Electrochemical Probing of in Vivo 5-Hydroxymethyl Furfural Reduction in Saccharomyces cerevisiae

ARTICLE *in* ANALYTICAL CHEMISTRY · NOVEMBER 2009

Impact Factor: 5.64 · DOI: 10.1021/ac901402m · Source: PubMed

6 AUTHORS, INCLUDING:

Natalie Kostesha
Technical University of Denmark
19 PUBLICATIONS **188** CITATIONS
SEE PROFILE

João R. M. Almeida
Brazilian Agricultural Research Corporatio…
21 PUBLICATIONS **1,199** CITATIONS
SEE PROFILE

Arto Heiskanen
Technical University of Denmark
51 PUBLICATIONS **458** CITATIONS
SEE PROFILE

Marie F. Gorwa-Grauslund
Lund University
86 PUBLICATIONS **4,661** CITATIONS
SEE PROFILE

Anal. Chem. **2009,** *81,* 9896–9901

Electrochemical Probing of in Vivo 5-Hydroxymethyl Furfural Reduction in *Saccharomyces cerevisiae*

Natalie V. Kostesha,[†,‡,§] João R. M. Almeida,[†] Arto R. Heiskanen,[‡,§] Marie F. Gorwa-Grauslund,[†] Barbel Hahn-Hägerdal,[†] and Jenny Emnéus*[,§]

Department of Applied Microbiology, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden, Department of Analytical Chemistry, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden, and Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsteds Plads 345 East, DK-2800 Kgs. Lyngby, Denmark

In this work, mediated amperometry was used to evaluate whether differences in intracellular nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) level could be observed between a genetically modified *Saccharomyces cerevisiae* strain, engineered for NADPH dependent 5-hydroxymethyl-2-furaldehyde (HMF) reduction, and its control strain. Cells overexpressing the alcohol dehydrogenase 6 gene (ADH6 strain) and cells carrying the corresponding control plasmid (control strain) were each immobilized on Au-microelectrodes. The real-time dynamics of NAD(P)H availability in the two strains, preincubated with HMF, was probed using the menadione−ferricyanide double mediator system. A lower intracellular NADPH level as the consequence of more effective HMF reduction was observed for the ADH6 strain both with and without added glucose, which increases the overall cellular NADPH level. The mediated amperometric signal during real-time monitoring of the concentration dependent HMF reduction in living cells could be translated into the cellular enzyme kinetic parameters: $K_{M,cell}^{app}$, V_{MAX}, $k_{cat,cell}$, and $k_{cat,cell}/K_{M,cell}^{app}$. The results indicated that the overexpression of the *ADH6* gene gave a 68% decrease in $K_{M,cell}^{app}$ and 42% increase in V_{MAX}, resulting in a 4-fold increase in $k_{cat,cell}/K_{M,cell}^{app}$. These results demonstrate that the mediated amperometric method is useful for monitoring the short-term dynamics of NAD(P)H variations and determining cellular enzyme kinetic parameters in *S. cerevisiae* cells.

During the last 30 years, the yeast *Saccharomyces cerevisiae* has become one of the most prominent model organisms for cell biology, microbiology, biochemistry, and genetics; highlights in the nuclear genome sequence of the yeast now play a central role in the yeast metabolism research. The understanding of sugar metabolism is the major goal in the extension of the yeast industrial potential. Yeast sugar metabolism results in the formation and utilization of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH). Moreover, the intracellular NAD^+/NADH and $NADP^+$/NADPH balance has a critical importance in the central metabolism and leads to energy output and product formation related to the redox balances in the cell.[1−3] *S. cerevisiae* is very robust, readily genetically manipulated and has a high degree of conservation with respect to human genes,[4] which also makes it of high interest to use for drug screening.[4,5]

Mediated electrochemistry has been successfully utilized in cell-biosensor applications for studying enzyme kinetics of intact prokaryotic[6,7] and eukaryotic[8] cells. In these applications, the probed enzymes are, however, located in the plasma membrane and do not require any intracellular cofactor, such as NAD(P)H. On the other hand, determination of the NAD(P)H level or activity of NAD(P)H-dependent redox enzymes based on traditional in vitro assay systems is not possible without disruptive strategies to prepare subcellular fractions for isolating NAD(P)H[9] or cell extracts for enzyme activity assay.[10] None of these approaches allows one to probe the NAD(P)H level or redox enzyme activity in relation to the metabolism of living cells. The total in vivo levels of NADH and NADPH of eukaryotic and mammalian cells have been quantified using electrochemical[11−13] and to some extent

* Corresponding author. Phone: +45-45256867. Fax: +45-45887762. E-mail: Jenny.Emneus@nanotech.dtu.dk.

† Department of Applied Microbiology, Lund University.

‡ Department of Analytical Chemistry, Lund University.

§ Technical University of Denmark.

(1) Almeida, J. R. M.; Roder, A.; Modig, T.; Laadan, B.; Liden, G.; Gorwa-Grauslund, M. F. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 939–945.
(2) dos Santos, M. M.; Raghevendran, V.; Kotter, P.; Olsson, L.; Nielsen, J. *Metab. Eng.* **2004**, *6*, 352–363.
(3) van Dijken, J. P.; Scheffers, W. A. *FEMS Microbiol. Rev.* **1986**, *32*, 199–224.
(4) Hughes, T. R. *Funct. Integr. Genom.* **2002**, *2*, 199–211.
(5) Mager, W. H.; Winderickx, J. *Trends Pharmacol. Sci.* **2005**, *26*, 265–273.
(6) Ikeda, T.; Kato, K.; Maeda, M.; Tatsumi, H.; Kano, K.; Matsushita, K. *J. Electroanal. Chem.* **1997**, *430*, 197–204.
(7) Takayama, K.; Kurosaki, T.; Ikeda, T. *J. Electroanal. Chem.* **1993**, *356*, 295–301.
(8) Racek, J.; Musil, J. *Clin. Chim. Acta* **1987**, *162*, 129–139.
(9) Ting, H. Y.; Jacobson, E. L.; Jacobson, M. K. *Arch. Biochem. Biophys.* **1977**, *183*, 98–104.
(10) Katz, M.; Johanson, T.; Gorwa-Grauslund, M. F. *Yeast* **2004**, *21*, 1253–1267.
(11) Baronian, K. H. R.; Downard, A. J.; Lowen, R. K.; Pasco, N. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 108–113.
(12) Heiskanen, A.; Yakovleva, J.; Spegel, C.; Taboryski, R.; Koudelka-Hep, M.; Emneus, J.; Ruzgas, T. *Electrochem. Commun.* **2004**, *6*, 219–224.
(13) Rabinowitz, J. D.; Vacchino, J. F.; Beeson, C.; McConnell, H. M. *J. Am. Chem. Soc.* **1998**, *120*, 2464–2473.

10.1021/ac901402m CCC: $40.75
Published on Web 11/19/2009

fluorescence based detection systems.[14] However, these methods are unable to distinguish between the different cofactors and thus different metabolic pathways. This can be, however, achieved by either introducing different inhibitors that affect different NADH and NADPH dependent metabolic pathways[15,16] and/or using genetically engineered cells, in which certain enzymes have been, for instance, deleted.[17,18] Previously, we used a real-time amperometric method, based on the double mediator system, lipophilic menadione, and hydrophilic ferricyanide,[18] to study the intracellular difference between the NADH and NADPH levels in a deletion mutant strain of *Saccharomyces cerevisiae* lacking the gene encoding for phosphoglucose isomerase (PGI). With the supply of the PGI deletion strain and the control strain with different carbon sources (glucose and fructose)[18] as well as introduction of the inhibitor dicoumarol,[17] significant effects on the amperometric current could be observed, which enabled the probing of different metabolic pathways.

In the present work, we explore instead a genetically engineered strain of *S. cerevisiae*, in which the gene encoding for the NADPH dependent enzyme alcohol dehydrogenase 6 (ADH6) is overexpressed (ADH6 strain).[19] The ADH6 strain is able to ferment lignocellulose to ethanol. During pretreatment and liquefaction of lignocellulose, 5-hydroxymethyl-2-furaldehyde (HMF) is formed as a toxic byproduct[20,21] at relatively high concentration.[22,23] Since HMF is a substrate for ADH6, the ADH6 strain is extremely efficient in HMF detoxification and as a result leads to increased ethanol production from lignocellulose.[21,23]

The goal of this work was to evaluate the in vivo effect of HMF on the intracellular NAD(P)H level in the ADH6 strain and the control strain containing an empty plasmid. The evaluation was done using the previously developed real-time amperometric method based on the menadione-ferricyanide double mediator system. Figure 1 shows the proposed mechanism of the double mediator system in living yeast cells when HMF is present. Menadione penetrates into the intracellular compartment where it is reduced by NAD(P)H-dependent menadione reducing enzymes (MREs). The reduced form, menadiol, diffuses out of the cell and undergoes reoxidation to menadione by ferricyanide in the extracellular environment. Electrochemical reoxidation of the produced ferrocyanide to ferricyanide is then monitored as an anodic current at a yeast-Au-microband electrode (yeast-Au-MBE). By addition of HMF to the cells, the current decreases since some of the NADPH is consumed for the reduction of HMF instead of

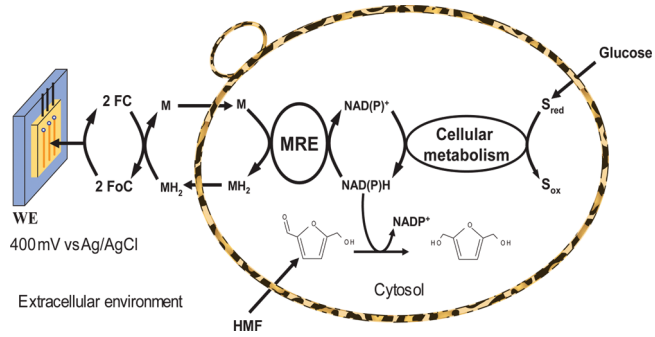

Figure 1. A schematic view of the putative mechanism of the generated current via the double mediator system in living yeast cells. Menadione (M) diffuses through the plasma membrane into the cell, where it is reduced by NAD(P)H-dependent menadione reducing enzymes (MREs) to menadiol (MH_2). MH_2 then diffuses back into the extracellular environment where it is reoxidized to M while reducing ferricyanide (FC) to ferrocyanide (FoC). The oxidation of FoC is recorded amperometrically at +400 mV vs Ag/AgCl. If HMF is added to the system, part of the NADPH pool is instead consumed for the reduction of HMF to 5-hydromethylfurfuryl alcohol by ADH6 in the presence of NADPH, which results in a decrease in the amperometric signal.

for reduction of menadione. This approach demonstrates the applicability of real-time amperometry in both screening of genetic modifications and determining enzyme kinetic parameters, $K^{app}_{M,cell}$, V_{MAX}, $k_{cat,cell}$, and $k_{cat,cell}/K^{app}_{M,cell}$, of live *Saccharomyces cerevisiae* cells. The method allows simultaneous determinations using a small number of cells of different *S. cerevisiae* strains without disrupting the cells, which is required in traditional in vitro enzyme assays.

EXPERIMENTAL SECTION

Chemicals and Media. Menadione (2-methyl-1,4-naphtoquinone), potassium ferricyanide, glucose, NADH, NADPH, 5-hydroxymethyl furfural (5-hydroxymethyl-2-furaldehyde, HMF), and sodium alginate were all purchased from Sigma Chemical Company. (St. Louis, MO). The Yeast Nitrogen Base (YNB) medium without amino acids was purchased from Difco (Sparks, MD). Yeast protein extraction reagent (Y-PER) and BCA Protein Assay Kit were obtained from Pierce (Rockford, IL). Glucose for culture media was purchased from BDH (Poole, U.K.). All other chemicals were of analytical grade and used without further purification. Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA).

Buffers and Reagents. For amperometric measurements, Tris-succinate buffer (TSB) was prepared in Milli-Q water using 10 mM Tris, 10 mM succinic acid, 100 mM KCl, and 10 mM $CaCl_2$; pH was adjusted to 5.0 with KOH. Potassium ferricyanide stock solution (500 mM) was prepared directly in TSB. Menadione stock solution was prepared by dissolution in either TSB (pH 5) or 100 mM phosphate buffer (pH 7) (PB) to give a concentration of 400 μM. Potassium ferricyanide and menadione stock solutions were stored in lightproof flasks at 4 °C. A stock solution of 1 M glucose was prepared in TSB and then sterile filtered (Millex-GS 0.22 μm, Millipore Co., Cork, Ireland) and stored at −20 °C. Stock solutions of NADH and NADPH (7.0 mM in PB) as well as HMF (4 M in either TSB or PB) were prepared immediately before use without filtration. All solutions were thermostatted at 30 °C before experiments.

(14) Zhao, J. S.; Wang, Z.; Wang, M.; He, Q. P.; Zhang, H. *Bioelectrochemistry* **2008**, *72*, 117–121.
(15) Zhao, J. S.; Wang, M.; Yang, Z. Y.; Yang, Z. Y. *Enzyme Microb. Technol.* **2005**, *37*, 246–253.
(16) Zhao, J. S.; Yang, Z. Y.; Lu, Y.; Yang, Z. Y. *Chin. Chem. Lett.* **2005**, *16*, 513–516.
(17) Heiskanen, A.; Spégel, C.; Kostesha, N.; Lindahl, S.; Ruzgas, T.; Emnéus, J. *Anal. Biochem.* **2009**, *384*, 11–19.
(18) Spegel, C. F.; Heiskanen, A. R.; Kostesha, N.; Johanson, T. H.; Gorwa-Grauslund, M. F.; Koudelka-Hep, M.; Emneus, J.; Ruzgas, T. *Anal. Chem.* **2007**, *79*, 8919–8926.
(19) Petersson, A.; Almeida, J. R. M.; Modig, T.; Karhumaa, K.; Hahn-Hagerdal, B.; Gorwa-Grauslund, M. F.; Liden, G. *Yeast* **2006**, *23*, 455–464.
(20) Palmqvist, E.; Almeida, J. S.; Hahn-Hagerdal, B. *Biotechnol. Bioeng.* **1999**, *62*, 447–454.
(21) Taherzadeh, M. J.; Gustafsson, L.; Niklasson, C.; Liden, G. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 701–708.
(22) Palmqvist, E.; Hahn-Hagerdal, B. *Bioresour. Technol.* **2000**, *74*, 25–33.
(23) Almeida, J. R. M.; Modig, T.; Petersson, A.; Hahn-Hagerdal, B.; Liden, G.; Gorwa-Grauslund, M. F. *J. Chem. Technol. Biotechnol.* **2007**, *82*, 340–349.

Yeast Strains and Growth Conditions. *S. cerevisiae* strains TMB3286 (ADH6 strain), overexpressing the *ADH6* gene on a multicopy plasmid,[19] and TMB3280 (control strain), carrying the corresponding empty plasmid,[19] were used for the study. The strains were kept on agar plates (VWR International, Stockholm, Sweden) containing YNB medium supplemented with 20 g/L glucose. Colonies were taken from agar plates and preinoculated in 5 mL of YNB medium supplemented with 20 g/L glucose in 50 mL conical Falcon tubes (BD Biosciences, San Jose, CA). After overnight incubation at 30 °C, the culture was diluted to an OD_{620} of 0.5, measured with a Hitachi U-1100 spectrophotometer (Hitachi Ltd. Tokyo, Japan). Cells were then grown in 50 mL of the same medium at 180 rpm and 30 °C until the early stationary phase. Cells were harvested by centrifugation for 5 min, using an Avanti J-25I centrifuge with a JA-10 rotor (Beckman Coulter Inc., Fullerton, CA) at 4 °C with a rotation speed of 4000 rpm. Cell pellets were first resuspended in 1 mL of Milli-Q water and washed twice by centrifugation, then resuspended in Milli-Q water to give a final OD_{620} of 70, which subsequently was used for immobilization on Au-MBEs and electrochemical assays.

Preparation of Crude Cell Extracts. For spectrophotometric assessment of ADH6 activity, cells were grown in 50 mL of YNB medium supplemented with 20 g/L glucose at 180 rpm and 30 °C until early stationary phase. Cells were harvested by centrifugation at 2500 rpm in an Avanti J-25I centrifuge with a JA-12 rotor (Beckman Coulter Inc., Fullerton, CA) for 5 min at 20 °C. The cell pellet was washed twice in Milli-Q water. Crude cell extract was prepared using Y-PER reagent. The protein concentration was determined with a Micro BCA Protein Assay Kit using bovine serum albumin as a standard.

Spectrophotometric Assessment of ADH6 Activity. The total menadione and HMF reducing capacity in crude cell extracts was determined by following the oxidation of NADPH and NADH via the change in absorbance at 340 nm over 60 s at 30 °C with a Hitachi U-2000 spectrophotometer (Hitachi Ltd. Tokyo, Japan). All assays were performed in the total volume of 1 mL using PB. The reagents were added in the following order: 250 μL of menadione stock solution in PB, 2.5 μL of HMF stock solution in PB, and 50 μg of crude cell extract. The reaction was initiated by the addition of 20 μL of 7.0 mM NADPH or NADH in PB. Experimental results are presented as an average ± standard error of mean (SEM) of three triplicate measurements using three independently grown cultures with three resulting extracts ($N = 3$, $n = 3$).

Immobilization of Cells on Au Microband Electrode (Au-MBE) Chips. A 0.5 mL cell suspension with an OD_{620} of 70 was gently mixed in a Falcon tube with 0.5 mL of 2% (w/v) sodium alginate. Yeast cells were then immobilized on Au-MBEs containing three individually addressable Au electrode bands, fabricated using standard UV lithography methods at the Department of Electrical Measurements, Lund University, Sweden (Si/SiO_2 substrate, Cr/Au (20 nm/200 nm), SU8 passivation layer (5 μm), microband dimension: width/length 25/1000 μm). The Au-MBEs were dipped into the yeast-alginate suspension, and excess material was removed by gently shaking the electrodes, creating a thin film of yeast/sodium alginate. To fix the yeast/alginate layer, the electrodes were immersed into a cross-linking solution (100 mM $CaCl_2$ in Milli-Q water) for 30 s to form a stable yeast/Ca-alginate film.

Electrochemical System. The tip of a working yeast-Au-MBE was introduced into a 30 °C thermostatted three-electrode electrochemical cell containing an Ag/AgCl (saturated KCl) reference and a Pt counter electrode. All experiments were performed with magnetic stirring at 250 rpm. The electrodes were connected to a CHI1000A multipotentiostat (CH Instruments Inc., Austin, TX) with an applied potential of +400 mV vs Ag/AgCl.

Electrochemical Assessment of ADH6 Activity. All experiments were performed in TSB with a total reaction volume of 20 mL. Estimation of the menadione reducing capacity (MRC) of the immobilized yeast cells was performed as follows: the yeast-Au-MBEs were preincubated for 15 min in TSB with or without 10 mM glucose present. Ferricyanide was then added to a final concentration of 2 mM and a baseline current recorded. After 30 s, menadione was added to a final concentration of 100 μM and a current−time response curve recorded (three data points for each measurement ($N = 3$), i.e., one from each of the three Au-microbands). All experiments were performed in triplicates ($n = 9$).

The effect of HMF on the MRC was evaluated in two ways: (1) static mode, yeast-Au-MBEs were preincubated for 15 min in TSB with or without 10 mM glucose present. HMF was added to a final concentration of 20 mM and incubated for 15 min. Ferricyanide and menadione were then added to a final concentration of 2 mM and 100 μM, respectively. All experiments were performed in triplicates ($N = 3$, $n = 9$). (2) Dynamic mode, yeast-Au-MBEs were incubated with TSB containing 10 mM glucose for 15 min, followed by the addition of ferricyanide and menadione to a concentration of 2 mM and 100 μM, respectively. When the current reached a steady state, HMF was added stepwise every 15 min to final concentrations of 5, 20, 40, 60, 80, and 100 mM in the solution. The current−time traces were recorded after each addition ($N = 3$, $n = 9$).

Generation of Concentration Response Curves. The effect of HMF on the MRC of living yeast cells is presented in the form of concentration response curves. The data was obtained from the steady state current−time response curves and reported as the difference between the initial steady state current before HMF addition and the new obtained steady state current after each HMF addition ($|\Delta i|$). The data was fitted to the four-parameter logistic equation (eq 1) (hyperbolic dose−response) using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA). The error bands represent the 95% confidence interval for each concentration response curve ($N = 3$, $n = 9$).

$$|\Delta i| = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + \left(\frac{10^{\log(EC_{50})}}{10^{\log[HMF]}}\right)^{\text{Hill slope}}} \qquad (1)$$

RESULTS AND DISCUSSION

Spectrophotometric Assessment of ADH6 Activity. Spectrophotometric assays were first run for the ADH6 strain and the control strain with menadione as the only substrate in order to check that ADH6 was not involved in the reduction of menadione. The assays were performed using NADH or NADPH and extracts of glucose-grown cells harvested during the early stationary phase.

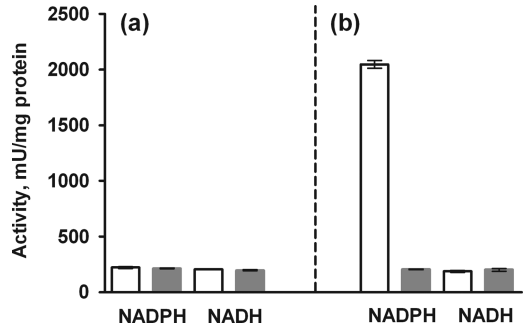

Figure 2. Spectrophotometric assessment of ADH6 activity in crude cell extracts from the ADH6 strain (black bars) and the control strain (gray filled bars) after addition of NADPH or NADH in the presence of (a) 100 μM menadione and (b) 10 mM HMF and 100 μM menadione. Experimental results are presented as the average ± SEM of three independently grown cultures with three resulting extracts and triplicate measurements for each extract ($N = 3$, $n = 9$).

The conditions were designed to mimic the real-time electrochemical assessment, i.e., in the presence of 100 μM menadione. The obtained NADPH- and NADH-dependent activities (Figure 2a) for the ADH6 strain were 214 ± 3 and 222 ± 8 mU/mg of protein, respectively, and for the control strain 205 ± 3 and 197 ± 5 mU/mg of protein, respectively. For both strains, the NADPH- and NADH-dependent activities are equal according to the *t*-test at 95% confidence level ($P = 0.374$ (ADH strain) and $P = 0.199$ (control strain)), which clearly indicates that menadione is not a substrate for ADH6.

Spectrophotometric assays, in the copresence of 10 mM HMF and 100 μM menadione, were run in the same way as above to compare the ADH6 activities in the two strains. As seen in Figure 2b, the measured NADPH-dependent HMF reduction for the ADH6 strain (2050 ± 40 mU/mg of protein) was 10 times higher than for the control strain (205 ± 3 mU/mg of protein). This is comparable with previously reported data,[19] although it should be noted that in the current work the measured change in NADPH concentration during the assay also comprises the contribution of mendione reduction. The absence of NADH-dependent ADH6 activity with respect to HMF was also confirmed. The determined activities were 188 ± 8 mU/mg of protein for the ADH6 strain and 200 ± 10 mU/mg of protein for the control strain. According to the *t*-test at 95% confidence level, these activities are equal ($P = 0.820$) and comparable to the determined activities in the absence of HMF in Figure 2a.

Electrochemical Assessment of ADH6 Activity. The MRC of the immobilized ADH6 strain and control strain were compared, using previously optimized menadione and ferricyanide concentrations of 100 μM and 2 mM, respectively.[18] Both strains were preincubated for 15 min with or without the presence of 10 mM glucose. The amperometric current was recorded after the addition of ferricyanide and menadione, reflecting the availability of NAD(P)H for menadione reduction by MREs. As seen in Figure 3a, higher steady state currents were recorded for both strains when cells were preincubated with glucose, confirming the increased availability of NAD(P)H for menadione reduction through metabolic glucose oxidation. Moreover, the obtained steady state currents reached similar levels for both strains, again indicating that ADH6 is not involved in menadione reduction.

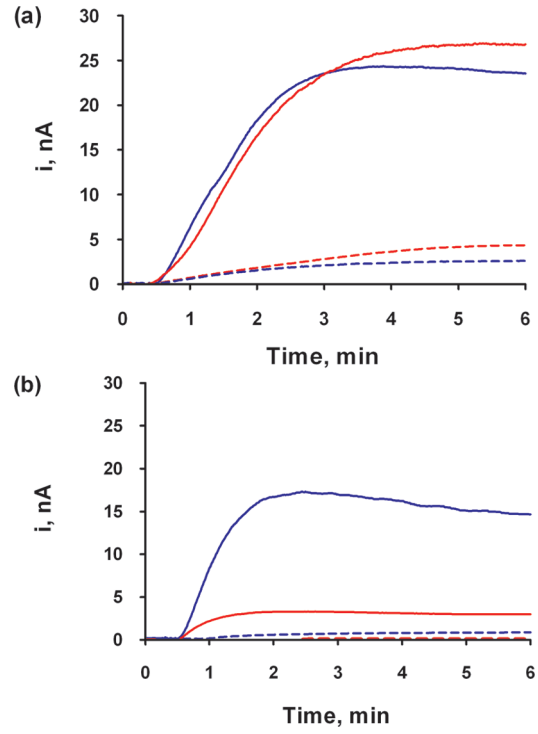

Figure 3. Electrochemical assessment of the MRE activity with and without incubation in the presence of HMF: (a) Current−time traces obtained after addition of the mediators (2 mM ferricyanide and 100 μM menadione) for the ADH6 strain (red lines) and control strain (blue lines). (b) Current−time traces obtained after incubation with 20 mM HMF for 15 min followed by addition of the mediators (2 mM ferricyanide and 100 μM menadione) for the ADH6 strain (red lines) and control strain (blue lines). In both parts a and b, cells were preincubated for 15 min either with (solid lines) or without (dashed lines) glucose (10 mM).

The impact of HMF reduction on the availability of intracellular NAD(P)H was investigated by comparing the amperometric response of the ADH6 and control strain. Two kinds of experiments were conducted, i.e., (1) static mode, conducted as above by preincubation with HMF and (2) dynamic mode, involving successive addition of HMF and recording of the change in the current after each addition. The static mode in the electrochemical assay was designed and performed for fast screening of the genetic modifications using living cells instead of crude cell extracts. The versatility of the method was demonstrated by monitoring the response of the ADH6 and control strain to glucose as well as to HMF, which functions as a substrate for ADH6. In the static mode, the in vivo competition for intracellular NADPH between MREs and the ADH6 was investigated by preincubating both strains for 15 min with or without 10 mM glucose, followed by 15 min incubation with 20 mM HMF, and finally addition of ferricyanide (2 mM) and menadione (100 μM). As seen in Figure 3b, the electrochemical response was again very low for both strains in the absence of glucose due to the limited availability of NAD(P)H for the MREs. When glucose was added, the steady state current increased for both strains, but a significantly lower steady state current was obtained for the ADH6 strain, indicating a shortage of NAD(P)H for menadione reduction by MREs, i.e., a larger pool of the available NADPH is used for HMF reduction in the ADH6 strain compared to the control strain.

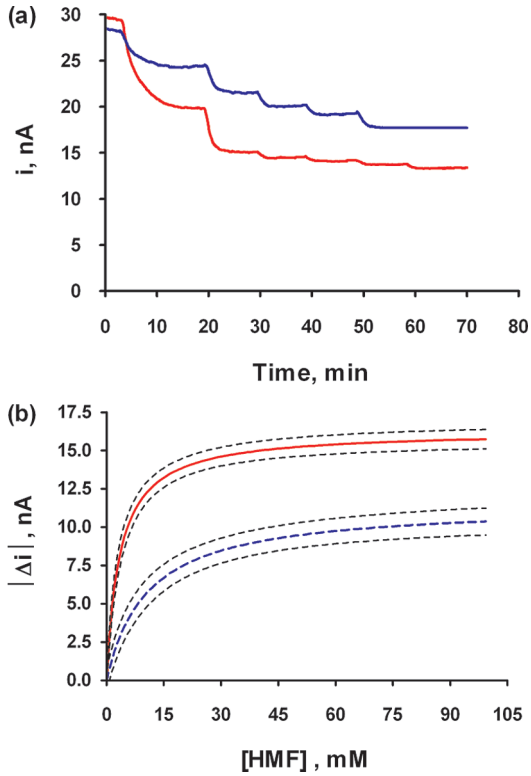

Figure 4. (a) In vivo amperometric response of the ADH6 strain (red line) and control strain (blue line) after incubation with 10 mM glucose. The initial steady-state was obtained after addition of mediators (100 μM menadione and 2 mM ferricyanide), followed by successive additions of HMF to the final concentrations of 5, 20, 40, 60, 80, and 100 mM. (b) The data extracted from part a were fitted to the hyperbolic concentration response relationship (eq 1): ADH6 strain (red line) and control strain (blue line). The error bands (black dashed lines) represent the 95% confidence interval for each concentration response curve ($N = 3$, $n = 9$).

The dynamic mode provides an alternative that enables the evaluation of the concentration dependent effect of different chemicals on intracellular cofactor availability. In the dynamic mode, the in vivo competition for intracellular NADPH between MREs and ADH6 was investigated by monitoring the amperometric response of MREs upon titration with increasing levels of HMF in the ADH6 strain and the control strain. Both strains were preincubated with 10 mM glucose for 15 min, and the steady state signal recorded after the addition of 2 mM ferricyanide and 100 μM menadione. When stable steady-state currents were reached, HMF was successively added into the system and the resulting currents for each addition to the two strains were recorded. As seen in Figure 4a, the addition of HMF resulted in an almost immediate reduction of the current response for both strains and the decrease was proportional to the level of HMF added. Moreover, it is clearly seen that the ADH6 strain is capable of reducing HMF more efficiently than the control, showing a much stronger decrease in the current response. A leveling off of the current was observed, when the concentration of HMF approached 100 mM, after which significant changes of the current could not be distinguished. This can be explained by the findings of Modig et al. and Liu et al.[24,25] who observed toxic effects of HMF on genetically modified yeast when the concentration of HMF rose above 100 mM; HMF is known to inhibit important metabolic enzymes, such as alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase.

In Figure 4b, the data were fitted to the four parameter logistic equation, which under the conditions, Hill slope = 1 and bottom = 0, is simplified to a hyperbolic dose−response relationship (eq 2),[26] where $[HMF]_b$ is the bulk concentration of HMF.

$$|\Delta i| = \frac{\text{top}[HMF]_b}{EC_{50} + [HMF]_b} \tag{2}$$

It should be noted that the amperometric steady state current measured in the electrochemical system is a result of the in vivo reduction of menadione by MREs of the yeast cells and that menadione was shown not to be a substrate for ADH6 (Figures 2a and 3a). This means that the decrease in the steady state current due to addition of HMF is not a direct effect on the MREs but rather an indirect effect due to a competition between MREs and ADH6 for the available NADPH to enable the reduction of their respective substrates, menadione and HMF. The dose−response curves in Figure 4b can thus be seen as actually mirroring the apparent enzyme kinetics of ADH6 for its substrate HMF in the presence of NADPH, assuming pseudo first order reaction kinetics. The hyperbolic dose−response relationship can in fact easily be translated into the Michaelis−Menten relationship from which the apparent Michaelis−Menten constant ($K^{app}_{M,cell}$) and the maximum current (I_{MAX}) for HMF reduction of the two strains can be extracted (eq 3).

$$|\Delta i| = \frac{I_{MAX} \times [HMF]_b}{K^{app}_{M,cell} + [HMF]_b} \tag{3}$$

A clear difference in I_{MAX} for the two strains can be seen, showing that the ADH6 strain as expected was able to reduce HMF at a much higher velocity than the control strain. I_{MAX} values with a 95% confidence interval were calculated from the hyperbolic dose−response curves for the two strains: 16.3 ± 0.4 nA for the ADH6 strain and 11.5 ± 0.7 nA for the control strain The $K^{app}_{M,cell}$ values for the ADH6 and control strain were 3.5 ± 0.6 and 11 ± 3 mM, respectively. Despite the fact that the amperometrically determined HMF reducing activity of both strains reflects the behavior of ADH6, the observed difference in $K^{app}_{M,cell}$ is explicable. Petersson et al.[19] demonstrated that aside from the increased enzyme activity, overexpression of the ADH6 gene also renders the cells 4 times more effective in uptake of HMF. Since $K^{app}_{M,cell}$ reflects the behavior of ADH6 in the entire cellular context, a limitation in substrate uptake also affects the kinetics.

Using Faraday's law of electrolysis, it is possible to convert the I_{MAX} values into V_{MAX} values, reflecting the number of moles of consumed substrate per unit time by the cells (eq 4):

$$V_{MAX} = \frac{n_{HMF}}{\tau} = \frac{60 I_{MAX}}{nF} \tag{4}$$

(24) Liu, Z. L. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 27–36.
(25) Modig, T.; Liden, G.; Taherzadeh, M. J. *Biochem. J.* **2002**, *363*, 769–776.
(26) Motulsky, H.; Christopoulos, A. *Fitting Models to Biological Data Using Linear and Nonlinear Regression: A Practical Guide to Curve Fitting*, 3rd ed.; GraphPad Software Inc.: San Diego, CA, 2003.

where n_{HMF} is the number of moles of HMF reduced per unit time, τ is the unit time for the enzymatic rate (min), I_{MAX} is given in Coulomb/second (C/s), n is the number of transferred electrons, and F is Faraday's constant (96 485 C/s). Considering the fact that the recorded current is obtained due to oxidation of ferrocyanide and that two ferrocyanide ions are formed for each consumed NADPH molecule (corresponding indirectly to each consumed HMF molecule), $n = 2$. Computation yields the following V_{MAX} for the two strains: 5.1 ± 0.1 (ADH strain) and 3.6 ± 0.2 pmol HMF/min (control strain). Taking into consideration that *S. cereviciae* cells are immobilized in a ~100 μm thick layer of alginate on the electrode surface,[27] the V_{MAX} values can be expressed in nanomoles of HMF per minute square centimeter to obtain a standardized measure of cellular HMF reducing capacity in the utilized immobilization matrix. When converted to nanomoles of HMF per minute square centimeter, the corresponding V_{MAX} are 20.2 ± 0.4 and 14.3 ± 0.9 nmol HMF/(min cm^2), respectively. On the basis of the determined V_{MAX} values alone, the overexpression of the ADH6 gene increases the cellular HMF reducing capacity by 42%.

As was mentioned earlier, the HMF uptake of the control strain is lower than that of the ADH6 strain, which increases the $K^{app}_{M,cell}$ of the control. This fact also influences the cellular HMF reducing capacity, which becomes especially pronounced at HMF concentrations that are much lower than the $K^{app}_{M,cell}$. Under such conditions and by combining eqs 3 and 4, the rate equation may be written (eq 5).

$$v_0 = \frac{V_{MAX}[HMF]_b}{K^{app}_{M,cell}} = \frac{K_{cat,cell}}{K^{app}_{M,cell}}[cell]_T[HMF]_b \qquad (5)$$

$k_{cat,cell}$ is the catalytic constant (i.e., the turnover number) that gives how many catalytic cycles each cell performs per unit time. Since V_{MAX} was expressed as nanomoles of HMF per minute square centimeter, the total cell density, $[cell]_T$, on the active electrode surface (a gold microband) can be expressed as the number of moles of cells per square centimeter. Previously, it has been shown that the number of cells in contact with a gold microband is about 5700 cells corresponding to 23×10^6 cells/cm^2.[27] Upon use of this cell density in the calculations and seconds as the unit of time, the following values are obtained for $k_{cat,cell}$ of the ADH6 strain and the control strain: $(8.9 \pm 0.2) \times 10^6$ s^{-1} and $(6.3 \pm 0.4) \times 10^6$ s^{-1}, respectively. In enzyme kinetics, k_{cat}/K_M is used as a measure of the catalytic efficiency[28] of an enzyme. In an analogous way, $k_{cat,cell}/K^{app}_{M,cell}$ can provide a measure of the cellular HMF reducing capacity that reflects the combined effect of the turnover and the apparent cellular Michaelis−Menten constant. The calculated values of this ratio are $(2.5 \pm 0.1) \times 10^9$ M^{-1} s^{-1} (ADH6 strain) and $(0.6 \pm 0.1) \times 10^9$ M^{-1} s^{-1} (control strain). These values are greater than expected in a system solely under diffusion control. However, in the present work, the calculated values were obtained in a system with a thin layer of cells on electrodes under strong forced convection, which provides mass transfer different from conventional enzyme assays based on diffusion. Furthermore, $K^{app}_{M,cell}$ is related to the bulk concentration of HMF, i.e., the dose the cells are exposed to, which is different from the actual concentration the cells have taken up. Because of these considerations, the values of $k_{cat,cell}/K^{app}_{M,cell}$ are not directly comparable to values of the ratio k_{cat}/K_M, which are directly related to the substrate concentration applied in the enzymatic reaction. However, $k_{cat,cell}/K^{app}_{M,cell}$ serves as a factor that can be used to compare different cell types in a manner that takes into consideration the whole cellular context, including both the enzyme activity of interest and the cellular capacity of substrate uptake. In this study, the ADH6 strain shows about 4-fold greater efficiency in reducing HMF than the control strain. This in vivo determined relation between the efficiencies is in agreement with the previously published in vitro enzyme assays[19] and clearly shows the applicability of amperometric assessment of intracellular redox enzyme activity.

CONCLUSIONS

In this work, real-time amperometry, using the double mediator system menadione-ferricyanide, was applied for determining the effect of overexpressing in *S. cerevisiae* the gene encoding for alcohol dehydrogenase 6 (ADH6) on the intracellular NAD(P)H level and the enzyme kinetic parameters of ADH6 in the presence of 5-hydroxymethyl-2-furaldehyde. On the basis of the obtained results, the mediated amperometric method is useful for fast screening of genetic modifications and determining enzyme kinetic parameters in relation to the intracellular metabolism in eukaryotic cells, reflecting changes in the intracellular NAD(P)H levels. This in vivo method has the ability to accurately follow, in real-time, the dynamics of cellular processes and to quantify the activity of redox enzymes in living cells that can be used for screening of compounds with potential oxidation and reduction properties. In practice, it can be applied for either rapid reduction/oxidation control of industrial processes involved in fermentation, toxicological testing, or medical diagnostic research in the area of drug discovery for cancer and cardio- and neurological diseases.

ACKNOWLEDGMENT

The Marie Curie EST (BIONEL), the Network of Excellence Nano2Life, and the EU FP7 NMP Project EXCELL are kindly acknowledged for financial support. The authors would like to thank Professor Lo Gorton (Department of Biochemistry, Lund University) for valuable advice.

Received for review June 26, 2009. Accepted October 25, 2009.

AC901402M

(27) Kostesha, N.; Heiskanen, A.; Spégel, C.; Gorwa-Grauslund, M. F.; Hahn-Hägerdal, B.; Emnéus, J. *Bioelectrochemistry* **2009**, *76*, 180−188.

(28) Voet, D.; Voet, J. G., Pratt, C. W. *Fundamentals of Biochemistry*; John Wiley & Sons, Inc.: New York, 1999.