteine, GSH or GSSG (Osuji, 1979; Udeh & Achremowicz, 1997) inhibited respiration, and compounds reducing GSH concentration, e.g. BSO (Meister, 1995b), either altered the oscillation period or elevated respiration during perturbation. Therefore glutathione or a product of glutathione metabolism directly perturbs oscillation.

Inhibition of glutathione reductase with NF produced a damped respiratory oscillation, indicating that the activity of glutathione reductase influences oscillation. These experiments indicate that the mechanism of oscillation regulation involves the redox cycling of GSH and GSSG. The perturbation phase delay observed when GSH or GSSG was injected at high and low dissolved oxygen did not occur when the BSO, GSH and NF, or GSH and NF cocktails were injected: therefore, glutathione reductase plays an important role in regulating the oscillation.

It is probable that the NAD^+/NADH redox couple is not involved directly in either the regulation or the population synchronisation of respiratory oscillation, because during GSH perturbation glutathione redox dropped from -0.30 to -0.35 mV. This indicated that conditions were more reducing; however, during the same period NAD(P)H fluorescence fell. This also indicated that the respiratory chain was not blocked at the NADH-Q reductase. GSH perturbation caused an immediate cessation of ethanol uptake; however, NAD(P)H fluorescence and acetaldehyde concentration decreased. This indicated direct inhibition of alcohol dehydrogenase; inhibition may occur by disruption at the zinc finger (Crow *et al.*, 1995).

Regulation of the respiratory oscillation occurs during ethanol metabolism and at a site in the respiratory chain beyond NADH-Q reductase. The synchronisation factor(s) must influence these regulation points. Period frequency may be caused by slow thiol-transfer rates and slow protein-folding rates observed *in vivo* (Raina & Missiakas, 1997). Pantothenate and pyridoxal-HCl both cause enhanced respiration, and glutathione and cysteine both caused reduced respiration during perturbation. These cofactors (Wainwright, 1969) and metabolites are involved in sulphur metabolism in yeast. Temperature and aeration rate but not dissolved oxygen (within limits) (Keulers *et al.*, 1996a, b), influence population synchrony, suggesting that the synchronisation mechanism may involve a volatile component. The volatile compound is a product of intracellular glutathione cycling, and therefore probably contains a thiol group. Further evidence for this was supplied by the perturbation of oscillation with GSH precursors cysteine and glutamate, i.e. cysteine injection produced a greater perturbation than glutamate.

In conclusion, the respiratory oscillation in ethanol-grown cultures of *S. cerevisiae* is regulated by intracellular redox potential. The regulation mechanism relies on the intracellular cycling of glutathione and glutathione reductase activity. It appears to have at least two sites of action, i.e. ethanol catabolism and the respiratory chain post NADH-Q reductase. The synchronisation mechanism probably involves a volatile compound with a thiol group. Respiratory oscillation provides an excellent opportunity to study the influence of intracellular redox on metabolism *in vivo*. Further work is being carried out to elucidate the molecular mechanisms involved in the regulation of respiratory

activity in the oscillatory state and the mechanism of population synchronisation.

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