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ORIGINAL PAPER

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Detection of two distinct substrate-dependent catabolic responses in yeast cells using a mediated electrochemical method

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Abstract Mediated electrochemical detection of catabolism in prokaryotic cells is well documented; however, the application of this technique to eukaryotic cells has received less attention. Two catabolic substrate-dependent mediated electrochemical signals were detected in the yeast *Saccharomyces cerevisiae*. The signal using a single hydrophilic mediator (ferricyanide) is small whereas the response using a double mediator system comprising a hydrophilic and a lipophilic mediator (ferricyanide and menadione) is up to three orders of magnitude larger. The behaviour of each response during cell ageing is different: the single mediator response increases whereas the double mediator response decreases. This difference indicates that the two signals originate at different points in the catabolic pathways. In *S. cerevisiae* the double mediator response is proposed to originate from the reduction of the lipophilic mediator by NADPH produced in the pentose phosphate pathway. The single mediator signal arises from reduction of the hydrophilic mediator by an extracellular redox species produced in response to the presence of glucose.

Introduction

Monitoring catabolism by measuring oxygen consumption is a commonly used technique and is applicable to any aerobically respiring cell (bacteria, fungi, protozoa, or algae cells, and plant or animal tissue). An oxygen probe can be used to measure the change in oxygen concentration over an elapsed period, as in the 5-day biochemical oxygen demand (BOD_5) assessment, or for real time analysis of oxygen consumption, for example in rapid BOD sensors (Tan et al. 1992; Tanaka et al. 1994; Chan et al. 1999; Lehmann et al. 1999) and for the rapid biochemical characterisation of organisms (Subrahmanyam et al. 2001).

More recently, catabolism in bacteria has been monitored by replacing oxygen with redox mediators that capture electrons from a redox molecule in the electron transport chain (Ramsay and Turner 1988; Richardson et al. 1991; Ertl et al. 2000a, 2000b; Pasco et al. 2000; Ertl and Mikkelsen 2001). An oxidised mediator is reduced in its interaction with a reduced electron transport chain molecule and the reduced mediator is then quantified by an electrochemical method such as amperometry, voltammetry or coulometry.

Mediators are small molecules that can undergo redox reactions with a cellular component. Hydrophilic mediators are soluble in the aqueous environment of the cell but do not cross the cell membrane to enter the cytoplasm. Examples include potassium hexacyanoferrate(III) (ferricyanide), ruthenium hexamine and ferrocene carboxymethylate. Lipophilic mediators are soluble in the cell membrane and can enter the cytoplasm to interact with electron transport chains located within internal membranes. Examples of lipophilic mediators include quinones such as 2-methyl-1,4-naphthoquinone (menadione), benzoquinone and 1,2-napthoquinone, and benzoamines such as 2,3,5,6-tetramethyl-1,4-phenylenediamine (TMPD) and *N*,*N*-dimethyl-*p*-phenylenediamine (Fultz and Durst 1982; Chaubey and Malhotra 2002).

Lipophilic mediators have been used to detect metabolism in both prokaryotic and eukaryotic cells (Rawson et al. 1987; Rabinowitz et al. 1998). In contrast, hydrophilic mediators are not expected to be able to mediate catabolism in eukaryotic cells. Their use would seem to be restricted to the detection of catabolism in prokaryotic cells that have respiratory electron transport redox proteins located in the cell membrane and accessible from the periplasm.

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An elegant demonstration of the different properties of hydrophilic and lipophilic mediators is provided by the use of various mediators to image mammalian and bacterial cell features using scanning electrochemical microscopy (Liu et al. 2001; Cai et al. 2002). Topographic imaging of the cell was performed using hydrophilic mediators such as ferricyanide and ferrocene carboxymethylate, which cannot pass through the cell membrane. Mapping of intracellular redox activity was obtained using lipophilic mediators, such as menadione and 1,2-napthoquinone, which can cross the cell membrane.

A double mediator system comprising lipophilic and hydrophilic mediators was used for the electrochemical detection of intracellular redox activity in mammalian cells (Rabinowitz et al. 1998). The function of the lipophilic mediator menadione was to shuttle electrons from intracellular redox sites to the cell surface for reaction with the hydrophilic mediator ferricyanide.

We are interested in the use of yeast as the biocomponent in mediated electrochemical biosensors. In this work, we investigate the detection of catabolism in yeast and in particular the detection of the catabolic response to exogenous substrate. Because yeasts are eukaryotic organisms, a lipophilic mediator was anticipated to be necessary to interact with the intracellular redox molecules associated with catabolism. We have investigated the use of the double mediator system comprising lipophilic menadione and hydrophilic ferricyanide for the detection of catabolism. Menadione was selected because it is reported to interact with NADPH as well as with elements of the electron transport chain (Rabinowitz et al. 1998). Thus, it should be useful for the detection of catabolism in eukaryotic cells that operate in both respiratory and fermentative modes. During the course of this work we were intrigued by a report describing the detection of a ferricyanide-mediated electrochemical response dependent on exogenous substrate in a species of *Candida* (Trosok et al. 2001). Hence the study was extended to include investigation of both single and double mediator responses in yeast cells.

Materials and methods

Microorganism cultivation and preparation

S. cerevisiae (NCTC 10716) was maintained on yeast extract peptone dextrose (YEPD) agar at 4°C. Yeast cultures for experimental use were grown in 100 ml YEPD broth in shake flasks rotated at 150 rpm for 16 h at 28°C. Cells were harvested by centrifugation at 4,000 *g* for 5 min at 10°C. The cells were washed twice in phosphate buffer (PB, 0.05 M K_2HPO_4/KH_2PO_4, pH 7) and re-suspended in phosphate buffered saline (PBS, 0.05 M K_2HPO_4/KH_2PO_4, pH 7, 0.1 M KCl). Cell density was adjusted to an optical density at 600 nm (OD_{600}) of 2.25 using an LKB Novaspec II spectrophotometer. Cells were used immediately or were aged by placing in 250 ml shake flasks and incubating in PBS at 25°C, 150 rpm, for 7 or 14 days.

Mediators and substrate

Potassium ferricyanide (K_3FeCN_6, BDH AnalaR) was dissolved in distilled water to give a 0.25 M solution, filter-sterilised and stored at 4°C. Menadione (Sigma) was dissolved in 96% ethanol to give a 20 mM solution, filter-sterilised and stored in a light-proof container at 4°C. TMPD (Aldrich) was dissolved in 96% ethanol to give a 20 mM solution, filter-sterilised and stored at 4°C. Glucose (BDH AnalaR), 1 M in distilled water was sterilised at 121°C for 15 min and stored at 4°C.

Incubation of cells with mediator(s) and substrate

A total volume of 20 ml incubation suspension was prepared for each trial. The standard incubation suspension comprised: 18.15 ml cell suspension in PBS, 1.6 ml ferricyanide solution (final concentration 20 mM), 150 µl glucose solution (final concentration 7.5 mM) or 150 µl PBS, 100 µl menadione solution (final concentration 100 µM) or 100 µl PBS. In the trials with various concentrations of menadione or TMPD, the incubation was with 40 mM ferricyanide to ensure that ferricyanide was in excess. Incubation of cells with mediator(s) and substrate was for 1 h at 28°C under oxygen-free nitrogen sparging. At the completion of incubation, the cells were pelleted by centrifugation (4,000 *g*, 25°C, 10 min) and the supernatant removed for analysis. Unless stated otherwise, all trials were performed in triplicate.

Electroanalysis of the supernatant

Steady-state voltammetry was performed using a 100 µm diameter Pt microdisc working electrode, a Ag/AgCl reference electrode, and a Pt wire auxiliary electrode (all BioAnalytical Systems) controlled by a EG&G Princeton Applied Research, Model 364 polarographic analyser. An AD Instruments Maclab 2e and an Apple computer were used for signal processing. The Pt microdisc electrode was polished on a Lecloth polishing cloth (Leco) using a slurry of 0.3 µm alumina (Leco) prior to each scan. Steady-state voltammograms were obtained at a scan rate of 5 mV s^{-1} scanning from 425 to 100 mV. The steady-state current at 425 mV was measured and the mean current from three repeat voltammograms was calculated. The measured currents varied by less than 10%. The mean currents from each trial replicate were used to calculate the mean current and standard deviation for each experiment. The standard deviations are shown as error bars on the plotted data.

Results

Electrochemical detection of catabolism using steady-state voltammetry

Steady-state voltammetry is a convenient method for determining the amounts of oxidised and reduced forms of an electroactive species in solution. The position of the voltammogram on the current axis gives an immediate indication of the proportions of each oxidation state, and the anodic and cathodic plateau currents allow quantitation of each redox form. In this work, ferricyanide is converted to ferrocyanide by yeast catabolism. Figure 1 (curve a) shows that before incubation, the mediator is fully in the oxidised form (the voltammogram shows only cathodic current due to reduction of ferricyanide). After incubation for 1 h with yeast, glucose, ferricyanide and menadione, the voltammogram (curve b) has shifted up the current axis and there is both anodic current (arising from oxidation of ferrocyanide) and cathodic current (arising from reduction of ferricyanide). The steady-state anodic plateau current measured at E =425 mV was used as a relative measure of the amount of ferrocyanide pro-

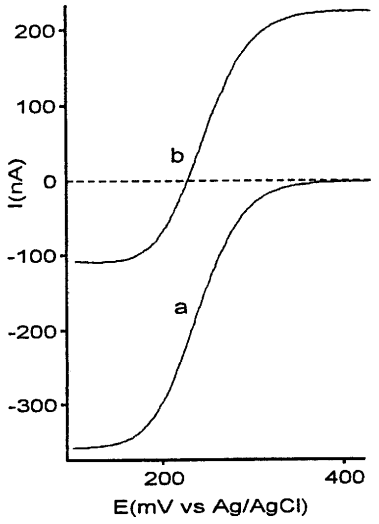

Fig. 1 Steady-state voltammograms of 20 mM ferricyanide, 7.5 mM glucose and 100 μM menadione in phosphate-buffered saline (PBS) (*curve a*) and the same solution after incubation for 1 h with *Saccharomyces cerevisiae* (*curve b*)

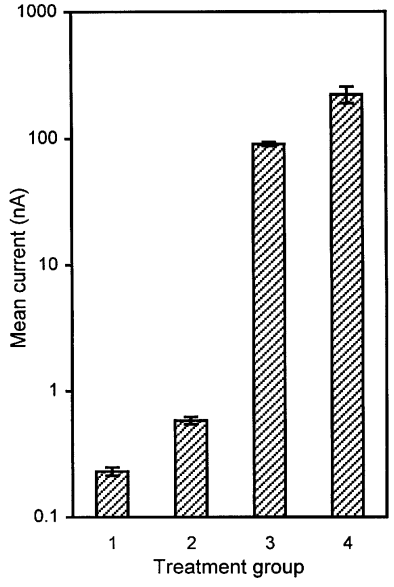

Fig. 2 Mean current for the oxidation of ferrocyanide after incubation of fresh *S. cerevisiae* cells for 1 h with: *1* 20 mM ferricyanide, *2* 20 mM ferricyanide and 7.5 mM glucose, *3* 20 mM ferricyanide and 100 μM menadione, *4* 20 mM ferricyanide, 7.5 mM glucose and 100 μM menadione. Each treatment was performed in triplicate and each sample was analysed in triplicate. *Error bars* Standard deviations

duced, and hence the amount of yeast catabolism. Note that although the current at E =425 mV can be measured without recording the full voltammogram, the full voltammogram provides a check of the reliability of the measurement, because any problems such as reduced sensitivity due to electrode fouling are easily detected.

At pH 7, the lipophilic mediators menadione and TMPD have E^{o} values significantly more negative than ferricyanide and hence will not interfere with the ferricyanide voltammogram. In fact, at the concentrations used in this work, meaningful voltammograms could not be obtained for either menadione or TMPD.

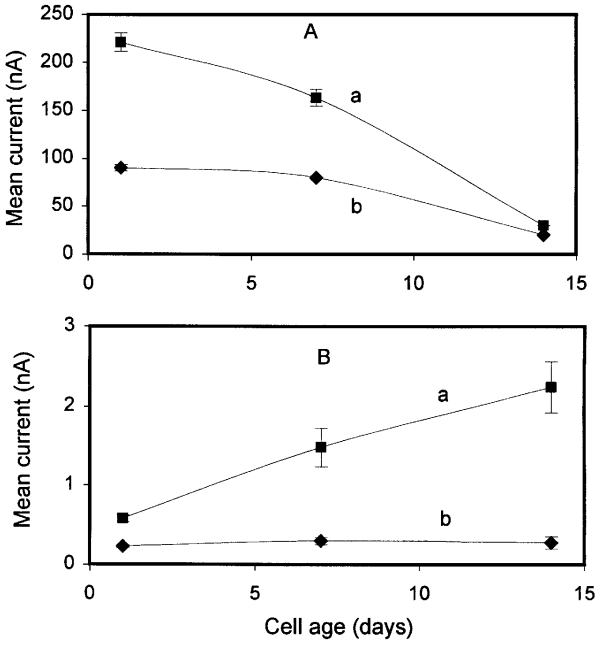

Fig. 3 Effect of cell age on the mean currents for oxidation of ferrocyanide after incubation of *S. cerevisiae* cells for 1 h with **A** 20 mM ferricyanide and 100 μM menadione in the presence (*a*) and absence (*b*) of 7.5 mM glucose and **B** 20 mM ferricyanide in the presence (*a*) and absence (*b*) of 7.5 mM glucose. Each treatment was performed in triplicate and each sample was analysed in triplicate. *Error bars* Standard deviations

Single and double mediator responses in the presence and absence of exogenous substrate

Fresh *S. cerevisiae* cells were incubated in two treatment groups: hydrophilic mediator (ferricyanide) and hydrophilic mediator plus lipophilic mediator (menadione) with two levels of glucose in each treatment group (0 mM and 7.5 mM). The responses of each treatment group are presented in Fig. 2. Note that lipophilic mediators were not used as the sole mediator because their low aqueous solubilities limit the mediator concentration and hence the magnitude of the signal. Furthermore, TMPD is the reduced form of the redox couple and hence relies on oxidation by ferricyanide to become functional.

Effect of culture age on single and double mediator responses

S. cerevisiae cells were aged by incubation in PB at 25°C. After time intervals of 1, 7 and 14 days, cells were removed and subjected to the experimental procedure described in the section above. Figure 3A shows the results obtained in the presence of ferricyanide and mena-

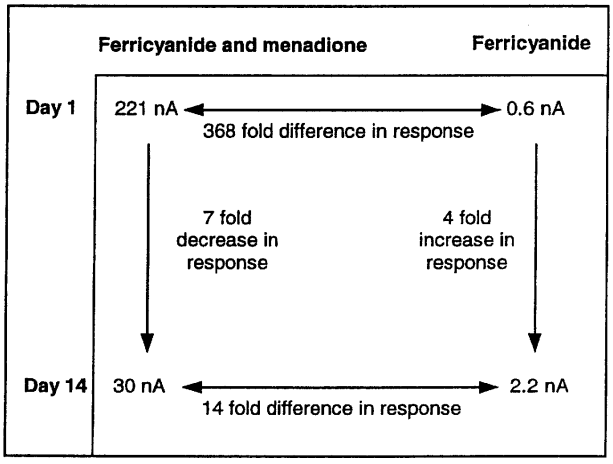

Fig. 4 Summary of the effects of cell ageing on double and single mediator responses

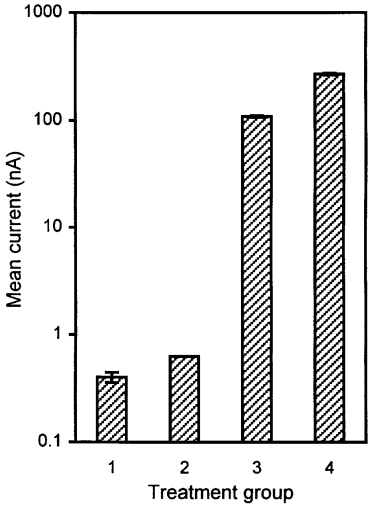

Fig. 5 Mean current for the oxidation of ferrocyanide after incubation of 21-day-old *S. cerevisiae* cells for 1 h with *1* 20 mM ferricyanide, *2* 20 mM ferricyanide and 7.5 mM glucose, *3* 20 mM ferricyanide and 100 µM menadione, *4* 20 mM ferricyanide, 7.5 mM glucose and 100 µM menadione. Each treatment was performed in triplicate and each sample was analysed in triplicate. *Error bars* Standard deviations

dione, and Fig. 3B shows the results obtained in the presence of ferricyanide alone.

The trends in the data presented in Fig. 3 are emphasised in Fig. 4, which shows the relationships between the signal magnitudes detected with single and double mediator systems over 14 days. *S. cerevisiae* cells were also stored in PB at 4°C for 21 days and subjected to single and double mediator trials. The results are shown in Fig. 5.

Effects of menadione and TMPD concentrations on the signal detected in the double mediator system

Steady-state voltammetry of the supernatant after incubation indicates the amounts of reduced and oxidised

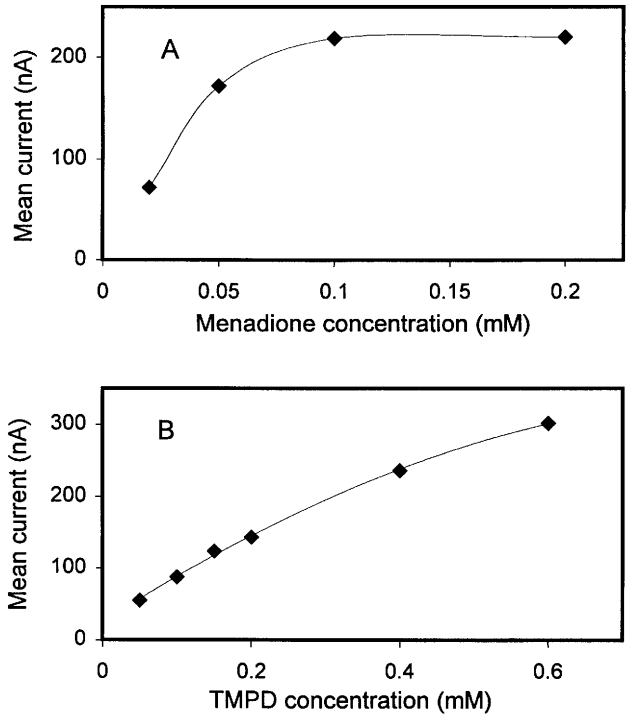

Fig. 6 Mean current for the oxidation of ferrocyanide after incubation of fresh *S. cerevisiae* cells for 1 h with 40 mM ferricyanide and 7.5 mM glucose together with **A** menadione (0.02–0.2 mM), or **B** 2,3,5,6-tetramethyl-1,4-phenylenediamine (TMPD; 0.05–0.6 mM). Each treatment was performed in duplicate and each sample was analysed in triplicate

ferricyanide. Thus, monitoring the concentration of this mediator to ensure over-supply is straightforward. However, the optimal concentration of the lipophilic mediator can only be determined indirectly. Fresh *S. cerevisiae* cells were incubated with four concentrations of menadione in the presence of excess (40 mM) ferricyanide giving the results presented in Fig. 6A. Also shown (Fig. 6B) are the results of similar experiments using TMPD in place of menadione. As added to the incubation mix, TMPD is in the reduced form and must be oxidised by ferricyanide prior to functioning as a mediator. However, at the relative concentrations of the two mediators used in the incubation experiments, the contribution of this pre-oxidation by ferricyanide accounts for <4% of the total signal. (The oxidation of TMPD by ferricyanide was confirmed using steady-state voltammetry; results not shown.)

Discussion

Catabolic substrate dependent responses were observed in both the single and double mediator trials, both in the presence and absence of exogenous glucose. However, in fresh *S. cerevisiae* cells, using the standard incubation suspension, the response to glucose of the double mediator system (menadione and ferricyanide) was approximately three orders of magnitude greater than that of the

single mediator system (ferricyanide only). Similar results were obtained when menadione was replaced with TMPD as the lipophilic mediator.

In order to gain insight into the origins of the responses, cell ageing experiments were conducted. Cell ageing was found to affect the responses of both systems in a markedly different manner. In the single mediator system, and as observed in a previous study (Trosok et al. 2001), the response to exogenous glucose increased significantly with cell age. In the present work, the endogenous response was also monitored and was found to remain constant. In contrast, in the double mediator system both the exogenous and endogenous responses decreased with cell age. Over 14 days, the ratio of exogenous:endogenous responses increased for the single mediator system and decreased for the double mediator system. In contrast, cells stored for 21 days at 4°C retained the same single and double mediator responses seen in fresh cells.

The different behaviour of the responses in the single and double mediator trials points to different origins for the two responses. Considering the double mediator response, we note that in mammalian cells the signal was shown to arise from menadione oxidation of either NADPH produced in the pentose phosphate pathway, or reduced molecules at the proximal end of the electron transport chain (Rabinowitz et al. 1998). In the present work, the concentration of glucose in the growth and incubation medium is above the concentration required to initiate catabolite repression in *S. cerevisiae* (Walker 1998). Thus, the organisms will be operating in fermentative mode and it is likely that menadione is reduced by NADPH from the pentose phosphate pathway and not by electron transport chain components.

The origin of the single hydrophilic mediator response is more uncertain. The effects of cell ageing suggest that the single mediator response arises from a branch of the respiratory pathway. *S. cerevisiae* cells aged in the absence of glucose will become increasingly catabolically derepressed with time, resulting in a fully functional respiratory pathway. When glucose is added to the starved cells, respiration of glucose will occur, at least for a short period of time. It is likely that a redox active molecule from the respiration of glucose diffuses out through the cell membrane where it reduces ferricyanide.

The persistence of the fresh cell responses in cells stored for 21 days at 4°C supports the notion that ageing the cells under aerobic conditions has resulted in a change in the pathways associated with the catabolism of glucose. Attempts to further elucidate the details of yeast-mediator interactions are continuing.

Yeast species show potential as the biocomponent of mediated electrochemical whole cell biosensors. It is not yet clear whether the single or double mediator system would be more appropriate for this application. On the one hand, the double mediator responses are very much larger than the single mediator responses and the maximum signal from the double mediator system is obtained without cell ageing. Further, there is no change in cell response after storage of the cells for 21 days. On the other hand, the most favourable exogenous: endogenous response ratio is obtained with the single mediator system. A relatively small endogenous response is advantageous for biosensor applications because of the improved signal:background ratio. Experiments are planned to determine the stability of the responses of both systems to cell storage and multiple cell use.

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