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Reengineered glucose oxidase for amperometric glucose determination in diabetes analytics

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ABSTRACT

Glucose oxidase is an oxidoreductase exhibiting a high β-D-glucose specificity and high stability which renders glucose oxidase well-suited for applications in diabetes care. Nevertheless, GOx activity is highly oxygen dependent which can lead to inaccuracies in amperometric β-D-glucose determinations. Therefore a directed evolution campaign with two rounds of random mutagenesis (SeSaM followed by epPCR), site saturation mutagenesis studies on individual positions, and one simultaneous site saturation library (OmniChange; 4 positions) was performed. A diabetes care well suited mediator (quinone diimine) was selected and the GOx variant (T30V I94V) served as starting point. For directed GOx evolution a microtiter plate detection system based on the quinone diimine mediator was developed and the well-known ABTS-assay was applied in microtiter plate format to validate oxygen independency of improved GOx variants. Two iterative rounds of random diversity generation and screening yielded to two subsets of amino acid positions which mainly improved activity (A173, A332) and oxygen independency (F414, V560). Simultaneous site saturation of all four positions with a reduced subset of amino acids using the OmniChange method yielded finally variant V7 with a 37-fold decreased oxygen dependency (mediator activity: 7.4 U/mg WT, 47.5 U/mg V7; oxygen activity: 172.3 U/mg WT, 30.1 U/mg V7). V7 is still highly β-D-glucose specific, highly active with the quinone diimine mediator and thermal resistance is retained (prerequisite for GOx coating of diabetes test stripes). The latter properties and V7's oxygen insensitivity make V7 a very promising candidate to replace standard GOx in diabetes care applications.

1. Introduction

Diabetes mellitus is a metabolic disease that affects 366 million people in the world in 2011 with an expected increase to 552 million till 2030 (Whiting et al., 2011). Diabetes patients require a precise control of their blood-glucose level through administered insulin to avoid long-term effects through hyperglycemia or life-threatening hypoglycemia. Glucose determination systems have a market-size of $8.8 billion (Hughes, 2009) and several enzyme- (e.g. through oxidoreductases) and non-enzyme based systems (e.g. platinum or non-catalytic binding proteins) have been developed (Ferri et al., 2011; Hoenes et al., 2008; Jin et al., 2011; Lee et al., 2009; Park et al., 2006; Veetil et al., 2010; Ye and Schultz, 2003). Amperomentric systems employing glucose oxidases (GOx's) and glucose dehydrogenases (GDH's) are most commonly used in diabetes care due to their specificity and activity (Freckmann et al., 2012; Hoenes et al., 2008). In the latter systems, electrons are transferred from the redox center of GOx or GDH to the working electrode through a mediator compound or mediator cascade system. The recorded electron flux is thereby proportional to the glucose concentration in the investigated blood sample (Wang, 2008). Main challenges in accurate amperometric glucose determination are competitions between the electron acceptor oxygen and employed mediators (especially for GOx) and the specificity of the employed oxidoreductase for β-D-glucose conversion (mainly for GDHs). GOxs are in general highly specific for β-D-glucose whereas GDHs convert often maltose, galactose and xylose to an extend that is analytically relevant (Adams et al., 1960; Hauge, 1960; Olsthoorn and Duine, 1998; Raba and Mottola, 1995). For instance, Icodextrin is used as an osmotic agent during peritoneal dialysis (Mistery and Gokal, 1994). Icodextrin is a cornstarch derived high molecular weight polymer which is metabolized after absorption from the peritoneal cavity yielding an average metabolite concentration of 300 mg/dL (sum of maltose, maltotriose and maltotetraose) in serum compared to a blood glucose

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concentration of <100 mg/dL (a healthy person when fasting) (Alsop, 1993; Davies, 1993; Flore and Delanghe, 2009; Garcia-Lopez and Lindholm, 2009; Moberly et al., 2002; Tsai et al., 2010). The natural activity of GOx with oxygen as an electron acceptor leads to a competitive electron transfer in amperometric devices employing GOx (Bankar et al., 2009; Bentley and Neuberger, 1949; Gibson et al., 1964; Pazur and Kleppe, 1964) what can cause a underestimating of β-D-glucose concentrations (Ginsberg, 2009; Oberg and Ostenson, 2005). Pronounced oxygen effects occur at pO_2 levels >100 Torr which are relevant for critically ill patients (Dungan et al., 2007; Kost et al., 1998; Tang et al., 2001).

In order to develop sensitive and in accuracy improved β-D-glucose detection systems, GOxs and GDHs have extensively been reengineered along with controversial discussions on needs and performances (Dungan et al., 2007; Mahoney and Ellison, 2007). GDH-PQQ was reengineered to improve substrate specificities (50-times reduced maltose activity) (Boenitz-Dulat et al., 2006; Kratzsch et al., 2002) and alternatively, flavin adenine dinucleotide (FAD) dependent GDHs were employed in glucose measurements (0.3% residual activity for maltose) (Sode et al., 1996; Tsujimura et al., 2006; Yamaoka and Sode, 2007; Zafar et al., 2012).

GOx has various applications in textile bleaching, preserving food, low alcohol wine production, and bioprocesses (glucose sensing) (Bankar et al., 2009). Protein engineering campaigns by directed evolution or rational design aimed mainly to increase activity and/or stability (Holland et al., 2012; Prodanovic et al., 2011, 2012; Zhu et al., 2006, 2007).

Glucose oxidase reengineering for mediated applications comprise two reports. The glucose oxidase T30V-I94V was found in a directed evolution experiment employing a ferrocenemethanol mediator and exhibits a 2-fold increased k_{cat}-value (69.5/s WT; 137.7/s T30V-I94V) as well as a slightly improved thermal-resistance and a shifted pH activity profile. Horaguchi et al. (2012) found a 6-fold higher dehydrogenase/oxidase ratio for the GOx of *Aspergillus niger* and a 11-fold higher dehydrogenase/oxidase ratio for the GOx of *Penicillium amagasakiense* in a rational protein engineering experiment employing phenazine methosulfate as electron mediator (Horaguchi et al., 2012).

None of these reports refers to a determination of β-D-glucose in serum and in diabetes care (Horaguchi et al., 2012; Zhu et al., 2007). None of the employed mediator systems (ferrocenemethanol, phenazine methosulfate) in the latter two reports is to our best knowledge relevant for β-D-glucose determination in diabetes. The quinone diimine/phenylenediamine mediator system is in contrast to the previous mediator systems employed in various test stripe systems (Hoenes et al., 2008). Quinone diimines are attractive for diabetes analytics due to reduced unspecific interferences with serum compounds that can be attributed to a low redox potential and specific mediator chemistry. Additionally, quinone diimines are applicable and stable on test stripes when immobilized in form of N-substituted *p*-nitrosoaniline, a storage-stable precompound of the corresponding quinone diimine (Becker, 2005; Hoenes, 1990; Hoenes et al., 2008).

In order to develop an ideal enzyme system for amperometric β-D-glucose determination on test stripes, the GOx variant (T30V I94V) from *A. niger* was reengineered to yield for the first time an oxygen insensitive GOx which operates efficiently with a diabetes relevant mediator system (quinone diimine/phenylendiamine).

2. Material and methods

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Taufkirchen, Germany), Applichem (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany). All enzymes were purchased from New England Biolabs (UK) and Sigma-Aldrich (Taufkirchen, Germany). The pYES2 shuttle vector and *Escherichia coli strain DH5α* were purchased from Invitrogen (Karlsruhe, Germany). The *Saccharomyces cerevisiae* strain (ngd29) was provided by Roche diagnostics (Lehle et al., 1995). Nucleotide analogs dPTPαS and dGTPαS and polymerases along with respective buffers (SeSaM-Taq and 3D1) were obtained from SeSaM Biotech GmbH (Aachen, Germany). All other nucleotides were purchased from Fermentas (St. Leon-Rot, Germany). DNA was quantified using a NanoDrop photometer (NanoDrop Technologies, Wilmington, DE, USA). Sequencing and Oligonucleotide synthesis was done by Eurofins MWG Operon, Ebersberg, Germany. Primers and employed media are summarized in Tables S2 and S3 (Supplementary Information).

2.1. Generation of random mutagenesis libraries

Random mutagenesis of the GOx was performed applying the Sequence Saturation Mutagenesis Method Tv-II (SeSaM-Tv-II) and Error Prone PCR (epPCR) using the pYES2-GOx T30V I94V plasmid as a DNA template (Zhu et al., 2007).

SeSaM library generation was carried out as previously described (Mundhada et al., 2011). DNA templates for SeSaM-TV-II-step 1 and step 2 were prepared by PCR. The 50 μL PCR mixture contained: 1x Phusion HF Buffer; (New England Biolabs); 0.2 mM of each dNTP; 12.5 pmol of each primer (forward and reverse); 2.5 U of Phusion polymerase (New England Biolabs) and 50 ng of plasmid as template. PCR protocol: 98 °C for 30 section (1 cycle); 98 °C for 10 s; 60 °C for 30 s; 72 °C for 1 min 20 s (20 cycles); 72 °C for 5 min (1 cycle). The primers P1 and P2 were used for step 1 template generation and the primer P3 and P4 for the step 3 template generation. The PCR-product of step-4 was subjected to a final PCR-amplification applying identical PCR-conditions as used for the SeSaM-Tv-II template preparation employing the primers P5 and P6.

For the epPCR library generation the following conditions were used: 94 °C for 2 min, 1 cycle; 94 °C for 1 min/65 °C for 1 min/72 °C for 2 min 25 s, 30 cycles; 72 °C for 10 min, 1 cycle (Cadwell and Joyce, 1994). The PCR-mix had a total volume of 50 μL and was composed as follows: 3 ng/μL pDNA template, 0.2 mM dNTP mix, 0.1 mM $MnCl_2$ for adjusting the mutation frequency, 10 pmol of each primer (P5 and P6) and 5 U Taq polymerase. The final PCR-product was purified using the NucleoSpin® Extract II – kit (MACHERY-NAGEL, Germany) and subjected to *Dpn*I digestion before cloning.

2.2. Multiple- and single site saturation mutagenesis

For multiple site saturation mutagenesis the OmniChange protocol was applied (Dennig et al., 2011). For Fragment 1 amplification, targeting A173, primers P7 and P8 were added in the master mix. While for A332 saturation, P9 and P10 were used. F414 site was targeted by using P11 and P12. The V560 site was included in vector backbone as it was amplified by P13 and P14. The PCR program for amplification of fragments was 98 °C for 45 section (1 cycle); 98 °C for 15 s; 65 °C for 30 s; 72 °C for 30 s (20 cycles); 72 °C for 5 min (1 cycle). For vector backbone amplification extension time of 4 min was applied. The fragments were digested and ligated as previously reported before transformation of chemical competent *E. coli* DH5α for library amplification (Dennig et al., 2011). For yeast transformation the gene library was isolated from *E. coli* DH5α using the plasmid isolation kit 'NucleoSpin Plasmid MACHERY-NAGEL GmbH & Co. KG Düren'.

For individual site saturation mutagenesis (SSM) a two-step PCR protocol has been followed (Sambrook and Russel, 2001). 50 μL PCR reaction mixture contained 1 ng/μL of plasmid template, 1 × Phusion buffer (New England Biolabs, Frankfurt, Germany), 1 U of Phusion polymerase and 300 μM dNTP's. The reaction

mixture was divided in two equal volumes and forward primer and reverse primer of the targeted site were added (reaction mixture 1: 400 nM forward primer; rection mixture 2: 400 nM reverse primer). PCR program was 98 °C for 45 section (1 cycle); 98 °C for 15 s; 65 °C for 30 s; 72 °C for 4 min (5 cycles); 72 °C for 5 min (1 cycle). After first step both reactions were pooled and the same program was carried out with 15 more repetitions. The following primers were used: P15/P16 for position 173, P17/P18 for position 332, P19/P20 for position 414 and P21/22 for position 560. The final PCR-product was purified using the NucleoSpin® Extract II – kit (MACHERY-NAGEL, Germany) and subjected to *Dpn*I digestion.

2.3. Gene cloning and yeast cell transformation

The cloning of mutated GOxs into the pYES2 vector backbone was performed according to a recombination method (Oldenburg et al., 1997). Firstly, 2 μg of pYES2-GOx T30V I94V were linearized by SalI/BamHI digestion (10 U/μg DNA, 6 h, 37 °C) and subsequently purified by gel-extraction employing the NucleoSpin® Extract II – kit (MACHERY-NAGEL, Düren, Germany). The primers, used for library amplification were homologous to the linearized vector backbone.

Secondly, the linearized pYES2-vector and the amplified gene library were mixed in a ratio of 1:3 (250 ng/750 ng). This DNA-mix was used for the transformation employing a lithium acetate method (Gietz and Schiestl, 2007). Transformants were grown on SC-U selective plates containing 2% glucose. For the transformation of closed plasmids 300 ng DNA was used. To obtain GOx-molecules with a reduced glycosylation level the glyco-engineered *S. cerevisiae* strain ngd29 was chosen as expression host (Lehle et al., 1995).

2.4. Cultivation and expression in 96-well plates

Single colonies were grown on SC-U selective agar plates containing 2% glucose and transferred into 96-well microtiter plates (100 μL SC-U media; 1% glucose; PS-F-bottom, Greiner Bio-One). The cultivation of the pre-culture took place in a microtiter plate shaker (900 rpm; 30 °C; 70% humidity; 24 h; Infors GmbH, Eisenach, Germany). A certain amount of pre-culture was transferred to each well of a deep well plate (500 μL SC-U media; 0.5% glucose; 2% galactose; riplate-rectangular, ritter). The main-culture was cultivated as the pre-culture with an elongated cultivation step (72 h). Cell supernatants were harvested after centrifugation (3300 g; RT; 20 min) and used for activity determinations.

2.5. Production and purification of recombinant GOx

Pre-cultivation: 500 mL SynY media (2% glucose) were inoculated to an $OD_{600}=0.2$ using an overnight-culture of *S. cerevisiae* ngd29/pYES2-GOx; cultivations took place in 5 L shaking flasks (12 h; 30 °C; 250 rpm).

Main-cultivation: 10 L SynY media (1% glucose) was inoculated with 500 mL pre-culture. For the cultivation a 10 L fermenter was used (30 °C; 400 rpm; 1 vvm; 48 h).

Cell separation and buffer exchange: The GOx containing supernatant was recovered by micro-filtration (Hydrosart 0.45 μm, Sartorius Stedim Biotech GmbH Göttingen) employing a tangential flow filtration setup. Recovered supernatants were subjected for ultra-filtration and buffer exchange (SARTOCON Slice, Hydrosart 10 kDa, Sartorius Stedim Biotech GmbH Göttingen) employing a diafiltration setup (50 mM NaH_2PO_4, pH 6; 7 diafiltration volumes).

For enzyme purification anion-exchange chromatography was employed, using the ÄKTA*pilot* system and a FinLine Pilot 35 column (GE Healthcare). The column was packed with 220 mL resin (Fractogel TSK DEAE-650 s Merck), a flow rate of 62.5 cm/h was selected; equilibration with 440 mL sodium phosphate buffer (50 mM; pH 6); sample load: 0.5 L retentate (from ultra-filtration); wash: 440 mL sodium phosphate buffer (50 mM, pH 6); elution with a step gradient using a mixture of sodium phosphate buffer (95%; 50 mM pH 6) and NaCl (5%; 1 M). The GOx containing fractions were pooled and followed by a final buffer exchange step against potassium phosphate (50 mM; pH 7; Amicon® Ultracel-30k Millipore Ireland).

2.6. Normalization of protein concentrations

An enzyme-linked immunosorbent assay (ELISA) was used for specific determination of GOx concentrations, employing two GOx specific polyclonal antibodies (the principle of the ELISA is shown in the Supplementary Information, Fig. S1). Each well of a streptavidin coated microtiter plate (Roche 11643673 001) was filled with 100 μL of a primary antibody solution (2 μg/mL; Rabbit PAK GOD-Biotin Acris R1083B; 1 h; RT; 400 rpm). After a subsequent washing step (4 times 350 μL 9 g/L NaCl_0.1% Tween 20) 100 μL of GOx sample was transferred into each 96-well of a microtiter plate (1 h; RT; 400 rpm). The washing step was repeated and the 96-well plate was filled with 100 μL of a secondary antibody solution (10 μg/mL; Rabbit PAK GOD-HRP Acris R1083HRP; 1 h; RT; 400 rpm). After a third washing step the 96-well plate was filled with 100 μL of a ABTS/H_2O_2 solution (11684302001 Roche Diagnostics GmbH Mannheim; 30 min; RT; 400 rpm), subsequently the absorption at 405 nm was determined. Glucose oxidase from *A. niger* was used as internal standard (0.075–20 ng/mL; G7141-50KU Sigma-Aldrich).

2.7. Mediator detection system

For the liquid handling multi-channel pipettes from Brand (Transferpette S-8) and Eppendorf (Research pro) were used. 75 μL of supernatant (see cultivation and expression in 96-well plates) or purified enzyme were transferred into a 96-well flat-bottom microtiter plate (Greiner Bio-One). 100 μL of mediator solution (19.05 mM N,N-bis(2-hydroxyethyl)-4-nitrosoaniline (Becker, 2005), Roche – Material no. 100088314; 5% (w/w) polyvinylpyrrolidone, Roche – Material no. 10003476964; pH 7) and 20 μL of 25 g/L phosphomolybdic acid (Roche, Genisys-nr.: 11889893001) were supplemented. Reactions were initiated by supplementing 25 μL substrate solution. The kinetic of phosphomolybdic acid reduction was monitored at 700 nm in microtiter plate reader (Tecan Sunrise; Tecan Trading AG, Switzerland). The amount of GOx that converts 1 μmol of substrate in 1 min was defined as 1 U of that enzyme.

2.8. ABTS detection system

For liquid handling multi-channel pipettes from Brand (Transferpette S-8) and Eppendorf (Research pro) were used. 75 μL of supernatant (see cultivation and expression in 96-well plates) or purified enzyme transferred to a 96-well flat-bottom microplate (Greiner Bio-One) containing 100 μL of phosphate buffer (0.2 M; pH 7). 20 μL of reaction mixture were supplemented to each well resulting in final concentrations of 0.91 U/mL HRP and 2.3 mM ABTS. Reactions were initiated by supplementing 25 μL substrate solution. The oxidation of ABTS was kinetically determined at 414 nm using the microplate reader FLUOstar Omega (BMG LABTECH). The amount of GOx that converts 1 μmol of substrate in 1 min was defined as 1 U of that enzyme.

2.9. Oxygen consumption assay

For the direct determination of the oxygen consumption (%/min) the optical oxygen probe 'Fibox3 – Minisensor Oxygen Meter' (Precision Sensing GmbH Regensburg) was used.

3. Results

3.1. Screening systems

The ABTS detection system was used as reported to measure the oxidative half-reaction of the glucose oxidase via the formation of hydrogen peroxide from oxygen (Zhu et al., 2006).

A quinone diimine mediator detection system (QDM assay) was used to detect variants with improved mediator over oxygen preference. The mediator reaction starts from the stable precompound *p*-nitrosoaniline (N,N-bis(2-hydroxyethyl)-4-nitrosoaniline) which is converted to the corresponding quinone diimine (Becker, 2005; Hoenes, 1990; Hoenes et al., 2008). The principle of the mediator detection system is described within the Supplementary Information (Fig. S2).

The ratio mediator to oxygen activity is a measure for the oxygen independency of GOx variants and termed as OI-ratio (Oxygen-Independent GOx ratio) in the whole manuscript.

3.2. Gene mutagenesis and screening of mutant libraries

Fig. 1 shows the employed directed evolution strategies by random and focused mutagenesis. The two iterative cycles of random mutagenesis were performed to identify GOx residues or regions which modulate oxygen and mediator activity. The first random mutagenesis library was generated by the SeSaM II method (Mundhada et al., 2011) employing the GOx-(T30V I94V) variant as reference due to its 100% improved activity towards ABTS (Zhu et al., 2006, 2007). Subsequently the best two variants from the SeSaM library (A173T, A332S) were used as template for an error-prone-PCR mutant library (epPCR; 0.1 mM $MnCl_2$) (Cadwell and Joyce, 1994). In both libraries approximately 30% of the clones showed GOx activity and in total 2288 clones were screened in 96-well plate format (Fig. 1).

The random mutagenesis round of evolution yielded four beneficial substitutions (A173T, A332S, F414L, V560A). V01 (A173T) and V02 (A332S) showed increased activities in the ABTS and the QDM assay (V01: 170% QDM assay, 165% ABTS assay; V02: 175% QDM assay. 135% ABTS assay). V03 (A173T, F414L) and V05 (A332S, V560A) showed a significant decrease in oxygen activity ($< 30\%$ than GOx reference) in combination with a slightly increased mediator activity (V03: 200%; V05: 160% of GOx reference).

All four positions were subjected to individual site saturation mutagenesis in order to identify the most beneficial substitution at each position in respect to oxygen and mediator activity and to elucidate the function of each position. Table 1 summarizes the sequencing results and shows that positions 173 and 332 contribute to increased activities in the mediator assay and the ABTS-assay. Substitutions at positions 414 led to an either reduced oxygen activity (F414M) or improved mediator activity (F414V). The position 560 is important for the oxygen dependency of GOx; all substitutions to L, P, T, W, and Q showed a substantial decreased oxygen activity while maintaining the mediator activity (see Table 1).

Results of individual site saturation experiments prove that the identified residues allow to improve jointly or independently oxygen sensitivity and mediator activity.

To study cooperative effects and to generate an 'ideal' variant that combines reduced oxygen activity and improved mediator activity an OmniChange multi-site saturation experiment was performed with a reduced subset of amino acids for each position (Table S1, Supplementary Information). The reduced OmniChange library consisted of 1152 codon variants and 2112 clones were screened (theoretically 94% coverage) (Firth and Patrick, 2005).

Table 2 summarizes the results of the OmniChange library screening which yielded five variants (V6-8; V10-11) with significantly more beneficial properties (higher mediator activity; less activity with oxygen) compared to the GOx variants from the random and single site saturation mutagenesis experiments. Table 2 contains the sequencing results of the 'best five' variants (V6-8; V10-11) and an assessment in respect to their performance (oxygen- and mediator activity at 34 mM β-D-glucose).

Specific GOx concentrations were additionally determined (V6-8; V10-11) by an ELISA assay (see Material and methods). All values in Table 2 were calculated using clarified cell supernatants of secreted GOx without purification steps; recorded values have therefore to be considered as apparent values which were used for selection of the oxygen-independent variant V7. V7 was purified and detailed characterization (ν_{max}, K_M; T_m) with the GOx wild type (GOx WT) and reference variant (GOx-T30V I94V).

V7 and V10 have an increased mediator activity (reference variant: 3.2 U/mg; V7: 14.1 U/mg; V10: 7.8 U/mg) as well as a significant decreased oxygen activity (reference variant: 296 U/mg; not detected for V7 and V10 under selected conditions). V7 was

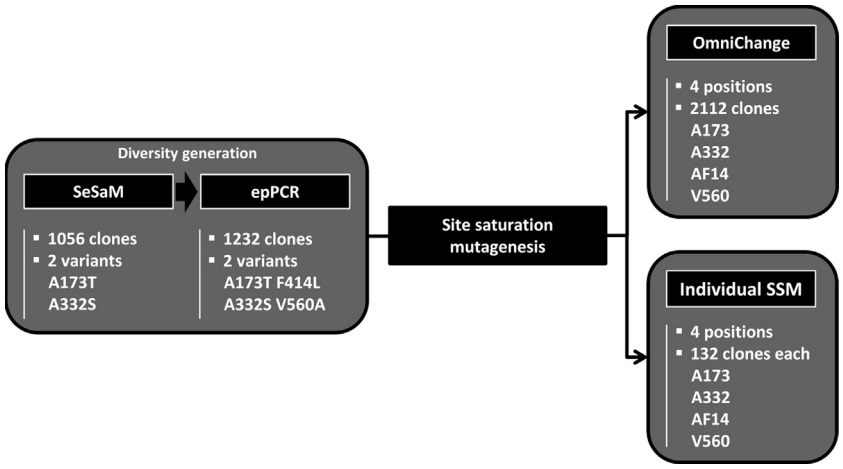

Fig. 1. Overview of the directed GOx evolution campaign. The campaign was performed to decrease the oxygen dependency and to increase the mediator activity. Two iterative cycles of random mutagenesis were performed yielding four beneficial positions (A173, A332, F414, and V560). All four positions were mutagenized either individually (QuikChange; –NNK) or simultaneously (OmniChange; reduced amino acid subsets).

Table 1
Sequence analysis of individual saturated positions in GOx variants and influence of identified positions (A173, A332, F414, and V560) on mediator and oxygen activity. Clones from the mutant libraries were selected based on increased dehydrogenase activity and/or decreased oxygen activity. Substitutions and the number of occurrence in 11 to 18 sequenced clones are given.

Residue wild type	No. seq. clones	Found substitution (No. of occurrence)	Property
A173	12	V (5); A (4); I (2); T (1)	Improved oxygen and mediator activity
A332	14	V (5); T (4); M (2); S (2); P (1)	Improved oxygen and mediator activity
F414	11	F (4); M (3); V (3); L (1)	Improved mediator activity/reduced oxygen activity (M)
V560	18	L (8); P (6); T (2); W (1); Q (1)	Significantly reduced oxygen activity

Table 2
Results of the OmniChange multi-site saturation library of the GOx reference variant(GOx-T30V I94V). GOx variants were selected according to mediator and/or lower oxygen activity. Sequencing results are reported for the positions 173, 332, 414, and 560 with apparent improvements in mediator and/or oxygen sensitivity. 34 mMβ-D-glucose was used in all activity determinations. Calculations are given in % as ratios of specific activities (U/mg) compared to the GOx reference variant (RV; T30V I94V). n.d.: not detected.

Variant	Substitutions				Residual activity (%)	
	A 173	A 332	F 414	V 560	Mediator activity	Oxygen activity
V6	I	S	L	V	160	30
V7	V	S	I	T	440	n.d.
V8	V	N	F	P	100	n.d.
V10	V	N	V	L	243	n.d.
V11	A	S	F	P	120	n.d.

finally selected for detailed characterization since it showed the highest improved mediator activity and a highly reduced oxygen activity. Before selecting V7 the substrate specificity and thermal stability was determined for all variants (V6-8; V10-11; data not shown) in order to ensure that the high selectivity of GOx for β-D-glucose is maintained. V7 has in the latter two properties (specificity Table 4; thermal resistance (Supplementary Information Fig. S4)) a comparable performance to WT and the RV (T30V I94V). In essence, V7 exhibits after pre-characterization all the desired properties of an 'ideal' oxidase for amperometric β-D-glucose determination.

3.3. Characterization of WT, reference variant (RV) and V7

GOx WT, RV and V7 were produced in *S. cerevsiae* ngd29 in 10 L fermentation scale, and subsequently purified as described in the Material and method section. Highly purified GOx-WT and variants (> 90% content) were employed in kinetic characterizations (50 mM potassium phosphate buffer; pH 7). Michaelis–Menten kinetics were recorded in the QDM- and the ABTS-assay for WT, RV, and V7 (Fig. S3, Supplementary Information).

Table 3 summarizes the kinetic parameters and residual activities of V7 in comparison to WT, and RV.

V7 has 638% residual activity in the QDM assay (WT: 7.4 U/mg; V7: 47.5 U/mg) and a 17% residual activity in the ABTS assay (WT: 172.3 U/mg; V7: 30.12 U/mg), resulting in a 37.5 times reduced oxygen dependency (Residual activity QDM assay/ residual activity ABTS assay - OI-ratio) when compared to WT and RV (OI-ratio WT: 1; OI-ratio T30V I94V: 1.4).

Oxygen activity was determined indirectly by applying the coupled ABTS-assay. In order to validate the ABTS results an oxygen probe (Oxygen Meter – Precision Sensing GmbH Regensburg) was used to directly measure oxygen consumption [%/min]. The direct oxygen consumption measurements yielded comparable results, for instance in case of V7 (17% ABTS assay; 22% oxygen probe). In detail, a rate of 3.9%/min was observed for V7, 17.4%/min for WT and 24.2%/min for RV.

Table 3
Kinetic parameters and residual activities of WT, RV, and V7.Kinetic parameters (v_{max}; K_M)were obtained by non-linear regression based on the Michaelis–Menten model using GraphPad Prism software package.

	Mediator assay			ABTS assay		
	V_{max} [U/mg]	Residual activity	K_M [mM]	V_{max} [U/mg]	Residual activity	K_M [mM]
WT	7.4 ± 0.1	100%	13.2 ± 1.0	172.3 ± 4.2	100%	14.2 ± 1.3
RV (T30V-I94V)	13.7 ± 0.2	184%	11.9 ± 0.9	221.6 ± 6.0	128%	8.7 ± 1.0
V7	47.5 ± 1.0	638%	28.2 ± 1.9	30.1 ± 0.2	17%	1.3 ± 0.1

Table 4
Residual activities of WT, RV, and V7 on β-D-maltose, maltotriose, β-D-galactose and D-xylose. Activity was determined using the QDM-assay and 182 mM of each sugar was employed in activity measurements. WT, RV, and V7 activity for β-D-glucose were set to 100% as reference value. n.d.: not detected.

	Residual activity [%]		
	WT	RV	V7
β-D-Glucose	100.0 ± 1.10	100.0 ± 2.10	100.0 ± 0.30
β-D-Maltose	n.d.	n.d.	n.d.
Maltotriose	0.2 ± 0.01	0.8 ± 0.03	0.3 ± 0.01
β-D-Galactose	1.1 ± 0.08	1.0 ± 0.02	0.4 ± 0.02
D-Xylose	0.4 ± 0.04	0.3 ± 0.02	1.7 ± 0.01

V7 is attractive for amperometric β-D-glucose determination if its specificity for β-D-glucose is kept during directed GOx evolution in order to avoid cross-reactivity with clinical relevant sugars (β-D-maltose, maltotriose, β-D-galactose, and D-xylose). Table 4 summarizes residual activities of GOx, WT, RV, and V7 for β-D-maltose, maltotriose, β-D-galactose, and D-xylose. WT, RV, and V7 activity for β-D-glucose were set to 100% as reference value and the QDM assay was employed in specificity studies.

Overall, the activities for β-D-maltose, maltotriose, β-D-galactose and D-xylose are less than 2% of the glucose activity in WT, RV and V7 and differ less than 1.3% among all investigated sugars. Therefore the specificities of WT, RV and V7 can be regarded as nearly unaltered for diabetes relevant sugars. V7 has a neglectable increased activity for D-xylose (from 0.38% to 1.66% residual activity) and interestingly the β-D-galactose activity is slightly reduced from 1.08% to 0.42%. Additionally none of the three GOx enzymes has a detectable activity on maltose (experimental setup; see Material and methods). Fig. S4 in the Supplementary Information shows that no alteration in thermal stability occurred.

4. Discussion

Oxygen dependency and substrate specificities are two key aspects to improve the performance, robustness and accuracy of

glucose sensing in diabetes care employing GOxs or GDHs. In test stripes analytics the employed mediator has furthermore to match application demands in terms of storage stability and efficient electron transfer. Goal of the directed evolution campaign was to design an optimal GOx for diabetes analytics. To the best of our knowledge there is only one report in which GOx was engineered to be less oxygen dependent employing the phenazine methosulfate as electron mediator (Horaguchi et al., 2012). Phenazine methosulfate is not employed in diabetes care applications. In this study a diabetes care relevant mediator system quinone diimine/phenylendiamine (QDM) was used in directed GOx evolution to reduce oxygen sensitivity and maintaining or improving properties that are relevant in diabetes care (glucose specificity and activity as well as thermal resistance).

Within two iterative cycles of directed GOx evolution (one with epPCR, one with SeSaM) four positions (A173, A332, F414, and V560) were identified which effect GOx activity and/or oxygen sensitivity. Fig. 2 shows all four positions, the FAD and the catalytic active residues H516 and H559 in the GOx (1cf3) X-ray structure.

All four positions were investigated in two focused mutagenesis experiments to elucidate the role of each position in increasing activity and/or reducing oxygen sensitivity (site saturation mutagenesis with QuikChange) and to generate an 'ideal' GOx variant (simultaneous site saturation with OmniChange). The OmniChange approach was performed since two positions are located in close vicinity of the active site (414, 560) or within 14.8 Å of the FAD (173, 332 Fig. 2) so that cooperative interactions are likely to occur. The latter expectation was confirmed since the 'best' identified variant, V7, was found in a simultaneous site saturation mutagenesis of RV harboring the four substitutions A173V, A332S, F414I, and V560T. V7 has an approximately 2.5 times higher OI-ratio than the best variant obtained in random mutagenesis experiments (data not shown). V7 shows a 6.4-fold increase in specific activity with the quinone diimine mediator (WT: 7.4 U/mg; EZ07: 47.5 U/mg) and a 5.7-fold reduced activity with oxygen (WT: 172.3 U/mg; EZ07: 30.12 U/mg) resulting in a 37-fold reduced oxygen dependency (OI-ratio). The only reported GOx from *A. niger* which was engineered for reduced oxygen sensitivity was the GOx-T110A with a 6-fold reduced oxygen dependency (Horaguchi et al., 2012).

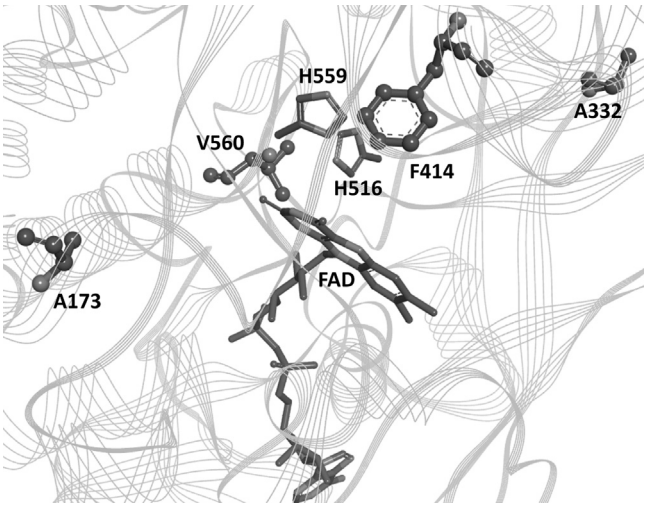

Fig. 2. Ribbon representation of GOx from *A. niger* based on the X-ray structure 1cf3 (Wohlfahrt et al., 1999). F414, V560, A173, and A332 are identified 'Hot-spots' for high mediator activity and reduced oxygen activity after two rounds of directed GOx evolution. H516 and H559 are highlighted as catalytic active residues along with FAD in the active site. Ribbon representation was prepared using the Discovery Studio software package (Accelrys Software, 2007).

Individual site saturation mutagenesis studies discovered that substitutions at A173 and A332 lead to an increase in the mediator (up to 3-fold) as well as in oxygen activity (up to 3-fold). A332 is located at the substrate entrance channel with a distance of 14.6 Å to the flavin isoalloxazine and residue A173 is located at the surface of the GOx and 14.8 Å away from the FAD and catalytic histidines. The contributions of the individual substitutions and how the best performing variants (A173V and A332V) improve mediator activity and oxygen activity remains to be studied by in-depth computational analysis. The latter will help to rationalize how conformational changes are relayed from the positions 173 and 332 to the active site. Position F414 is responsible for increased mediator activity by substitutions from F to M, V, L or I. F414 it is located 8.2 Å above the FAD (Fig. 2) in the substrate binding pocket and involved in the β-D-glucose binding (Leskovac et al., 2005). After gluconolactone formation and dissociation from the active site (Leskovac et al., 2005), an oxygen molecule or a mediator acts as electron acceptor from the reduced cofactor $FADH_2$. The substitutions from F to aliphatic M, V, L or I indicate that a change in flexibility and size of the side chain is mainly contributing to an increased mediator activity. The location of the position 414 supports the hypothesis, that the QDM mediator can directly bind in the binding pocket to reoxidized $FADH_2$ efficiently. The binding efficiency (mediator affinity) and the associated electron transfer rate is thereby very likely depending on the mediator chemistry, the mediator size and the amino acid side chains surrounding the binding pocket. The latter renders it likely that improved mediator activities are dependent on the identified amino acid substitutions and specific for the employed mediator system. Further structural and/or docking studies with QDM are required in order to clarify mediator binding and interplay with oxygen (see Table 1; A414M) as well as to investigate alternative electron transfer chains from the FAD to the protein.

The position V560 is important for 'oxygen activity' and substitutions to L, P or T drastically reduce oxygen activity. V560 neighbors the active site residues H516 and H559 which are involved in shuttling of protons from $FADH_2$ to oxygen. Replacement of V by T can perturb the catalytic hydrogen-bond network (FAD, H559, H516, E412) due to an additional hydroxyl group (V560T). Replacement of V560 by L or P might alter the orientation of the neighboring catalytic residue H559. Therefore the oxygen activation or binding between FAD and His 559 is perturbed by substitutions at position 560.

In essence, V7 is an excellent alternative to PQQ- and FAD-dependent GDH's since it has a drastically reduced oxygen activity (Table 3). The main advantage of GOx and V7 over GDH is the β-D-glucose specificity which is important for accurate glucose determinations in serum. PQQ-dependent GDHs convert also maltose ($>70\%$ of β-D-glucose activity) whereas V7 is not converting maltose to a detectable amount (see Table 4). The specificity of V7 ensures that concentrations of β-D-glucose are not overestimated for instance during peritoneal dialysis. The glucose versus maltose specificity was tackled by PQQ-GDH reengineering yielding GDHs variants with low activities on maltose ($<3\%$ residual activity). Alternatively FAD–GDH with low activity on maltose ($<3\%$ residual activity) were introduced for glucose determinations. However FAD–GDHs have in general a high activity on xylose ($>20\%$ of glucose activity) (Boenitz-Dulat et al., 2006; Kratzsch et al., 2002; Zafar et al., 2012). The latter might represent a further challenge for accurate β-D-glucose determinations (Schleis, 2007).

5. Conclusion

Four novel positions were identified and site saturation studies revealed that position 560 is a key residue for oxygen sensitivity

that positions 173 and 332 increase mediator and oxygen activity, and that position 414 influences mediator activity and increases or reduces oxygen activity depending on the substitution. The glucose oxidase variant V7 has a 37-fold reduced oxygen dependency by maintained β-D-glucose specificity and thermal resistance. V7 was successfully evolved to operate with diabetes relevant mediator system (quinone diimine/phenylendiamine) and comes from our point of view close to an 'ideal enzyme' for accurate diabetes analytics. The identified positions have to further be studied in depth to understand the communication between mediator, oxygen, and GOx. The latter is a prerequisite to novel design concepts for ideal mediator/protein couples in diagnostics.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.06.029.

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