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NOTE

Regulation of Branched-Chain, and Sulfur-Containing Amino Acid Metabolism by Glutathione during Ultradian Metabolic Oscillation of *Saccharomyces cerevisiae*

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Autonomous ultradian metabolic oscillation ($T \cong 50$ min) was detected in an aerobic chemostat culture of *Saccharomyces cerevisiae*. A pulse injection of GSH (a reduced form of glutathione) into the culture induced a perturbation in metabolic oscillation, with respiratory inhibition caused by H_2S burst production. As the production of H_2S in the culture was controlled by different amino acids, we attempted to characterize the effects of GSH on amino acid metabolism, particularly with regard to branched chain and sulfur-containing amino acids. During stable metabolic oscillation, concentrations of intracellular glutamate, aspartate, threonine, valine, leucine, isoleucine, and cysteine were observed to oscillate with the same periods of dissolved O_2 oscillation, although the oscillation amplitudes and maximal phases were shown to differ. The methionine concentration was stably maintained at 0.05 mM. When GSH (100 μM) was injected into the culture, cellular levels of branched chain amino acids increased dramatically with continuous H_2S production, whereas the cysteine and methionine concentrations were noticeably reduced. These results indicate that GSH-dependent perturbation occurs as the result of the promotion of branched chain amino acid synthesis and an attenuation of cysteine and methionine synthesis, both of which activate the generation of H_2S. In a low sulfate medium containing 2.5 mM sulfate, the GSH injections did not result in perturbations of dissolved O_2, NAD(P)H redox oscillations without burst H_2S production. This suggests that GSH-dependent perturbation is intimately linked with the metabolism of branched-chain amino acids and H_2S generation, rather than with direct GSH-GSSG redox control.

Key words: cellular amino acid concentrations, hydrogen sulfide, GSH dependent respiratory inhibition, *Saccharomyces cerevisiae*, ultradian metabolic oscillation

Cellular oscillations are appropriate objects for investigations into the structure and regulatory properties of biochemical networks. The cell cycle, the Ca^{++} cycle, and glycolytic oscillations are among the most intensively studied cellular oscillations. Autonomous ultradian metabolic oscillation has been previously shown to occur in aerobic chemostat cultures of *Saccharomyces cerevisiae* (Satroutdinov *et al.*, 1992; Sohn and Kuriyama, 2001a; Klevecz *et al.*, 2004). This ultradian metabolic oscillation originates from the metabolic sum of individual cells within the population (Keulers and Kuriyama, 1998), and can be characterized by the consecutive changes of a host of metabolites and parameters, including dissolved O_2, NAD(P)H fluorescence (Murray *et al.*, 1998, 1999), CO_2 production rate, oxygen uptake rate, intracellular pH (Keulers *et al.*, 1996a, 1996b), H_2S production rate, sulfate uptake rate (Sohn *et al.*, 2000, 2001b) as well as the concentrations of ethanol, GSH, and cysteine (Murray *et al.*, 1998; Sohn *et al.*, 2001b). The rhythmicity of this process appears to be independent of the cell cycle, glycolysis, environmental triggering or dark-light transitions, and appears to be dependent on nutrients, air flow rate, dilution rate, and pH levels (Keulers *et al.*, 1996a; Murray *et al.*, 1999). On the basis of the free-running rhythm and

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the properties of temperature compensation under different culture conditions, this oscillation has been identified to function as a biological clock (Murray *et al.*, 2001; Lloyd *et al.*, 2003; Klevecz *et al.*, 2004).

The consecutive production of H_2S by sulfite reductase in the sulfate assimilation pathway performs a vital function in population synchrony and redox homeostasis during metabolic oscillation; population synchrony is mediated via the consecutive inhibition of mitochondrial cytochrome *c* oxidase by cyclic H_2S production (Sohn and Kuriyama, 2001b; Lloyd *et al.*, 2002), and the timing and amount of H_2S production are dependent on cellular oxidative stress levels (Kwak *et al.*, 2003; Henson, 2004). Because H_2S is located at the junction of the sulfate assimilation and carbon metabolism pathways, the production of H_2S may be multiply regulated by the level of sulfite, which functions as a cellular source of sulfur (Sohn and Kuriyama, 2001b), *O*-acetylhomoserine, which acts as a sulfide acceptor (Sohn and Kuriyama, 2001a), the cellular redox states (Murray *et al.*, 1999; Kwak *et al.*, 2003), and the cellular levels of various amino acids, including threonine, methionine, and cysteine (Ono *et al.*, 1999; Hansen and Johannesen, 2000; Sohn and Kuriyama, 2001a). The concentrations of intracellular amino acid have been intimately linked with the regulation of metabolic oscillation. For example, H_2S and *O*-acetylhomoserine formed homocysteine, which, in turn, was transformed into either cysteine or methionine, both of which regulate sulfate assimilation (Fig. 1). Because threonine is a repressor of aspartate kinase, aspartate–semialdehyde dehydrogenase, and homoserine dehydrogenase, the addition of threonine is expected to reduce the supply of homoserine from aspartate (Fig. 1). Therefore, perturbations in cellular metabolic oscillation occur as the result of pulse injections of cysteine, methionine, or threonine (Sohn and Kuriyama, 2001a), and the feedback inhibition of sulfate uptake high cysteine concentrations has been considered to be a primary source of metabolic oscillation (Ono *et al.*, 1999; Wolf *et al.*, 2001; Henson, 2004).

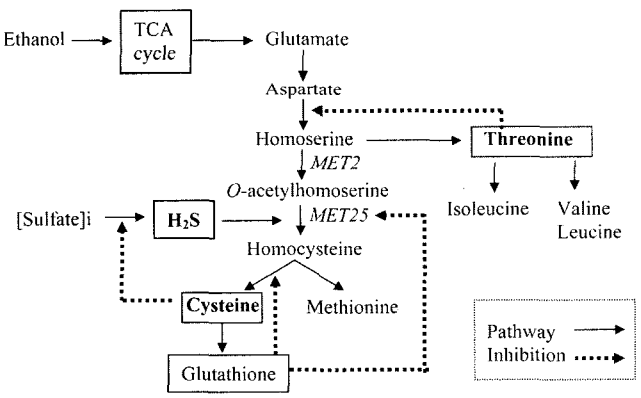

Fig. 1. Simplified diagram of the sulfate assimilation pathway, and sulfur-containing, and branched-chain amino acid synthesis from glutamate in *S. cerevisiae*. Dotted lines indicate the inhibition of specified pathways by threonine, cysteine, and GSH. [Sulfate]i, intracellular sulfate.

Pulse injections of GSH, a major antioxidant and a thiol compound (Lee *et al.*, 2003; Shin *et al.*, 2003), into cell cultures have been shown to elicit perturbations of metabolic oscillation, along with changes in the cellular redox potential (Murray *et al.*, 1999). These perturbations were accompanied by the burst production of H_2S, thus severely attenuating cellular respiration during GSH-dependent perturbation (Sohn *et al.*, 2000). However, although the GSH/GSSG redox coupling has been linked with apoptosis, and has been proposed to be a regulator of the ultradian clock during metabolic oscillation (Murray *et al.*, 1999; Lloyd *et al.*, 2003), the mechanism underlying the production of H_2S resulting from pulse injections of GSH remains incompletely understood. In order, then, to gain insight into the mechanisms responsible for GSH-dependent H_2S production during the perturbation of metabolic oscillation, the present study sought to determine the effects of GSH on amino acid metabolism, particularly with regard to the metabolism of branched chain and sulfur-containing amino acids, since cellular levels of threonine, cysteine, and methionine have been implicated in the direct control of H_2S production in cultures (Fig. 1) (Ono *et al.*, 1999; Sohn and Kuriyama, 2001a).

Using the polyploid wild-type *S. cerevisiae* strain, IFO-0233 (IFO, Institute of Fermentation, Japan), we conducted batch and continuous chemostat cultures, as was previously described (Sohn *et al.* 2000; Kwak *et al.*, 2003). The medium contained 340 mM ethanol and 46 mM sulfate (Sohn and Kuriyama, 2001b). The fermentor (BioFlo, USA) was operated at 30°C, with an agitation rate of 800 rpm, a working volume of 1.2 l, an air flow rate of 180 cm^3/min, and a dilution rate of 0.085/h. The amount of dissolved O_2 in the culture was employed as an indicator of respiratory activity. Amino acid concentrations were determined by the use of an amino acid analyzer (L-8500A, Hitachi, Japan). Two ml of culture broth were withdrawn from the culture, and the cells were rapidly collected via centrifugation at 4°C. The pellets were then washed in ice-cold phosphate buffer (5 mM, pH 6.0) containing 1 mM EDTA, and suspended with 1 ml of phosphate buffer (5 mM, pH 6.0). After 15 min of boiling at 100°C, the supernatants were collected and used for amino acid analysis. The cytosolic volume was assumed to be 1.6 ml/g-dry cell weight (Keulers *et al.*, 1996b) and the results were presented as the mean value of triplicate trials. We determined H_2S concentrations using a Gas Chromatograph (Shimadzu GC-14A, Japan) equipped with an OPND column (Shimadzu, 25% CW, Japan) and a FPD detector (Shimadzu, detection limit 50 nM, Japan). The analytical techniques used in this study were identical to those reported previously (Sohn *et al.*, 2000; Kwak *et al.*, 2003).

During ultradian metabolic oscillation, a 55-min period, the dissolved O_2 concentration oscillated between 72 to 185 μM, and the H_2S concentration oscillated between 0

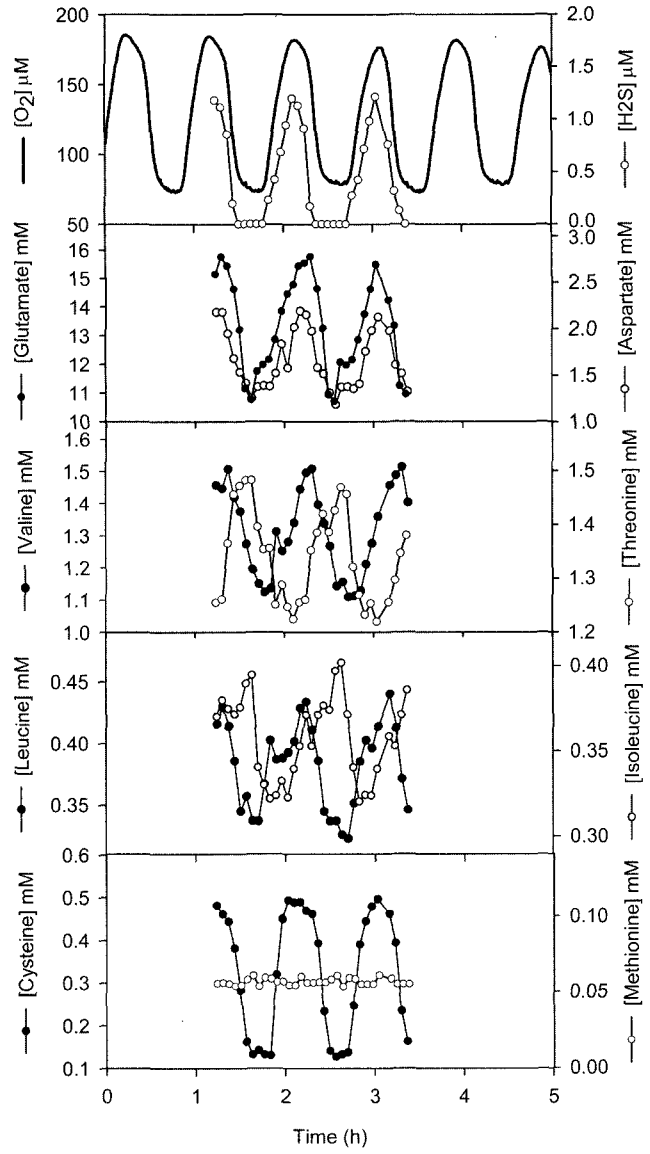

Fig. 2. Oscillatory changes in the levels of dissolved O_2 and H_2S, and the dynamics of intracellular concentrations of glutamate, aspartate, threonine, branched-chain amino acids (valine, leucine, and isoleucine) and sulfur-containing amino acids (cysteine and methionine) during the ultradian metabolic oscillation of *S. cerevisiae*.

to 1.2 μM, in phase with the fluctuation of dissolved O_2 levels (Fig. 2). Intracellular concentrations of glutamate, aspartate, threonine, branched-chain amino acids, and sulfur-containing amino acids exhibited significant and complex dynamic behavior; the concentration of amino acids varied, from 0.05 to 15.8 mM, depending on the amino acid species and on the respiration state of the cells. Glutamate was detected at the highest concentrations, whereas methionine was found at the lowest concentrations, as has been previously reported in cultures of *S. cerevisiae* (Kitamoto *et al.*, 1988; Hans *et al.*, 2001, 2003). With the exception of methionine, the concentrations of the amino acids oscillated in phase with the oscillation of the levels of dissolved O_2. Concentrations of glutamate, aspartate, valine, leucine, and cysteine were found to oscillate in phase with the dissolved O_2 levels, whereas the threonine and isoleucine concentrations oscillated out of phase with the oscillations in the dissolved O_2 levels. Oscillation of dissolved O_2, a period of 100 min, and dynamic changes in cellular concentrations of amino acids have been reported during the autonomous cell cycle oscillation of *S. cerevisiae* (Hans *et al.*, 2003). During cell cycle oscillation, intracellular aspartate concentrations were shown to oscillate in phase with dissolved O_2, but valine and leucine concentrations fluctuated out of phase with the oscillations in the levels of dissolved O_2, contrary to what was observed in our ultradian metabolic oscillation. Furthermore, threonine and isoleucine levels did not exhibit any oscillatory behavior, and changes in the concentrations of glutamate occurred in a chaotic pattern during cell cycle oscillation (Hans *et al.*, 2003). The different profiles of amino acid concentration oscillation observed during ultradian metabolic oscillation and during cell cycle oscillation indicate that the regulation mechanisms involved during these processes are also different, although the cellular amino acid levels changed in a dynamic fashion during both processes.

These dynamic oscillatory changes in amino acid metabolism complements have been previously reported in conjunction with oscillatory changes in the TCA cycle (Satroutdinov *et al.*, 1992; Keulers *et al.*, 1996b) and periodic protein synthesis/degradation (Klevecz *et al.*, 2004) occurring during ultradian metabolic oscillation. Interestingly, concentrations of threonine were inversely related with the concentrations of cysteine. During the respiratory inhibition phase (H_2S production phase), cysteine concentrations increased, while threonine concentrations decreased. During the active respiration phase, threonine and isoleucine levels increased, while cysteine concentrations decreased. These findings suggested that periodic flux changes from homoserine to cysteine or to threonine are linked with the regulation of H_2S generation (Fig. 1), as homoserine removes H_2S, threonine reduces homoserine concentrations, and cysteine inhibits sulfate assimilation (Mountain *et al.*, 1991; Sohn and Kuriyama, 2001a; Marina *et al.*, 2004).

In order to characterize the effects of GSH on amino acid metabolism during ultradian metabolic oscillation, a final concentration of 100 μM of GSH was pulse-injected into our culture. The GSH was dissolved in 2 ml of deionized water, then injected into the cultures through a sterile filter (0.22 μm). The cultures were maintained at a pH of 3.4 after the injection. During stable metabolic oscillation, which persisted for a period of 46 min, the concentration of dissolved O_2 oscillated between 52 to 168 μM, and the concentration of H_2S oscillated between 0 to 1.26 μM, in phase with the oscillation in dissolved O_2 levels (Fig. 3). The shoulder of dissolved O_2 was comparable with that of the H_2S concentration. GSH pulse injections at the min-

imum dissolved O_2 concentration were observed to perturb metabolic oscillation, along with H_2S burst production, and the maximum H_2S concentration increased to 8.28 μM after 38 min. The uptake of GSH by the cells occurred within 12 min (Miyake *et al.*, 1999; Murray *et al.*, 1999; Bourbouloux *et al.*, 2000). After the GSH injection, transient increases in the concentrations of glutamate and cysteine were observed for 28 min. The glutamate concentration increased by 3 mM (from 14.2 to 17.2 mM) and the cysteine concentration increased by 0.3 mM (from 0.1 to 0.4 mM). Aspartate concentrations were observed to increase from 1.55 mm to 1.99 mM within 36 min after the GSH injection, and were maintained between 1.74 and 1.86 mM during the perturbation of the oscillation. During the prolonged respiratory inhibition phase (1.5 ~ 2.8 h) established by continuous H_2S production, glutamate and cysteine concentrations were maintained at minimal levels, whereas the valine, leucine, and isoleucine concentrations increased dramatically. When the cysteine concentrations were increased, and the threonine, valine, leucine and isoleucine concentrations decreased (3.3 h), respiration activity was observed to recover along with decreased production of H_2S. Although the exact mechanism underlying this time-delay effect of GSH remains fairly unclear, our results indicated that GSH activates the synthesis of branched-chain amino acids, and attenuates

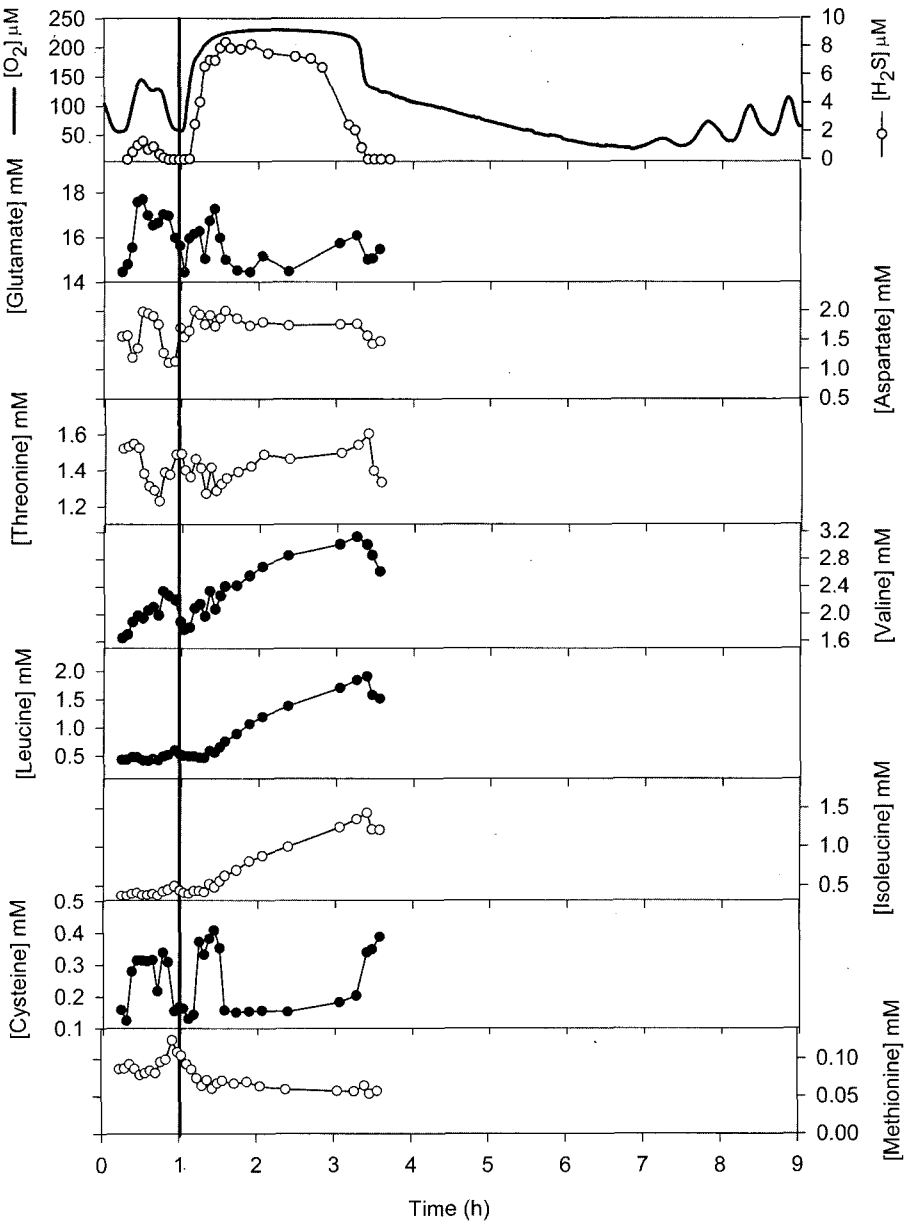

Fig. 3. Perturbation of dissolved O_2 oscillation, H_2S concentration in culture, and intracellular concentrations of glutamate, aspartate, threonine, branched-chain amino acids (valine, leucine, and isoleucine) and sulfur-containing amino acids (cysteine and methionine) as the result of a pulse injection of 100 μM of GSH in an aerobic chemostat *S. cerevisiae* culture. The vertical line indicates the GSH injection time.

cysteine synthesis. These results also suggested that the homoserine generated from aspartate is converted to threonine and branched-chain amino acids, rather than to cysteine, during GSH-dependent perturbation. Under conditions in which sulfate assimilation is activated by lower cysteine levels, and the supply of homoserine is limited as the result of increased branched amino acid synthesis, H_2S can be overproduced (Fig. 3). These findings suggest that the possible regulation loci of ultradian metabolic oscillation include a bifurcate homoserine pathway (homoserine to homocysteine or homoserine to threonine).

The H_2S production observed as the result of GSH pulse injection was confirmed in a chemostat culture, using a low-sulfate medium (LSM). The LSM, which contained 2.5 mM sulfate, was prepared by omission of sulfuric acid, and replacement of all sulfates with corresponding chloride salts (Sohn and Kuriyama, 2001b). In LSM, the period of metabolic oscillation was shortened to 32 ~ 35 min due to sulfate shortage (Sohn and Kuriyama, 2001b). During stable oscillation, the amounts of dissolved O_2 in the culture oscillated between 88 to 150 μM, and the H_2S levels oscillated between 0 to 1.8 μM, in phase with the fluctuations in the concentrations of dissolved O_2 (Fig. 4a). The concentration of sulfates in the LSM culture was between 0.6 and 0.7 mM, whereas that of the normal medium was between 35 and 40 mM (Sohn and Kuriyama, 2001b). Consistently with our expectations, the pulse injection of GSH (100 μM) into the LSM culture did not result in prolonged respiratory inhibition and burst H_2S production. In fact, the injection of GSH into the LSM culture reduced H_2S production, similar to the results seen after the pulse injection of antioxidants, including *N*-acetylcysteine or ascorbic acid, into normal medium (Kwak *et al.*, 2003). These results indicate that GSH-dependent perturbation is the result of H_2S production from sulfate assimilation, which is multiply regulated by cellular cysteine and threonine levels (Sohn and Kuriyama, 2001a). In a while, NAD(P)H fluorescence was measured after GSH injection, using a fiber-optic probe (Biomedical Instrumentation Group, University of Pennsylvania). Unlike the substantial perturbation of NAD(P)H fluorescence observed in the chemostat culture using a medium containing 46 mM sulfate (Murray *et al.*, 1999), the injection of GSH into the LSM medium did not result in a perturbation of the cellular redox rhythm (Fig. 4b). This suggests that GSH-dependent perturbation can be confidently linked with the regulation of branched-chain and sulfur-containing amino acid metabolism and the sequestration of H_2S, rather than with direct GSH/GSSG redox control.

In conclusion, GSH regulates the dynamics of amino acid metabolism during ultradian metabolic oscillation, and GSH injection activates branched-chain amino acid synthesis and reduces cysteine synthesis, which induces burst H_2S production and a severe reduction of the cellular redox state. Further studies into the repression and inhibition of the bifurcate homoserine pathway by GSH and the regulation of homoserine *O*-acetyltransferase (*MET2*) and *O*-acetylhomoserine sulfhydrylase (*MET25*) by cysteine or GSH are necessary.

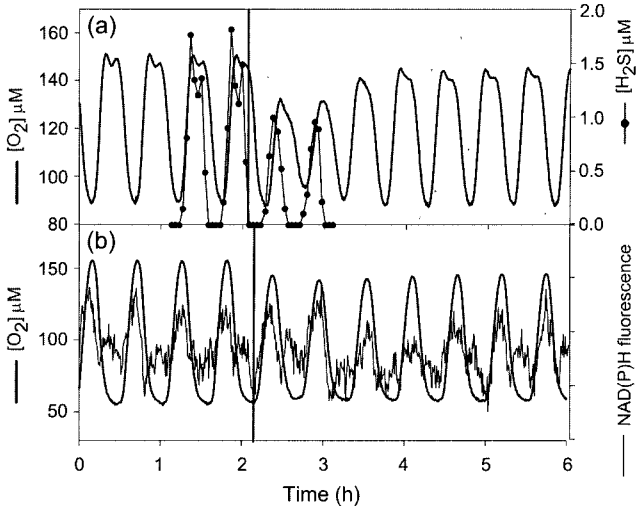

Fig. 4. Changes in (a) dissolved O_2 oscillation and (b) NAD(P)H fluorescence as the result of a pulse injection of 100 μM of GSH into an LSM culture containing 2.5 mM sulfate. The vertical line indicates the GSH injection time.

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