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Making glucose oxidase fit for biofuel cell applications by directed protein evolution

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Abstract

Progress in miniature chip-design raises demands for implantable power sources in health care applications such as continuous glucose monitoring of diabetic patients. Pioneered by Adam Heller, miniaturized enzymatic biofuel cells (mBCs) convert blood sugars into electrical energy by employing for example glucose oxidase (GOx) on the anode and bilirubin oxidase on the cathode. To match application demands it is crucial to increase lifetime and power output of mBCs. The power output has been limited by the performance of GOx on the anode. We developed a glucose oxidase detection assay (GODA) as medium-throughput screening system for improving GOx properties by directed protein evolution. GODA is a reaction product detection assay based on coupled enzymatic reactions leading to NADPH formation which is recorded at 340 nm. The main advantage of the assay is that it detects the production of D-gluconolactone instead of the side-product hydrogen peroxide and enables to improve bioelectrochemical properties of GOx. For validating the screening system, a mutagenic library of GOx from *Aspergillus niger* (EC 1.1.3.4) was generated and screened for improved activity using *Saccharomyces cerevisiae* as host. Directed evolution resulted in a GOx mutant I115V with 1.4–1.5-fold improved activity for β-D-glucose (V_{max} from 7.94 to 10.81 μmol min^{-1} mg^{-1}; $K_m \sim 19$–21 mM) and oxygen consumption kinetics correlate well [V_{max} (O_2) from 5.94 to 8.34 μmol min^{-1} mg^{-1}; K_m (O_2) from 700 to 474 μM]. The developed mutagenic protocol and GODA represent a proof-of-principle that GOx can be evolved by directed evolution in *S. cerevisiae* for putative use in biofuel cells.

Keywords: Biofuel cell; Glucose oxidase; Directed evolution; High-throughput screening; GODA

1. Introduction

Enzymatic biofuel cells are promising power sources to drive miniaturized electronic devices and biosensors within the human body (Heller, 2004). A main advantage of miniature biofuel cell (mBC) is its simplified design compared to a conventional fuel cell (Drews et al., 2001; Latham et al., 2004). This design omits the need for a case to confine fuel, electrodes and membranes and will likely allow miniaturizing enzymatic biofuel cells further than conventional power sources (Chen et al., 2001).

mBCs employ enzymes for converting chemical energy into electrical energy (Barton et al., 2004). In recent years research has been focused on design of miniature enzyme-based biofuel cells and electrically "wiring" of enzymes through conducting polymers (Chen et al., 2001). The choice of enzymes for implantable biofuel cells is limited by blood composition, physiological conditions (e.g. pH, temperature and ions) and requirements for a cathode and an anode. On the cathode mainly blue copper-containing oxidases such as laccase or bilirubin oxidase (BOD) have been employed (Kang et al., 2004). On the anode glucose oxidase from *Aspergillus niger* (*A. niger*; GOx; EC 1.1.3.4) and glucose dehydrogenase from *Acinetobacter calcoaceticus* (PQQGDH; EC 1.1.99.17) have preferentially been used as biocatalysts (Yuhashi et al., 2005).

The blood composition in arteries (glucose concentration ~15 mM, NaCl 100 mM, pH 7.2) differs significantly from the optimal performance conditions of GOx from *A. niger* which converts β-D-glucose to D-gluconolactone. GOx has an optimum pH between 5.5–6.0 and at pH 7 the activity of GOx is reduced by ~20% (Kenausis et al., 1997). The pH dependence and low chloride resistance of the GOx have been studied in detail (Bright and Appleby, 1969; Weibel and Bright, 1971). GOx from *A.*

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niger has a molecular weight around 155–160 kDa in glycosylated form and consists of two identical subunits (Hecht et al., 1993). The K_m value for β-D-glucose of GOx from *A. niger* has been reported to be between 11 and 41.8 mM (Bohmhammel et al., 1993). Current biofuel cells are limited by GOx in terms of power output and lifetime (Heller, 2004).

Directed evolution has become a powerful method for biocatalyst engineering to tailor enzyme properties to application demands (Arnold and Volkov, 1999; Cirino and Arnold, 2002). A directed protein evolution experiment comprises two main steps: generating diverse mutant libraries (Wong et al., 2004b) and screening for improved protein variants (Wong et al., 2004a, 2005). An analysis of improved mutants assists in understanding fundamental structure–function relationships and developing novel hypotheses for rational or semi-rational protein engineering.

A validated screening system is the key to success in any directed protein evolution experiment (Wong et al., 2005). Here we report for the first time a product based medium-throughput screening system for the directed evolution of GOx. The glucose oxidase detection assay (GODA) is based on NADPH formation in coupled enzyme reactions and was validated by improving the activity of GOx by directed protein evolution. The GODA medium-throughput screening protocol will especially be useful for evolving mediated or direct electron transfer properties of GOx in which oxygen is not used as electron acceptor and reduced to hydrogen peroxide. The GODA performance was further analyzed by comparing NADPH formation with hydrogen peroxide production using the well-known ABTS dye for quantification (Sun et al., 2001).

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical reagent grade or higher quality and purchased from Sigma–Aldrich Chemie (Taufkirchen, Germany) and Applichem (Darmstadt, Germany). Toyopearl Butyl-650S chromatographic resin was purchased from TOSOH BioScience GmbH (Stuttgart, Germany). T7 Gene 6 Exonuclease was purchased from Amersham Biosciences Europe GmbH (Freiburg, Germany). pYES2 shuttle vector, *Escherichia coli* strain DH5α and *Saccharomyces cerevisiae* strain INVScI were purchased from Invitrogen (Karlsruhe, Germany). Nucleotides and all other enzymes were purchased from Fermentas (St. Leon-Rot, Germany) if not stated otherwise.

2.2. Generation of mutant library of GOx

pYIP5 shuttle vector harboring *MFα1s*-GOx gene from *A. niger* was obtained from Professor Van Rensburg (Stellenbosch University, South Africa) and used as template for mutant library generation by error-prone PCR (epPCR). epPCR was performed using a thermal cycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany) and had the following conditions: 94 °C for 4 min, 1 cycle; 94 °C for 1 min/56 °C for 1 min/72 °C for 2.25 min, 29 cycles; 72 °C for 10 min, 1 cycle. PCR mix in thin-wall PCR tubes (Multi-Ultra tubes 0.2 ml; Carl Roth, Germany) had a total volume of 50 μl and consisted of 0.2 mM dNTP mix, 40 ng pYIP5 template, 0.05 mM $MnCl_2$ (Cadwell and Joyce, 1992) for adjusting the mutation frequency and 10 pmol of each primer (forward primer: 5′-GGGAATATTAAGCTTGG TAC*C*AGGATGAGATTTCCTTC-3′ and reverse primer: 5′-TAGTGGATCCGAGCTCGGTA*A*ATCACTGCATGGAA-GC-3′). Nucleotides marked with a (*) contain a phosphothioate bond which terminates T7 Gene 6 Exonuclease digestion (Zhou and Hatahet, 1995). PCR was hot started when the reaction mix reached 94 °C by adding 2.5 U *Taq* polymerase. PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

Ligation of mutated *MFα1s*-GOx gene into pYES2 shuttle vector was performed according to a ligase free cloning method (Zhou and Hatahet, 1995). Firstly, 1 μg pYES2 plasmid was linearized by KpnI digestion (5 U/μg DNA, 6 h, 37 °C) and purified with QIAquick Gel Extraction Kit (Qiagen). Secondly, 3 μg PCR product and 250 ng linearized pYES2 vector were treated with T7 Gene 6 Exonuclease (5 U/μg DNA, 15 min, 37 °C) in order to generate complementary overhangs of ~20 nucleotides. T7 Gene 6 Exonuclease was subsequently inactivated by heat treatment (10 min, 80 °C). Ligation was then performed by mixing digested PCR product and linearized pYES2 vector in a ratio of 12:1 (3000:250 ng). The ligation mix was heated (15 min, 75 °C) and slowly cooled down to ambient temperature before transforming into *E. coli* strain DH5α using the TSS method (Chung et al., 1989).

The mutant library was resuspended with a Drigalski spatula from the LB_{amp} plate after adding 1 ml LB_{amp} media. Plasmids of the mutant library were extracted using a QIAprep Spin Miniprep Kit (Qiagen) and subsequently purified by gel extraction (QIAquick Gel Extraction Kit; Qiagen). Purified plasmids harboring the *GOx*-gene were then transformed for functional expression into the *S. cerevisiae* strain INVScI by using the lithium acetate method (Gietz et al., 1995) and denaturated salmon sperm DNA (Invitrogen). Transformants were grown on SC-U selective plates containing 2% glucose.

2.3. Cultivation and expression of GOx in S. cerevisiae

Colonies grown on SC-U selective agar plates containing 2% glucose were picked with toothpicks and transferred into 96-deep well plates (BRAND, Wertheim, Germany) containing 1 ml SC-U growth media (containing 2% glucose) in each well and grown to saturation (48 h, 30 °C, 700 rpm) using a Multitron II incubator shaker (Infors GmbH, Einsbach, Germany). GOx production was induced by removing SC-U growth media through centrifugation (Eppendorf 5810R, 10 min, 4 °C, 3220 × *g*) followed by resuspension of cell pellets in 1 ml induction media (SC-U medium containing 2% galactose) using a Liquidator (Steinbrenner GmbH, Wiesenbach, Germany). Recombinant GOx expression into the media was performed using Multitron II incubator shaker (24 h, 30 °C, 700 rpm). Cells were centrifuged (Eppendorf 5810R, 10 min, 4 °C, 3220 × *g*) and 100 μl of the clear supernatant from each well was used for activity determination.

2.4. GODA in 96-well format

Liquidator (Steinbrenner GmbH) was used for all liquid handling in the GODA and ABTS assay in 96-well flat-bottom microplates (Greiner Bio-One GmbH, Frickenhausen, Germany).

A 100 μl supernatant containing recombinant GOx was added to 100 μl acetate buffer (30 mM, pH 5.5). The bioconversion of β-D-glucose to D-gluconolactone was initiated by adding 100 μl β-D-glucose (1 M) and quenched after 2–3 h by adding 20 μl of sodium hydroxide 200 mM to reach pH 10 (final concentration 12.5 mM). D-gluconolactone hydrolyzes to D-gluconate at pH 10 within 1 h at ambient temperature. To detect NADPH formation, 100 μl of hydrolyzed reaction mixture was pipetted into a fresh microtiter plate containing 200 μl assay mix (0.37 M triethanolamine, pH 7.5; 0.99 mM $NADP^+$; 3.62 mM ATP; $MgSO_4$ (concentration not specified in the kit); 72 mU/ml 6-phosphogluconate dehydrogenase; 8.55 mU/ml gluconate kinase). This D-gluconate detection procedure based on NADPH formation follows the D-gluconic acid detection kit (R-Biopharm AG, Darmstadt, Germany), except that a 20-fold lower concentration of both 6-phosphogluconate dehydrogenase and gluconate kinase were used. In these coupled enzymatic reactions, the kinetic of NADPH formation was recorded at 340 nm (ambient temperature; $\varepsilon = 6300\,M^{-1}\,cm^{-1}$) using a FLASHScan S12 microplate reader (Analytik Jena AG, Jena, Germany).

2.5. ABTS assay in 96-well format to validate GODA

ABTS assay was used (Sun et al., 2002) with the following modifications to determine GOx activity. A 100 μl of supernatant (containing recombinant GOx) was transferred to a 96-well flat-bottom microplate (Greiner Bio-One GmbH) and 200 μl assay mix was added into each well resulting in the following final concentrations: 330 mM β-D-glucose, 3.3 mM ABTS, and 0.25 U horseradish peroxidase (HRP) in acetate buffer (100 mM, pH 5.5). Activity was measured by change in absorbance at 414 nm at ambient temperature using a FLASHScan S12 microplate reader (Analytik Jena AG).

2.6. Production and purification of recombinant GOx

S. cerevisiae clones carrying wild-type and mutated GOx variant were grown to an $OD_{600\,nm}$ of 0.4 in 1 l shaking flasks containing 250 ml of SC-U growth media (48 h, 30 °C, 250 rpm) using a Multitron II incubator shaker (Infos GmbH). *S. cerevisiae* cells were centrifuged (Eppendorf 5810R; 10 min, 4 °C, 3220 × g) in 50 ml Falcon tubes (Greiner Bio-One), resuspended in 250 ml induction media (SC-U containing 2% galactose) and grown in a shaking incubator (Multitron II; Infors GmbH; 12 h, 30 °C, 250 rpm). Cells were centrifuged (Eppendorf 5810R; 10 min, 4 °C, 3220 × *g*) and the supernatant containing recombinant GOx was purified by hydrophobic interaction chromatography (Sasaki et al., 1982) using an ÄKTA purifier (Amersham Pharmacia Biotech, Freiburg, Germany) and Toyopearl Butyl-650S (TOSOH BioScience GmbH) in a 15/125 mm column (KronLab GmbH, Sinsheim, Germany). The supernatant containing recombinant GOx was first filtered using a 0.45 μM hydrophilic polypropylene membrane filter (VWR International GmbH, Darmstadt, Germany). $(NH_4)_2SO_4$ was added to the cleared supernatant until the conductivity reached a value of 183 mS/cm (Laboratory conductivity meter 703; Knick GmbH, Berlin, Germany) and subsequently loaded on Toyopearl Butyl-650S column (TOSOH Bioscience) that was pre-equilibrated at pH 5.0 with 50 mM sodium acetate buffer containing 1.5 M $(NH_4)_2SO_4$. The unabsorbed proteins were washed out of the column with same buffer (10 column volumes; flow rate 5 ml/min) and GOx was finally eluted by a step gradient [$(NH_4)_2SO_4$ 1.5 to 0 M; 50 acetate buffer pH 5.5; three column volumes; flow rate 5 ml/min). GOx elution was monitored at 280 nm (protein), and 382/452 nm (FAD). Collected GOx fractions were subsequently desalted and concentrated using centrifugal filters with 50 kDa cut-off membrane (Centricon YM-50; Millipore, Schwalbach, Germany).

Total protein content was measured according to BCA™ assay kit (Cat No. 23225; Pierce, Bonn, Germany) using BSA for calibration within a range from 0 to 250 μg/ml. This calibration curve was further used for determining kinetic constants.

2.7. Determination of kinetic parameters of β-D-glucose and oxygen conversion

Maximum reaction rate (V_{max}), Michaelis–Menten constant (K_m) and turnover number (k_{cat}) were determined for β-D-glucose and oxygen consumption using the ABTS assay and a Michaelis–Menten model.

ABTS based kinetic measurements of purified wild-type GOx and mutant I115V were performed identically to the ABTS assay in 96-well format, except that final concentrations of β-D-glucose ranged between 0 and 300 mM. The reactions were performed at ambient temperature for 5 min.

Kinetics of oxygen consumption were recorded using a Fibox 3 fiber optical probe (PreSens, Regensburg, Germany) within a sealed gas tight vial of 25 ml total volume. Oxygen concentration in the liquid phase inside the sealed vial has been controlled (0–1100 μM) via oxygen partial pressure by supplying pure nitrogen or pure oxygen. The steady state oxygen consumption was initiated by injecting 100 μl of supernatant (containing equal amounts of purified wild-type GOx and mutant I115V) into the sealed vial with 8 ml of 500 mM glucose solution in acetate buffer (pH 5.5, 50 mM; 5 min at ambient temperature).

2.8. Plasmid recovery from yeast and sequencing

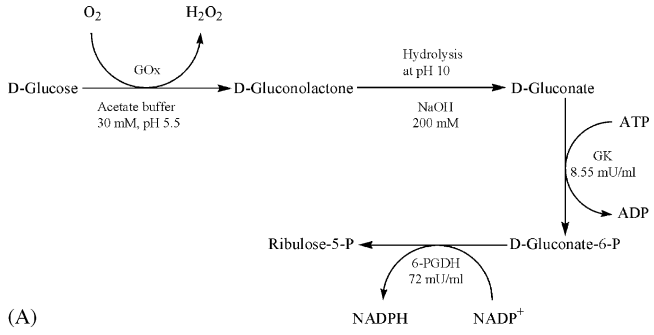
Plasmids harboring mutated *GOx* genes that encode GOx variants with improved activity were isolated from *S. cerevisiae* INVScI (Singh and Weil, 2002) and retransformed for amplification into *E. coli* strain DH5α using the TSS method (Chung et al., 1989). The clones harboring the plasmids were cultivated in LB_{amp} media (2 ml, 12 h, 37 °C, 220 rpm). Plasmids were

subsequently purified using QIAprep Spin MiniPrep Kit (Qiagen) and sequenced (MWG-Biotech AG; Ebersberg, Germany) using the following sequencing primers: forward primer 1: 5′-GCAGACTCTCCTTGTGAGC- TCG-3′, forward primer 2: 5′-GACACCGGCGATGACTATTCTCC-3′, forward primer 3: 5′-GATTCCACAACACCACCGCCTTGC-3′ and reverse primer: 5′-GCCACTTTCGATGACGAGCACACT-3′.

3. Results

3.1. Reaction principle and linear detection range of NADPH formation

Fig. 1A shows the reaction sequence of GODA leading to NADPH formation which is recorded at 340 nm ($\varepsilon = 6300\ M^{-1}\ cm^{-1}$) (Mollering and Bergmeyer, 1967). Conversion of β-D-glucose by GOx is terminated by pH shift to 10. At the pH the reaction product D-gluconolactone is hydrolyzed to D-gluconate, which is subsequently phosphorylated by gluconate kinase (GK) and oxidized by 6-phosphogluconate dehydrogenase (6-PGDH) to ribulose-5-P (Fig. 1A). Fig. 1B shows the sensitivity of GODA for detecting D-gluconolactone. A linear range of up 2.0 absorbance units (data not shown) and up to 3 mM D-gluconolactone (Fig. 1B) has been recorded. Under assay conditions in microplates and 2 h incubation time concentrations down to 0.5 mM could be monitored.



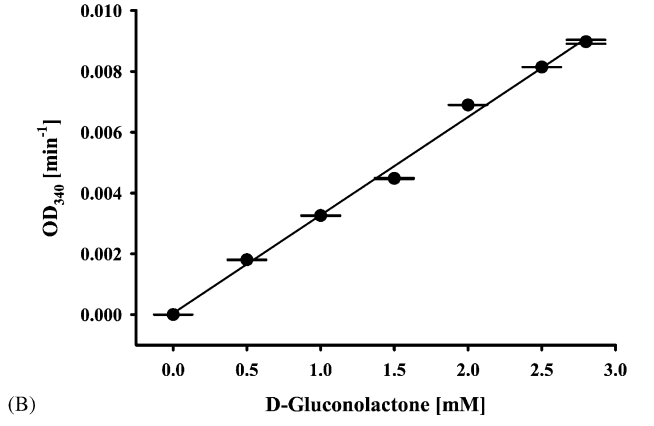


Fig. 1. (A) Shows the reaction scheme of glucose oxidase detection assay (GODA) leading to NADPH formation which is recorded at 340 nm (GOx: glucose oxidase; GK: gluconate kinase; 6-PGDH: 6-phosphogluconate dehydrogenase). (B) Shows performance of GODA at various D-gluconolactone concentrations. The calculated rates in ($OD_{340}\ min^{-1}$) are linear for a reaction time of at least 120 min.

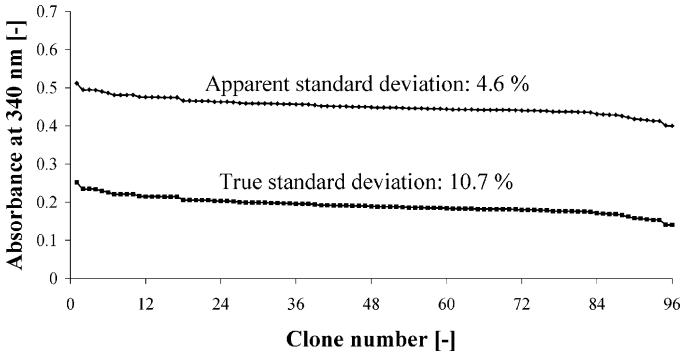
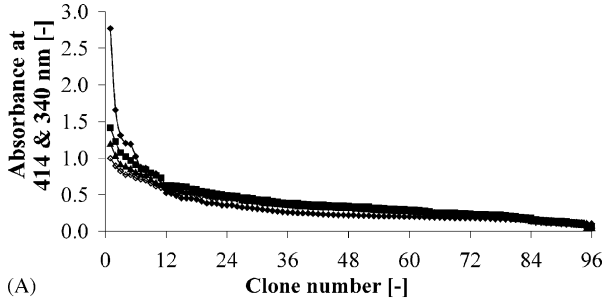


Fig. 2. Shows the activity profile of GOx wild-type expressed in *S. cerevisiae* in descending order of absorbance values in a 96-well plate. True standard deviation of GODA was calculated by subtracting absorbance background from apparent absorbance.

3.2. Standard deviation in 96-well plates

An important performance criterion for a screening system is the standard deviation of the wild-type enzyme in 96-well plate format. Fig. 2 shows the standard deviation (S.D.) of wild-type GOx before (4.6%; apparent S.D.) and after (10.7%; true S.D.) subtracting the background absorbance.

For validating GODA, a mutant library (2000 clones) was generated by epPCR and screened for improved activity towards β-D-glucose. Improved variants and potentially improved variants were combined on a 96-well master plate. Fig. 3 shows for this 96-well plate the performance of GODA compared to a modified ABTS assay (see Section 2), which detects the side-product hydrogen peroxide. D-gluconolactone and hydrogen peroxide



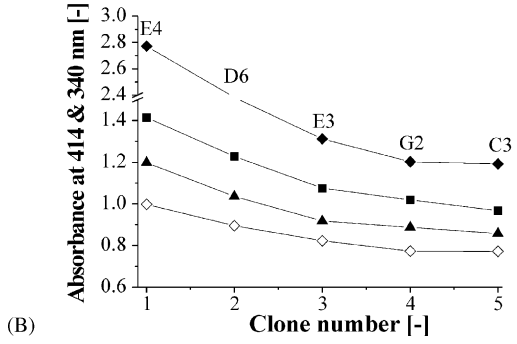


Fig. 3. (A) Compares a mutagenic GOx library in 96-well microplate format detected by: ♦-ABTS-assay (414 nm, 5 min); ■–GODA (340 nm, 120 min); ▲-GODA (340 nm, 75 min); ◇-GODA (340 nm, 50 min). (B) Shows the five most active GOx variants (E4, D6, E3, G2, and C3) of (A) in descending order of absorbencies in a 96-well plate.

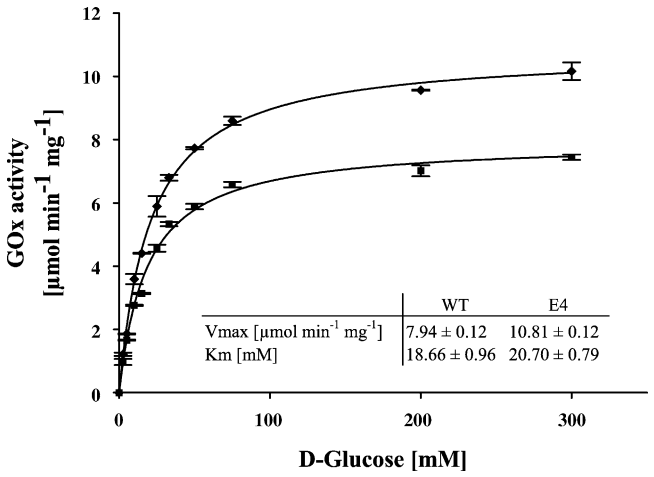


Fig. 4. Shows kinetics of β-D-glucose conversions (■-wild-type and ♦-E4 mutant) measured at an oxygen concentration ~280 μM by ABTS assay (414 nm) as described in Section 2.

formation correlates well in a 96-well plate (Fig. 3A). GOx variants with high activity show the same order (Fig. 3B) after at least 50 min incubation time. An incubation time of 2 h proved to be a suitable compromise between conversion and time consumption for detection of GOx activities. The absolute absorbance values differ however significantly for the most active mutants in terms of absolute values.

3.3. Directed protein evolution to identify GOx variants with improved activity toward β-D-glucose

The screening of a GOx mutant library resulted in variants with increased V_{max} and preserved K_m values. The most active mutant (E4) was isolated, sequenced, purified and the kinetic data for glucose conversion and oxygen consumption were determined. The GOx mutant E4 carries a I115V substitution and kinetic parameters were determined as follows: (a) β-D-glucose: $V_{max} = 10.81\ \mu mol\ min^{-1}\ mg^{-1}$ (versus wild-type $7.94\ \mu mol\ min^{-1}\ mg^{-1}$) and $K_m = 20.7$ mM (versus wild-type 18.66 mM), $k_{cat} = 21.5\ s^{-1}$ (versus wild-type $16\ s^{-1}$) and (b) oxygen consumption (Fig. 4): $V_{max} = 8.34\ \mu mol\ min^{-1}\ mg^{-1}$ (versus wild-type $5.44\ \mu mol\ min^{-1}\ mg^{-1}$) and $K_m = 474.2\ \mu M$ (versus wild-type 700.8 μM). The difference to reported kinetic constants of GOx from *A. niger* (Bohmhammel et al., 1993) can be attributed to the extend of FAD incorporation in recombinant GOx expressed in *S. cerevisiae* (data not shown).

4. Discussion

For advancing the field of miniature biofuel cells (mBCs) it is important to boost power output, improve long-term stability and avoid hydrogen peroxide production that might cause tissue damage. Specific objectives for in-body-applications of GOx in mBCs are derived from conditions in blood vessels and comprise: (A) improving activity of GOx for β-D-glucose, (B) reducing K_m value of GOx for β-D-glucose, (C) designing oxygen independent GOx, (D) improving electron transfer interface from electrode to GOx, and (E) improving chloride resistance and pH tolerance.

The commonly used GOx in mBCs is from *A. niger* and cannot functionally be expressed in rapidly growing *E. coli* cells. The yeast *S. cerevisiae* has been used for directed evolution of a few catalysts (Bulter et al., 2003), allows functional secretion of GOx into the media and was therefore selected as host for directed evolution of GOx.

For validating the GODA protocol we decided to improve the enzyme activity of GOx toward β-D-glucose as proof of principle. GOx variants with increased activity will improve power output in mBCs and allow a validation of GODA by hydrogen peroxide determination with the well-known ABTS assay (Sun et al., 2001). In contrast to the ABTS assay the GODA detects the product D-gluconolactone and allows improving bioelectrochemical properties in which GOx is not reoxidized by molecular oxygen as in biofuel cells. The product detection in GODA should further prevent directed evolution of GOx into an uncoupled hydrogen peroxide producer. Using surrogate substrates and conditions not close to application conditions resulted in bitter lessons that we learned in many directed protein evolution experiments (Woodyer et al., 2004).

GODA is a medium-throughput screening system with a S.D. of 10.7% and detection limit of 0.5–1 mM D-gluconolactone under assay conditions in microplates. Standard deviations around 11% have successfully been used for the directed evolution of monooxygenases, for example toward aromatic and heterocyclic compounds (Wong et al., 2005) and improved organic solvent resistance (Wong et al., 2004a). The reaction principle of the GODA assay is based on coupled enzyme reactions (Mollering and Bergmeyer, 1967) resulting in the formation of NADPH which is monitored at 340 nm. The respectively high sensitivity and low background is achieved due to secretion of GOx into the cultivation broth in which NADPH consuming side-reactions are minimized. GODA was validated by improving beyond statistical errors the activity of GOx; mutant I115V: (V_{max} (β-D-glucose) $10.81\ \mu mol\ min^{-1}\ mg^{-1}$ compared to $7.94\ \mu mol\ min^{-1}\ mg^{-1}$ of the wild-type; differences between K_m values are below 2 mM and k_{cat} values increased to $21.5\ s^{-1}$ compared to $16\ s^{-1}$ of the wild-type). Additionally, the improved activity of mutant I115V was validated by oxygen kinetics, showing that the maximal oxygen consumption rates (V_{max}) have been improved by ~1.5-fold. Amino acid position 115 is in close proximity to the catalytic FAD centers of the GOx dimer and additional studies are in progress to understand the activity modulating role of this residue.

5. Conclusions

With GODA a directed evolution protocol for GOx has been reported and validated by improving the activity of GOx toward β-D-glucose. Insufficient GOx properties (points (A)–(E); in Section 4) can in the future be improved with GODA to match demands of mBCs that are implanted in blood vessels and use β-D-glucose as fuel. GODA is in contrast to peroxide detection systems product-based and allows improving electrochemical properties of GOx in which oxygen is not used as electron accep-

tor. In course of ongoing GOx evolution we aim to improve the stated properties and to investigate subsequently the performance of evolved catalysts in mBCs. On the long run we aim to understand structure–function relationships to engineer GOx rapidly by rational or semi-rational design for use in mBCs and to shed light on underlying design principles.

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