Mol Biotechnol (2011) 47:253–261
DOI 10.1007/s12033-010-9339-5

RESEARCH

Characterization and Engineering of a Novel Pyrroloquinoline Quinone Dependent Glucose Dehydrogenase from *Sorangium cellulosum* So ce56

Michael Hofer · Kathrin Bönsch · Thomas Greiner-Stöffele · Meike Ballschmiter

Published online: 1 October 2010

Abstract A novel pyrroloquinoline quinone dependent glucose dehydrogenase like enzyme (PQQ GDH) was isolated from *Sorangium cellulosum* So ce56. The putative coding region was cloned, over expressed in *E. coli* and the resulting enzyme was characterized. The recombinant protein has a relative molecular mass of 63 kDa and shows 43% homology to PQQ GDH-B from *Acinetobacter calcoaceticus*. In the presence of PQQ and $CaCl_2$ the enzyme has dehydrogenase activity with the substrate glucose as well as with other mono- and disaccharides. The thermal stability and its pH activity profile mark the enzyme as a potential glucose biosensor enzyme. In order to decrease the activity on maltose, which is unwanted for a potential application in biosensors, the protein was rationally modified at three specified positions. The best variant showed a 59% reduction in activity on maltose compared to the wild type enzyme. The catalytic efficiency (k_{cat}/K_M) was reduced fivefold but the specific activity still amounted to 63% of the wild type activity.

M. Hofer · K. Bönsch · T. Greiner-Stöffele · M. Ballschmiter (✉)
Institute of Biochemistry, University of Leipzig, Deutscher Platz 5b, 04103 Leipzig, Germany
e-mail: ballschmiter@uni-leipzig.de

M. Hofer
e-mail: hofer@uni-leipzig.de

K. Bönsch
e-mail: boensch@uni-leipzig.de

T. Greiner-Stöffele
c-LEcta GmbH, Deutscher Platz 5b, 04103 Leipzig, Germany
e-mail: thomas.greiner@c-lecta.de

Keywords PQQ · Glucose dehydrogenase · *Sorangium cellulosum* · Biosensor · Substrate specificity

Introduction

Several enzymes have been used in glucose biosensors for the determination of glucose levels especially in blood samples from patients diagnosed with diabetes. Glucose oxidase (GOD) was used initially, followed by pyrroloquinoline quinone dependent glucose dehydrogenase B of *Acinetobacter calcoaceticus* (PQQ GDH-BAca) [1–3]. Although the usage of new enzymes, namely the FAD dependent glucose dehydrogenase from *Aspergillus terreus*, for this type of application was reported recently [4], PQQ GDH-BAca remains one of the most frequently used enzymes for glucose biosensor devices. Its high turnover number and insensitivity toward oxygen makes it superior in the determination of glucose concentrations [5, 6]. Besides its beneficial properties, there are still some drawbacks connected to PQQ GDH-BAca. For example, in addition to its natural substrate glucose this enzyme recognizes several mono- as well as disaccharides, especially maltose, which can lead to imprecise blood glucose measurements [7]. Several attempts have already been made to improve its substrate specificity [8–10] as well as its thermal stability [11, 12]. Despite of all these optimization efforts there is still no PQQ GDH-BAca variant available which fulfills all demands for an optimal biocatalyst in glucose biosensors. Thus, besides further improvements of PQQ GDH-BAca by enzyme engineering approaches the discovery of novel enzymes presenting alternatives for the application in glucose biosensors would be beneficial.

In 2007 the 13 Mbp genome sequence of *Sorangium cellulosum* So ce56 was published, which is one of the

largest bacterial genomes known to date [13]. This gram-negative myxobacterium is of great biotechnological interest since it produces novel classes of secondary metabolites. Among many interesting enzymes of potential industrial importance *S. cellulosum* So ce56 possesses a putative protein with high sequence similarity to PQQ GDH-BAca from *A. calcoaceticus* [14]. In this work the corresponding gene was cloned and over expressed in *E. coli*. After purification and characterization of the wild type enzyme, which was named PQQ GDH-BSce, rational enzyme engineering approaches were used to improve its substrate specificity for a potential application in biosensors for glucose monitoring.

Materials and Methods

Materials

All chemicals were purchased from Sigma–Aldrich (Munich, Germany). The strain *E. coli* NovaBlue *endA1hs dR17(*$r_{K12}-$ $m_{K12}+$*) supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proA*$^+$*B*$^+$ *lacI*^{q} *ZDM15::* Tn*10* (Tet^r)] (DE3) and expression vector pRSF-1b were obtained from Novagen (Darmstadt, Germany). For the use as PQQ GDH-BSce production and screening strain the chromosomal *gcd* gene coding for PQQ glucose dehydrogenase A was knocked out in the *E. coli* NovaBlue (DE3) strain by the method of Datsenko and Wanner resulting in the strain *E. coli* NovaBlue (DE3) Δ*gcd* [15].

Genomic DNA from *S. cellulosum* So ce56 was kindly provided by Prof. Rolf Müller, Department of Pharmaceutical Biotechnology, Saarland University, Saarbrücken, Germany.

Growth media for the production of PQQ GDH-BSce were Luria–Bertani medium (LB), pH 7.0, containing 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 2× YTM5 medium, pH 6.9 containing 16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 0.01% (v/v) glycerol.

Cloning and Expression

The pyrroloquinoline quinone dependent glucose dehydrogenase like gene *gdhB* (GeneID: 5806305, Locus Tag: sce0774) was amplified by PCR from genomic DNA of *S. cellulosum* So ce56 using the primer pair fwd 5′-TCATACATGTCGCCCTTTATCTCACGCAAC-3′ and rev 5′-CATGAAGCTTTCAGTGGTGGTGGTGGTGGTGGCCGCCGTGCGATGTCGTGTACTTG-3′. The primer pair included suitable restriction enzyme sites (underlined) and a C-terminal His_6-tag. The gene was cloned into the expression vector pRSF-1b, and *E. coli* NovaBlue (DE3) Δ*gcd* cells were transformed with the resulting plasmid.

Protein Production and Purification

For protein production cultures from single colonies were grown at 37°C in 800 ml LB medium containing 50 μg/ml kanamycin. After reaching an optical density of 0.6 at 600 nm (OD_{600}), 250 μM IPTG for induction and 500 nM PQQ were added and protein expression was carried out over night at room temperature. Cells were harvested by centrifugation at 3,000×*g* and 4°C for 15 min and the cell pellet was resuspended in 10 mM MOPS pH 7.0 containing 50 mM imidazole and 0.5 M NaCl. The following disruption of the cells was done by French Press at 10,000 psi (Thermo, Milford, USA). After centrifugation at 10,000×*g* and 4°C for 1 h, the pellet containing the cell debris and the main fraction of the protein in inclusion bodies was resuspended in 10 mM MOPS pH 7.0 containing 50 mM imidazole, 0.5 M NaCl, and 8 M urea. The suspension was sonicated for 5 min (Labsonic, Braun, Melsungen, Germany). Insoluble cell debris was removed by centrifugation for 1 h at 10,000×*g* and 4°C. The supernatant was applied to a 1 ml HisTrap HP column (GE Healthcare, Munich, Germany). The column was then washed with 20 column volumes (cv) of 10 mM MOPS pH 7.0 containing 50 mM imidazole, 0.5 M NaCl, and 8 M urea. After the washing step the urea containing buffer was exchanged within 30 cv against 10 mM MOPS pH 7.0 containing 0.5 M NaCl, 3 μM $CaCl_2$, and 50 mM imidazole by a linear gradient. Finally the protein was eluted within 30 cv 10 mM MOPS pH 7.0 containing 0.5 M NaCl, 3 μM $CaCl_2$, and 500 mM imidazole by a linear gradient. The fractions with the highest amounts of the target protein were collected and dialyzed three times against the 100 times volume of 50 mM sodium potassium phosphate buffer pH 7.5 containing 3 μM $CaCl_2$ and 2 μM PQQ. The progress of the purification was analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 2). Protein concentrations were determined by Bradford assay kit (Bio-Rad, Munich, Germany) with bovine serum albumin as the protein standard.

N-terminal Sequencing

After the SDS-PAGE analysis, PQQ GDH-BSce was electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using a semi-dry blotting apparatus (Thermo Scientific HEP-3, Waltham, MA, USA). The protein was stained with Ponceau S, excised from the PVDF membrane and subjected to *N*-terminal sequencing by Edman degradation (Proteome Factory, Berlin, Germany).

Activity Assay

The activity of PQQ GDH-BSce was assayed for 2 min spectrophotometrically by the reduction of nitrotetrazolium blue (NTB) at 570 nm in the presence of phenazine methosulfate (PMS). The assay mixture contained 42 mM sodium potassium phosphate buffer pH 7.5, 30 mM glucose, 0.2 mM PMS, 0.22 mM NTB, 0.1% (w/v) BSA, 0.1% (v/v) Triton X-100, and 3 μM $CaCl_2$. One unit of GDH activity was defined as the conversion of 1 μmol glucose per minute at 37°C which corresponds to the formation of 0.5 μmol of diformazan in the assay. For each measurement the amount of enzyme was adjusted to 0.01–0.3 U/ml. Furthermore, the enzyme was pre-incubated in 50 mM sodium potassium phosphate buffer (pH 7.5) containing 3 μM $CaCl_2$, 2 μM PQQ, 0.1% (w/v) BSA, and 0.1% (v/v) Triton X-100 for at least 30 min on ice before each measurement.

Enzyme Characterization

To determine the optimal pH and buffer conditions for the activity measurement of the enzyme 42 mM sodium potassium phosphate buffer (pH 5.5–8), 42 mM citrate phosphate buffer (pH 5–6.5), and 42 mM sodium borate buffer (pH 8–9) was used for the assay as well as for the enzyme solution.

All other measurements were performed in 42 mM sodium potassium phosphate buffer (pH 7.5) with calcium chloride concentrations below the precipitation level. The temperature profile of PQQ GDH-BSce activity was determined by heating the assay solution and the enzyme to the indicated temperatures and measuring the activity at these temperatures for 2 min. At temperatures above 40°C the assay solution became unstable.

Thermal stability was determined by heating the enzyme solution for 1 h to the indicated temperatures and subsequent activity measurement at 37°C. Before using the enzyme solution for the activity assay it was cooled on ice for at least 5 min.

Substrate specificity profiling was carried out by varying the substrate in the standard assay. In addition to glucose, the mono- and disaccharides D-maltose, D-lactose, D-mannose, 2-deoxy-D-glucose, D-allose, D-galactose, D-xylose, and L-arabinose were tested as substrates at a concentration of 30 mM.

For enzyme kinetics the activity measurement was carried out with glucose concentrations varying from 0.3 to 60 mM. For the determination of activity the initial rates within the linear range of the product time curve were used. The kinetic parameters k_{cat} and K_m were calculated by non linear regression fitting to the Michaelis–Menten equation using GraFit version 6.0.7 (Erithacus Software Ltd., Horley, Surrey).

Enzyme Engineering and Screening

Mutations were introduced into the *gdhB* (sce0774, GeneID 5806305) gene by site-directed mutagenesis (Stratagene, USA). The respective oligonucleotides are listed in Table 1. At the positions Q126 and Q219/F220 the NNK motif was used, resulting in libraries Q126 and Q219/F220, respectively. After digestion with DpnI *E. coli* NovaBlue (DE3) Δ*gcd* cells were transformed with both libraries individually. Single colonies were picked and transferred into 96 deep-well plates containing 1 ml 2× YTM5 medium. Cells were grown to an OD_{600} of 0.6 at 37°C. Protein expression was then induced by adding 1 mM IPTG at each well. After 3 h of incubation at 37°C cells were harvested by centrifugation and the cell pellets were resuspended in 50 mM sodium potassium phosphate buffer (pH 7.5) containing 1× Cell Lytic B solution (Sigma–Aldrich, Munich, Germany), 0.1 mg/ml lysozyme, and 0.1 mg/ml DNaseI for cell lysis. After 45 min incubation at 37°C cells were centrifuged at 3,000×*g* for 15 min and the supernatant was used as crude enzyme extract for activity screening. The crude enzyme extract was split in half and diluted each with 1 volume of 50 mM sodium potassium phosphate buffer (pH 7.5) containing 2 μM PQQ and 3 μM $CaCl_2$. After incubation at room temperature for 30 min 2 volumes of 50 mM sodium potassium phosphate buffer (pH 7.5) containing 1.2 mM PMS and 1.2 mM 2,6-dichlorophenolindophenol sodium salt hydrate (DCIP) were added. Finally, either glucose or maltose was added to a final concentration of 30 mM and the reduction of DCIP was followed spectrophotometrically at 595 nm for 45 min [16]. Relative activities on maltose were deduced for each enzyme variant by calculating the ratio of activity on maltose compared to activity on glucose.

Table 1 Oligonucleotide sequences for site-directed mutagenesis. The mutated codons are marked in italics

Name	Sequence 5′ → 3′
126_fwd	GTTCCAGTCGGGGGGT*NNK*GACGGGCTGCTGGCCCTG
126_rev	CAGCAGCCCGTC*MNN*ACCCCCCGACTGGAACG
219/220_fwd	GATCAGGGGAACAAC*NNKNNK*GCCAGGATGTGCAAC
219/220_rev	GTTGCACATCCTGGC*MNNMNN*GTTGTTCCCCTGATCCCCTATG

Modeling of the Tertiary Structure

The protein sequence of PQQ GDH-BSce (YP_0016 11411.1) was used for an iterative PSI-Blast search [17]. The best 500 Hits were used to create a scoring matrix and an alignment sequence for the modeling software using ClustalW2 [18]. The software program MOE 2008.10 (Chemical Computing Group Inc., Montreal, Quebec, Canada) was then used to generate the 3-D model of PQQ GDH-BSce using the crystal structure of PQQ GDH-BAca (pdb accession code 1cq1) together with the scoring matrix and the alignment sequence. The substrate glucose was modeled into the substrate binding pocket afterwards. Finally PyMOL (DeLano Scientific LLC, Palo Alto, CA, USA) was used to render figures.

Results and Discussion

Sequence Analysis of PQQ GDH-BSce

The 1518 base pair gene *gdhB* (sce0774, GeneID 5806305) from the genome of *Sorangium cellulosum* So ce56 codes for a protein of 505 amino acids, which will be referred to as PQQ GDH-BSce. The translated protein sequence shows high similarity to the functional regions of soluble glucose dehydrogenases (s-GDH) which require the cofactor pyrroloquinoline quinone (PQQ) to oxidize glucose to gluconolactone. 43% of all amino acids are identical to the best characterized s-GDH, the PQQ GDH-B from *Acinetobacter calcoaceticus* (PQQ GDH-BAca, Fig. 1). In the sequence of PQQ GDH-BSce seven of the nine amino acids forming the binding site for the cofactor PQQ in PQQ GDH-BAca are conserved (Fig. 1) [19]. Positions R432, R430, K401, L400, A374, N253, and R252 from PQQ GDH-BAca are identical to the corresponding positions of PQQ GDH-BSce. Only the position Q270, where glutamine is exchanged by histidine in PQQ GDH-BSce and the position Q47, where glutamine is exchanged by valine, differ from the PQQ-binding pocket of PQQ GDH-BAca. Also the active sites among both enzymes are highly conserved (Fig. 1). At positions corresponding to W370, Y367, R252, Q192, H168, D167, and Q100 in PQQ GDH-BAca no exchanges can be observed. Only at position L193, leucine is replaced by phenylalanine in PQQ GDH-BSce.

More differences can be found in the signal peptide sequences of both enzymes. The first 24 amino acids of PQQ GDH-BAca form a signal peptide I which directs the protein from the cytoplasm to the periplasm of *A. calcoaceticus* [14]. Computational analysis with SignalP 3.0 [20] and LipoP 1.0 [21] of PQQ GDH-BSce revealed that its first 28 amino acids might form a putative type II signal peptide. This indicates that PQQ GDH-BSce might be a lipoprotein of *S. cellulosum* So ce56.

Cloning, Expression, and Refolding

For the characterization of PQQ GDH-BSce the *gdhB* gene from *S. cellulosum* So ce56 was cloned into the expression vector pRSF-1b and expressed as his_6-tagged protein in *E. coli* NovaBlue (DE3) Δ*gcd* cells which lack the gene encoding the endogenous PQQ glucose dehydrogenase A [22]. The recombinant protein accumulated in inclusion bodies with only a minor soluble fraction in the cytoplasm (Fig. 2). Since the reduction of the expression time, variations of growth temperature (16–37°C) as well as the reduction of the amount of IPTG used to induce expression resulted in inclusion bodies (data not shown), a solubilization and subsequent refolding of the protein was essential.

Solubilization and purification of PQQ GDH-BSce via immobilized metal affinity chromatography yielded a protein preparation of >95% purity. The dominant band had an apparent mass of 63 kDa as analyzed by SDS-PAGE, whereas the mass deduced from the primary structure was 56 kDa (Fig. 2). The identity of the band as PQQ GDH-BSce was verified by N-terminal protein sequencing. The aberrant electrophoretic performance of the enzyme was reproducible and occurred in all states of purification.

Protein refolding was started on the column during elution in the presence of Ca^{2+}. Refolding was finished by dialysis against 50 mM sodium potassium phosphate buffer pH 7.5 containing 3 μM $CaCl_2$ and 2 μM PQQ. These conditions led to a high dehydrogenase activity of PQQ GDH-BSce with the substrate glucose. Refolding without Ca^{2+} and PQQ led to an inactive enzyme. The activity could only be restored by incubation with both factors. Olsthoorn and Duine have observed that the presence of Ca^{2+} and PQQ is also essential for the reconstitution and activity for PQQ GDH-BAca, where Ca^{2+} is needed for the correct folding and for the functionalization of PQQ as cofactor [23]. In case of PQQ GDH-BAca, Ca^{2+} plays a crucial role in the formation of the homodimer complex as well as for the activation of the cofactor PQQ. Four ions are located at the homodimer interface stabilizing the formation of this complex. It is speculated that the interaction of two ions with the 4CD loop brings together distant parts of the loop in each monomer which helps to stabilize the homodimer. Additionally, one calcium ion is located in the active site of each monomer, playing a crucial role in the catalytic process [19]. It can be deduced that in PQQ GDH-BSce Ca^{2+} has the same function.

```
A. calcoaceticus      1 mnkhllakial-lsavqlvtlsafa----------dvpltpsqfakakse
S. cellulosum         1 mspfisrnlavvlarvgllttaswsltgcgqagppdeplesdaqaivaee

A. calcoaceticus     40 ----------------nfdkkviqsnlnkphallwgpdnqiwlteratgk
S. cellulosum        51 aedpgdtdevcpgeeptftsreivrdlsfpmrivwgpdgylwltertakr
                                          P
A. calcoaceticus     74 ilrvnpesgsvktvfqvpeivndadgqngllgfafhpdf---knnpyiyi
S. cellulosum       101 vvrvrpedglqktaitipeafq-sgggdgllalalhpdllkhkgkdyvyv
                                             A
A. calcoaceticus    121 sgtfknpkstdkelpnqtiirrytynkstdtlekpvdllaglpsskdhqs
S. cellulosum       150 sftyn--vaadpavdrrtkirrytynrvtetlgspvdvitglpgsddhns
                                                                  AA
A. calcoaceticus    171 grlvigpdqkiyytigdqgrnqlaylflpnqaqhtptqqelngkdyhtym
S. cellulosum       198 arllfgpdgklyytigdqgnnqfarmcnpnraqelptaaevaaqdwqkyv
                                      AA
A. calcoaceticus    221 gkvlrlnldgsipkdnpsfngvvshiytlghrnpqglaftpngkllqseq
S. cellulosum       248 gkilrlnvdgsipkdnpkidgvrshiysyghrnaqgltfgrgdklyaneh
                                                    *P                P
A. calcoaceticus    271 gpnsddeinlivkggnygwpnvagykddsgyayanysaaanksikdlaqn
S. cellulosum       298 gpksddelnliqagknygwpyvagyqddqaytydnwsassptpcnelsws

A. calcoaceticus    321 gvkvaagvpvtkesewtgknfvpplktlytvqdtynyndptcgemtyicw
S. cellulosum       348 dfapppsvprqlesafshpdfkeplltfytvpndyvffdwacgfsqyicw
                                                                  A  A
A. calcoaceticus    371 ptvapssayvykggkkaitgwentllvpslkrgvifrikldptysttydd
S. cellulosum       398 ptiapsslhyypagh-girgwdhsllmtslkkgavyqvklspngktvege
                           P                      PP
A. calcoaceticus    421 avpmfksnnryrdviaspdgnvlyvltdtagnvqkddgsvtntlenpgsl
S. cellulosum       447 pavlfqttnryrdlamspdrktfyiitdtagntsgptggvttvldnpgai
                              P P
A. calcoaceticus    471 ikftykak-
S. cellulosum       497 lefkyttsh
```

Fig. 1 Sequence alignment of PQQ GDH-BAca from *A. calcoaceticus* [14] and PQQ GDH-BSce from *S. cellulosum* So ce56. Identical amino acids are marked with *gray background*. Amino acids of the PQQ-binding site in PQQ GDH-BAca are marked with "P" and amino acids of the active site are marked with "A". R252 belongs to both sites and is marked with "*"

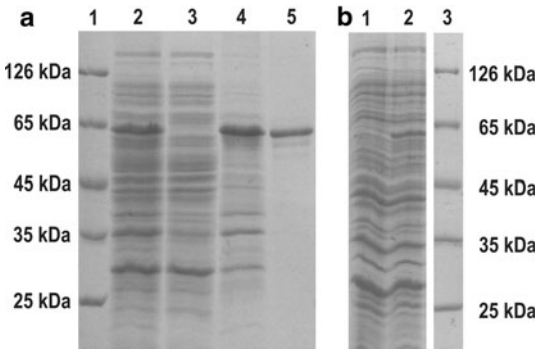

Fig. 2 **a** SDS-PAGE analysis of PQQ GDH-BSce. 1, protein marker; 2, *E. coli* NovaBlue (DE3) Δ*gcd* cells after expression of PQQ GDH-BSce; 3, soluble fraction after cell disruption with French Press; 4, pellet after cell disruption with French Press; 5, PQQ GDH-BSce after his_6-tag purification. **b** 1, *E. coli* NovaBlue (DE3) Δ*gcd* cells with pRSF1-b after expression; 2, *E. coli* NovaBlue (DE3) Δ*gcd* cells with pRSF1-b containing PQQ GDH-BSce after expression; 3, protein marker

Enzyme Characterization of PQQ GDH-BSce

The refolded protein PQQ GDH-BSce shows a PQQ dependent activity with glucose (1961 U/mg), clearly marking this enzyme as a novel PQQ dependent glucose dehydrogenase enzyme. However, in addition to glucose, PQQ GDH-BSce accepts various mono- and disaccharides as substrates (Table 2). The highest activity was observed with glucose, but the relative activity of the recombinant enzyme on most other substrates is 70% or higher compared to glucose, whereas the lowest activity was found with the substrate 2-deoxyglucose. This is in accordance with other soluble GDH-B enzymes. In the group of PQQ dependent GDH enzymes only the membrane-bound glucose dehydrogenase (PQQ GDH-A) shows high activity on 2-deoxy-D-glucose [24, 25].

PQQ GDH-BSce was further characterized with respect to pH dependency, temperature range, and temperature

Table 2 Relative activities of PQQ GDH-BSce and PQQ GDH-BAca [7, 8] on different substrates

Substrate	PQQ GDH-BSce (%)	PQQ GDH-BAca (%)
D-Glucose	100	100
L-Arabinose	97	35
D-Lactose	91	65
D-Xylose	86	20
D-Allose	85	47
D-Maltose	71	87
D-Galactose	71	30
D-Mannose	36	13
2-Deoxy-D-glucose	3	5

All activities were calculated in respect of the activity on glucose which was set to 100%

stability. The pH dependency was tested in three buffers with a variety of pH values (Fig. 3). The pH optimum of PQQ GDH-BSce is pH 7.5 with the enzyme showing activity from pH 5.5 to 9. The highest activity was observed with 42 mM sodium potassium phosphate buffer at pH 7.5. Consequently this buffer was used for all activity measurements.

PQQ GDH-BSce revealed the highest activity at a temperature of 37°C (Fig. 4a). At 25°C the enzyme activity was reduced to 50%, whereas at 40°C the activity was only reduced to 90% compared to the activity at 37°C. The thermostability was tested by incubating the enzyme solution at the indicated temperature for 1 h. The enzyme showed only a slight decrease in activity in the range of 25–45°C, with 100% activity at 25°C and still 80% activity at 45°C (Fig. 4b). After incubation at 60°C for 1 h the enzyme still had 27% of its initial activity while loosing nearly all activity after treatment at 65°C.

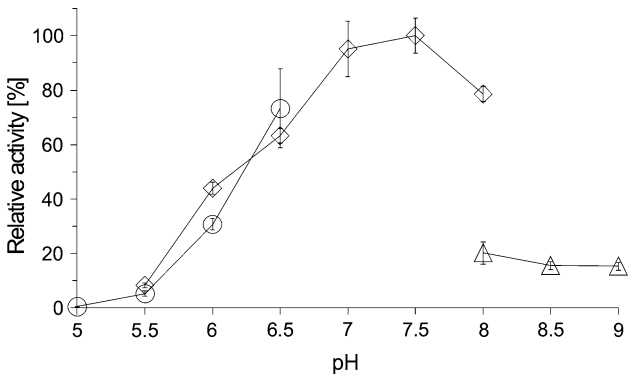

Fig. 3 pH and buffer dependencies of PQQ GDH-BSce. Relative enzyme activity was determined in different buffers and at different pH values using the same amount of enzyme in a 2-min assay. *Circle* 42 mM citrate phosphate buffer, *diamond* 42 mM sodium potassium phosphate buffer, and *triangle* 42 mM sodium borate buffer

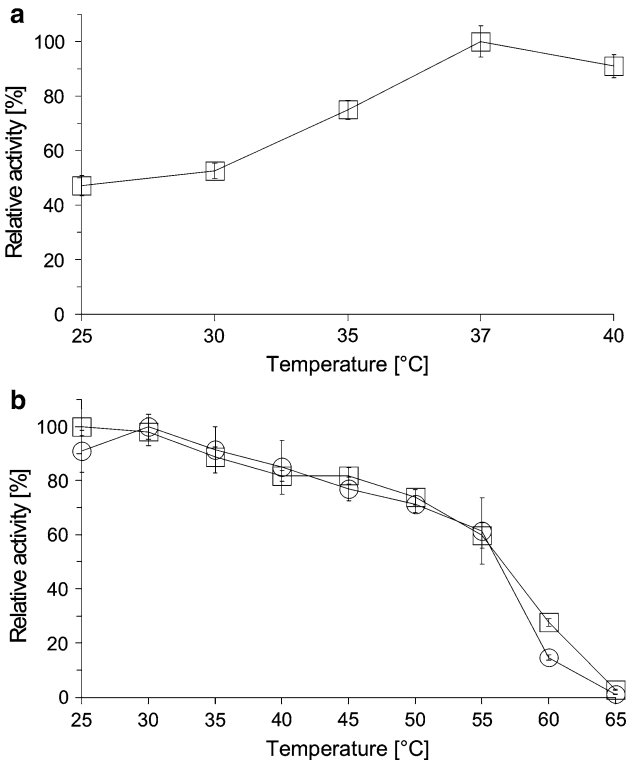

Fig. 4 Thermal characteristics of PQQ GDH-BSce. **a** Activity at various temperatures. **b** Temperature stability of PQQ GDH-BSce (*square*) and variant Q219K/F220K (*circle*) after 1 h heat treatment at the indicated temperature. The activity was determined in a 2-min assay in 42 mM sodium phosphate buffer pH 7.5 with 30 mM glucose as substrate at 37°C

Comparison with PQQ GDH-B

The high sequence similarity of PQQ GDH-BAca and PQQ GDH-BSce already indicated that both enzymes might also be similar in their biochemical properties. Beside their need for the cofactors PQQ and Ca^{2+} for the dehydrogenase activity on glucose both enzymes accept a broad range of different substrates.

The enzymes differ in their specific activity with glucose. PQQ GDH-BAca has a specific activity of 7400 U/mg, whereas PQQ GDH-BSce has only a specific activity of 1961 U/mg [23]. The relative activity on maltose (71%) of PQQ GDH-BSce is slightly lower than that of PQQ GDH-BAca (87%). PQQ GDH-BSce shows higher activities on xylose, arabinose, lactose, allose, mannose, and galactose (Table 2) compared to PQQ GDH-BAca [7, 8]. Also remarkable is the slightly better thermal stability of PQQ GDH-BSce with 27% residual activity after a 1-h treatment at 60°C compared to PQQ GDH-BAca, which has a residual activity below 20% after incubation for 1 h at 55°C [11].

These data reveal that PQQ GDH-BSce and PQQ GDH-BAca have similar properties. But PQQ GDH-BSce has a

higher thermal stability and a lower activity on maltose, whereas PQQ GDH-BAca has a higher specific activity and is less active on other substrates.

Engineering and Kinetic Parameters

The high activity of PQQ GDH-BSce on maltose (Table 2) is disadvantageous for a feasible application in glucose biosensors. Normally the blood glucose concentration is 50 times higher than the blood maltose concentration [26, 27]. However, in the case of diabetes patients receiving infusions with high maltose concentrations, this ratio is massively changed, leading to false glucose measurements. For an optimal glucose biosensor the maltose activity has to be reduced as much as possible while retaining high specific activity and thermal stability.

For the well characterized PQQ GDH-BAca several amino acid positions are known for their high influence on the substrate specificity [9, 10, 28]. Based on these reports and our own data of PQQ GDH-BAca variants with improved substrate specificity (unpublished data), three promising sites corresponding to Q100, Q192, and L193 in PQQ GDH-BAca were chosen as targets for improvement of the substrate specificity of PQQ GDH-BSce. Like Q100, Q192, and L193 in PQQ GDH-BAca the amino acids Q126, Q219, and F220 in PQQ GDH-BSce enclose the substrate glucose as shown in a modeled tertiary structure (Fig. 5). These sites were substituted by saturation mutagenesis. The resulting two libraries contained all 20 possible amino acids at position Q126 in one library and all 400 possible amino acid combinations at positions Q219 and F220 in a second library. In accordance with the formula for library coverage by Patrick et al. [29] 99% of all variants from both libraries were screened with respect to a reduced relative activity on maltose. The seven enzyme variants with the highest reduction in maltose activity were purified for kinetic analysis (Table 3).

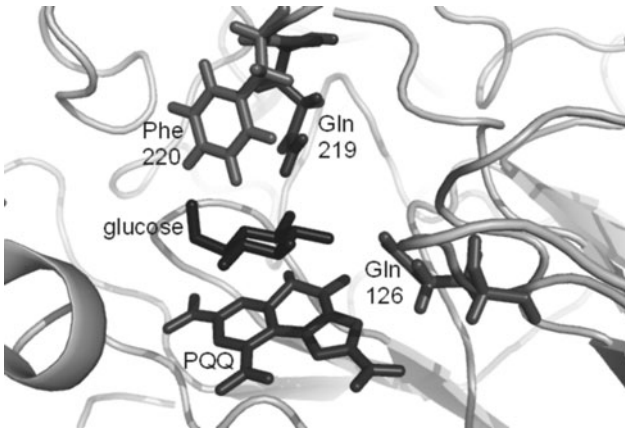

Fig. 5 Model of the active site of wild type PQQ GDH-BSce. Shown are the cofactor PQQ, the substrate glucose and the amino acids positions Q126, Q219, and F220 which were chosen as targets for site-directed mutagenesis

Three variants had changes at position Q126 and four were double mutants with changes at position Q219 and F220. In all seven variants the introduction of polar amino acids was observed (Table 3). The wild type protein has an uncharged polar glutamine at position 219 and an uncharged and unpolar phenylalanine at position 220. In variants Q219K/F220K and Q219E/F220E, two amino acids with charged and polar side chains, either glutamic acid or lysine was introduced. Also in variant Q219N/F220K and in variant Q219K/F220C at least one charged polar amino acid was introduced. One of the three best variants from the single mutant library (Q126R) carries a charged polar amino acid instead of the wild type glutamine. The preferred introduction of polar and charged amino acid side chains may influence the substrate specificity either by polar and ionic interactions of the side chains with the substrate or they may lead to a conformational

Table 3 Kinetic parameters of PQQ GDH-BSce wild type and variants with reduced activity on maltose from library Q126 and library Q219/F220

	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	Relative activity on maltose (%)
Wild type	2.4	1778.8	744.0 (100%)	71.0
Q126E	11.0	197.1	18.0 (2.4%)	25.6
Q126S	22.6	445.1	19.7 (2.6%)	12.4
Q126R	41.4	341.6	8.2 (1.1%)	11.4
Q219E/F220E	11.3	305.9	27.0 (3.6%)	5.2
Q219K/F220K	7.6	1113.8	146.6 (19.7%)	29.3
Q219N/F220K	10.6	227.2	21.7 (2.9%)	11.7
Q219K/F220C	7.2	121.5	16.8 (2.3%)	20.0

Activity was determined in a 2-min assay in 42 mM sodium phosphate buffer pH 7.5 at 37°C with glucose concentrations varying from 0.3 to 60 mM. For the determination of activity the initial rates within the linear range of the product time curve were used. The last column shows the relative activity on maltose referring to the corresponding glucose activity of each variant which is set to 100%

change of the protein. It can be speculated that this conformational change leads to a decreased binding of substrates like maltose, which is bulkier than glucose.

The wild type enzyme has a high relative activity on maltose (71%) compared to glucose. All selected seven variants have an activity on maltose which is at least reduced to 30% (Q219K/F220K), with the best variant showing a reduction down to 5.2% (Q219E/F220E). In PQQ GDH-BAca saturation mutagenesis at the corresponding positions also had great influence on the activity with maltose. Substitution of Q100 in PQQ GDH-BAca with alanine, glutamic acid, phenylalanine, glycine, lysine, leucine, or serine left the enzyme almost inactive [10]. Variations at this position in PQQ GDH-BSce, however, led to a further decreased maltose activity ranging from 36.1 to 16% compared to wild type PQQ GDH-BSce. At positions Q192 and L193 of PQQ GDH-BAca the best variants showed a reduction to 22% (Q192G) and 39% (L193F) compared to wild type PQQ GDH-BAca [10]. Experiments of our own revealed that the combination of the Q192/L193 mutations of PQQ GDH-BAca in one library resulted in a variant Q192G/L193F with massively decreased maltose activity (8.9%) which is comparable to the PQQ GDH-BSce double mutants. These data show that the positions Q126, Q219, and F220 in PQQ GDH-BSce have as great influence on the substrate specificity as their counterparts in PQQ GDH-BAca.

In 2004, Igarashi et al. [30] additionally disclosed that variants of PQQ GDH-BAca at the position Q192 corresponding to position Q219 from PQQ GDH-BSce have an improved substrate specificity but decreased catalytic efficiency. The kinetic analysis revealed that although all PQQ GDH-BSce variants show improved substrate specificity, their v_{max} values drastically decrease and their K_M values increase, reducing their catalytic efficiency, too (Table 3). Variant Q219K/F220K shows only a fivefold reduction in catalytic efficiency compared to the wild type enzyme, whereas all other variants have at least a 27-fold reduction in catalytic efficiency. Also the specific activity with glucose of the variant Q219K/F220K was the highest of all characterized variants with 1240 U/mg (63% of wild type). The high remaining specific activity together with its good catalytic efficiency and a reduction of maltose activity to 29.3% make variant Q219K/F220K a promising candidate for a glucose biosensor or further engineering. Additionally this variant showed no reduction in thermal stability (Fig. 4b) compared to wild type PQQ GDH-BSce. A second auspicious candidate is variant Q219E/F220E, where maltose activity was minimized to 5.2% of the wild type activity. However, this reduction is accompanied by an unwanted loss of catalytic efficiency.

Based on these two successful variants further studies have to be carried out to show whether the desirable combination of high catalytic efficiency and maximal reduction in maltose activity can be achieved by manipulation of additional sites located further from the catalytic center of the enzyme PQQ GDH-BSce. It also remains to be tested if variant Q219K/F220K and variant Q219E/F220E of PQQ GDH-BSce perform better than the commonly used PQQ GDH-B from *A. calcoaceticus* as glucose biosensor proteins.

Acknowledgments This work was supported by the BMBF Innovationsinitiative Neue Länder (Unternehmen Region, InnoProfile). We would like to thank Bernd Gründig of Senslab GmbH for the helpful discussions, Christian Seidl for his help with the computer model, and Rolf Müller for his kind gift of *S.cellulosum* So ce56 genomic DNA.

References

1. D'Costa, E. J., Higgins, I. J., & Turner, A. P. (1986). Quinoprotein glucose dehydrogenase and its application in an amperometric glucose sensor. *Biosensors, 2*, 71–87.
2. Ling, Y., Haemmerle, M., Olsthoorn, A. J. J., Schuhmann, W., Schmidt, H.-L., Duine, J. A., et al. (1993). High current density "wired" quinoprotein glucose dehydrogenase electrode. *Analytical Chemistry, 65*, 238–241.
3. Laurinavicius, V., Razumiene, J., Ramanavicius, A., & Ryabov, A. D. (2004). Wiring of PQQ-dehydrogenases. *Biosensors and Bioelectronics, 20*, 1217–1222.
4. Tsujimura, S., Kojima, S., Kano, K., Ikeda, T., Sato, M., Sanada, H., et al. (2006). Novel FAD-dependent glucose dehydrogenase for a dioxygen-insensitive glucose biosensor. *Bioscience, Biotechnology, and Biochemistry, 70*, 654–659.
5. Turner, A. P. F., & Wilson, R. (1992). Glucose oxidase: An ideal enzyme. *Biosensors and Bioelectronics, 7*, 165–185.
6. Bankar, S. B., Bule, M. V., Singhal, R. S., & Ananthanarayan, L. (2009). Glucose oxidase—an overview. *Biotechnology Advances, 27*, 489–501.
7. Dokter, P., Frank, J., & Duine, J. A. (1986). Purification and characterization of quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus* L.M.D. 79.41. *Biochemical Journal, 239*, 163–167.
8. Igarashi, S., Ohtera, T., Yoshida, H., Witarto, A. B., & Sode, K. (1999). Construction and characterization of mutant water-soluble PQQ glucose dehydrogenases with altered K(m) values—site-directed mutagenesis studies on the putative active site. *Biochemical and Biophysical Research Communications, 264*, 820–824.
9. Igarashi, S., Hirokawa, T., & Sode, K. (2004). Engineering PQQ glucose dehydrogenase with improved substrate specificity. Site-directed mutagenesis studies on the active center of PQQ glucose dehydrogenase. *Biomolecular Engineering, 21*, 81–89.
10. Hamamatsu, N., Suzumura, A., Nomiya, Y., Sato, M., Aita, T., Nakajima, M., et al. (2006). Modified substrate specificity of pyrroloquinoline quinone glucose dehydrogenase by biased mutation assembling with optimized amino acid substitution. *Applied Microbiology and Biotechnology, 73*, 607–617.
11. Sode, K., Ootera, T., Shirahane, M., Witarto, A. B., Igarashi, S., & Yoshida, H. (2000). Increasing the thermal stability of the water-soluble pyrroloquinoline quinone glucose dehydrogenase by single amino acid replacement. *Enzyme and Microbial Technology, 26*, 491–496.
12. Tanaka, S., Igarashi, S., Ferri, S., & Sode, K. (2005). Increasing stability of water-soluble PQQ glucose dehydrogenase by

increasing hydrophobic interaction at dimeric interface. *BMC Biochemistry, 6*, 1.
13. Schneiker, S., Perlova, O., Kaiser, O., Gerth, K., Alici, A., Altmeyer, M. O., et al. (2007). Complete genome sequence of the myxobacterium *Sorangium cellulosum*. *Nature Biotechnology, 25*, 1281–1289.
14. Cleton-Jansen, A. M., Goosen, N., Vink, K., & van de Putte, P. (1989). Cloning, characterization and DNA sequencing of the gene encoding the Mr 50, 000 quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*. *Molecular and General Genetics, 217*, 430–436.
15. Datsenko, K. A., & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences USA, 97*, 6640–6645.
16. Cozier, G. E., Salleh, R. A., & Anthony, C. (1999). Characterization of the membrane quinoprotein glucose dehydrogenase from *Escherichia coli* and characterization of a site-directed mutant in which histidine-262 has been changed to tyrosine. *Biochemical Journal, 340*, 639–647.
17. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research, 25*, 3389–3402.
18. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics, 23*, 2947–2948.
19. Oubrie, A., Rozeboom, H. J., Kalk, K. H., Olsthoorn, A. J., Duine, J. A., & Dijkstra, B. W. (1999). Structure and mechanism of soluble quinoprotein glucose dehydrogenase. *EMBO Journal, 18*, 5187–5194.
20. Bendtsen, J. D., Nielsen, H., von Heijne, G., & Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology, 340*, 783–795.
21. Juncker, A. S., Willenbrock, H., von Heijne, G., Brunak, S., Nielsen, H., & Krogh, A. (2003). Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Science, 12*, 1652–1662.
22. Hommes, R. W. J., Postma, P. W., Neijssel, O. M., Tempest, D. W., Dokter, P., & Duine, J. A. (1984). Evidence of a quinoprotein glucose-dehydrogenase apoenzyme in several strains of *Escherichia-Coli*. *FEMS Microbiology Letters, 24*, 329–333.
23. Olsthoorn, A. J., & Duine, J. A. (1996). Production, characterization, and reconstitution of recombinant quinoprotein glucose dehydrogenase (soluble type; EC 1.1.99.17) apoenzyme of *Acinetobacter calcoaceticus*. *Archives of Biochemistry and Biophysics, 336*, 42–48.
24. Cleton-Jansen, A. M., Goosen, N., Vink, K., & van de Putte, P. (1989). Cloning of the genes encoding the two different glucose dehydrogenases from *Acinetobacter calcoaceticus*. *Antonie Van Leeuwenhoek, 56*, 73–79.
25. James, P. L., & Anthony, C. (2003). The metal ion in the active site of the membrane glucose dehydrogenase of *Escherichia coli*. *Biochimica et Biophysica Acta, 1647*, 200–205.
26. Wang, J. (2008). Electrochemical glucose biosensors. *Chemical Reviews, 108*, 814–825.
27. Davies, D. S. (1994). Kinetics of icodextrin. *Peritoneal Dialysis International, 14*, 45–50.
28. Sode, K., Igarashi, S., Morimoto, A., & Yoshida, H. (2002). Construction of engineered water-soluble PQQ glucose dehydrogenase with improved substrate specificity. *Biocatalysis and Biotransformation, 20*, 405–412.
29. Patrick, W. M., Firth, A. E., & Blackburn, J. M. (2003). User-friendly algorithms for estimating completeness and diversity in randomized protein-encoding libraries. *Protein Engineering Design & Selection, 16*, 451–457.
30. Igarashi, S., Okuda, J., Ikebukuro, K., & Sode, K. (2004). Molecular engineering of PQQGDH and its applications. *Archives of Biochemistry and Biophysics, 428*, 52–63.