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Further Insights into the Catalytical Properties of Deglycosylated Pyranose Dehydrogenase from *Agaricus meleagris* Recombinantly Expressed in *Pichia pastoris*

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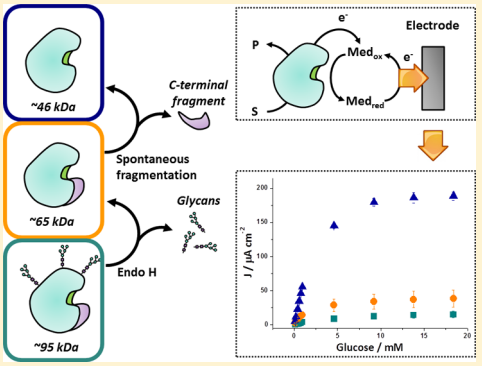
Supporting Information

ABSTRACT: The present study focuses on fragmented deglycosylated pyranose dehydrogenase (fdgPDH) from *Agaricus meleagris* recombinantly expressed in *Pichia pastoris*. Fragmented deglycosylated PDH is formed from the deglycosylated enzyme (dgPDH) when it spontaneously loses a C-terminal fragment when stored in a buffer solution at 4 °C. The remaining larger fragment has a molecular weight of ~46 kDa and exhibits higher volumetric activity for glucose oxidation compared with the deglycosylated and glycosylated (gPDH) forms of PDH. Flow injection amperometry and cyclic voltammetry were used to assess and compare the catalytic activity of the three investigated forms of PDH, "wired" to graphite electrodes with two different osmium redox polymers: [Os(4,4′-dimethyl-2,2′-bipyridine)$_2$(poly-(vinylimidazole))$_{10}$Cl]$^+$ [Os(dmbpy)PVI] and [Os(4,4′-dimethoxy-2,2′-bipyridine)$_2$(poly-(vinylimidazole))$_{10}$Cl]$^+$ [Os(dmobpy)PVI]. When "wired" with Os(dmbpy)PVI, the graphite electrodes modified with fdgPDH showed a pronounced increase in the current density with J_{max} 13- and 6-fold higher than that observed for gPDH- and dgPDH-modified electrodes, making the fragmented enzyme extraordinarily attractive for further biotechnological applications. An easier access of the substrate to the active site and improved communication between the enzyme and mediator matrix are suggested as the two main reasons for the excellent performance of the fdgPDH when compared with that of gPDH and dgPDH. Three of the four glycosites in PDH: N^{75}, N^{175}, and N^{252} were assigned using mass spectrometry in conjunction with endoglycosidase treatment and tryptic digestion. Determination of the asparagine residues carrying carbohydrate moieties in PDH can serve as a solid background for production of recombinant enzyme lacking glycosylation.



During the past few years, much attention has been drawn to pyranose dehydrogenase (PDH; EC 1.1.99.29), which is a glycosylated extracellular oxidoreductase produced by a small group of litter-degrading basidiomycete Agaricales.[1] PDH carries one flavin adenine dinucleotide (FAD) prosthetic group covalently bound to the polypeptide chain of the protein. The enzyme is able to oxidize a variety of nonphosphorylated sugars, primarily at the C-2, C-3, or C-2,3 positions, to their corresponding aldo and diketose derivatives.[1] It was previously reported that PDH displays a slight preference for oxidation at the C-3 over the C-2 position; however, recent studies by Tan et al. showed that C-2 is the preferred site for oxidation.[2] PDH belongs to the glucose-methanol-choline oxidoreductase family but has a number of properties, which are not common for other members of the same family. It is not able to utilize oxygen as electron acceptor [compared, for instance, to pyranose oxidase (PO*x*)] and has a broader substrate specificity and regioselectivity, which can be attributed to the unique structure of the region surrounding the C(4a) flavin pocket.[2,3] Both the lack of oxygen reactivity and the broad substrate tolerance make PDH attractive for the fabrication of amperometric biosensors and small self-powering devices: enzymatic biofuel cells (EBFCs).[4]

Tasca et al. reported on amperometric biosensors utilizing PDH isolated from the natural source (*Agaricus meleagris*) electrochemically "wired" with the osmium redox polymer {[Os(4,4′-dimethyl-2,2′-bipyridine)$_2$(poly(vinylimi-

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dazole)$_{10}$Cl$_2$]$^+$)} [Os(dmbpy)PVI].[5] The broad substrate specificity, superior sensitivity, and long operational stability of the PDH-based biosensor suggested that *Am*PDH is an excellent candidate for biotechnological applications. The work was extended by screening a number of osmium polymers with formal potentials ($E^{\circ\prime}$) ranging from below the potential of the bound FAD of *Am*PDH (150 mV vs NHE[6]) to several hundred millivolts higher in order to select polymer−enzyme pairs with maximized catalytic current density.[7] This approach was combined with the concept of multiple enzymes in the construction of a glucose biofuel cell anode reported by Tasca et al.[6] *Am*PDH was coimmobilized together with cellobiose dehydrogenase from *Myriococcum thermophilum* (*Mt*CDH) in an osmium polymer matrix in order to increase the Coulombic efficiency of the system. The concept was based on the fact that the reaction product of one enzyme can serve as a substrate for another. *Mt*CDH is an ascomycete enzyme able to catalyze the oxidation of mono-, di-, and oligosaccharides at the C(1) position to their corresponding lactones[8] and *Am*PDHmono- and dioxidizes, a variety of sugars at the C-2, C-3, and C-2,3 positions.[4,9,10] By combining these two enzymes, it is therefore theoretically possible to gain up to 6 electrons from one substrate molecule. This was shown in recent publications by Shao et al. that the current density and Coulombic efficiency of a bianode were further increased by entrapping these two enzymes with different types of Os-complex matrices.[11,12] Instead of using the native form of CDH consisting of one flavodomain containing FAD (DH_{CDH}) and one haem domain containing a *b*-type cytochrome (CYT_{CDH}) connected through a polypeptide linker region,[13,14] the free and deglycosylated DH_{CDH} domain of CDHs from various sources were coimmobilized with *Am*PDH in the Os-polymer matrix. In contrast to native PDH, native CDHs show facile direct electron transfer with electrodes through the CYT_{CDH}.[15,16] However, owing to the lower $E^{\circ\prime}$ of the FAD group in the free DH_{CDH} domain compared to that of the haem of CYT_{CDH} domain,[17] it was possible to deliver electrons to the electrode surface at a potential close to the $E^{\circ\prime}$ of the bound FAD of DH_{CDH}. The electron transfer was also improved due to the smaller size and lack of glycosylation on the DH_{CDH} domain compared to that of the native enzyme.[18−21] By these means, a higher voltage output for the respective EBFC was achieved when the free DH_{CDH} domain was used instead of the native enzyme.

Recently, the catalytic properties of glycosylated (gPDH) and deglycosylated PDH (dgPDH) from *A. meleagris*, recombinantly expressed in the methylotrophic yeast *Pichia pastoris*,[22] were investigated, and the pronounced effect of glycan depletion of gPDH on the current output was demonstrated.[23−25] The present study provides further insights into the properties of the deglycosylated PDH, as it was discovered that dgPDH loses an almost 20 kDa polypeptide from its C-terminus, resulting in the formation of a fragmented deglycosylated PDH (fdgPDH) with a much higher activity for glucose oxidation. In this contribution, the effect of the decomposition on the catalytic properties of fdgPDH was investigated. The fragmentation procedure was followed by gel electrophoresis (SDS−PAGE) and measurements of the enzyme activity [ferricenium (Fc^+)]. All three forms of the enzyme were electrically "wired" to an osmium-based redox polymer on the surface of graphite electrodes, mounted in a flow-injection system (FIA). The current output accompanying the glucose oxidation by the immobilized gPDH, dgPDH, and fdgPDH was compared using FIA and cyclic voltammetry.

The proposed glycosylation sites of PDH (NetNGlyc 1.0 Server) were investigated using Endo H treatment in combination with tryptic digestion followed by MALDI-MS measurements. Determination of the asparagine residues carrying the carbohydrate moieties opens up an interesting possibility for further improvement of the performance of the enzyme. Through the use of site-directed mutagenesis, it is possible to eliminate those asparagine residues, to which the glycans are attached and recombinantly express an enzyme with an altered glycosylation pattern.

EXPERIMENTAL SECTION

Chemicals. All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) unless stated otherwise and were of analytical grade. Glycosylated PDH (EC 1.1.99.29; sequence UniProt: Q3L245;[26] protein content 23.8 mg mL^{-1} [Bradford assay]; volumetric activity 335 units mL^{-1} [Fc^+ assay, 20 °C]) from *A. meleagris* was recombinantly expressed in *P. pastoris* according to the previously reported procedure.[22] Deglycosylation was performed with Endo Hf (1000000 NEB units mL^{-1}, New England Biolabs, Bionordiska AB, Stockholm, Sweden) as described in the Supporting Information. A stock solution of D-glucose (40 mM) was prepared in the running buffer and stored overnight to reach mutarotational equilibrium. The osmium redox polymers [Os(dmobpy)PVI] $E^{\circ\prime}$ = 140 mV vs NHE and [Os(dmobpy)-PVI] $E^{\circ\prime}$ = 320 mV vs NHE) were synthesized, according to a well-established procedure.[27,28] Dithiothretiol (DTT), iodoacetic acid (IAA), and 2,2,2-trifluoroethanol (TFE) were obtained from Fluka (Buchs, Switzerland). Deionized water was used throughout all experiments. It was purified with a Milli-Q purification system (EMD Millipore Corporation, Billerica, MA).

Enzyme Assay. The enzyme activity toward Fc^+ was measured with a UV-2401 PC spectrophotometer (Shimadzu Deutschland GmbH, Duisburg, Germany) at 20 °C, using a standard spectrophotometric method[29] (Supporting Information).

Electrode Preparation. Graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) were polished on wet fine emery paper (grit number P1200), rinsed thoroughly with deionized water, and dried under a flow of air. The electrode surface was first incubated for 10 min with a mixture of 2 μL of Os-polymer (5 mg mL^{-1} aqueous solution) and 1 μL of poly(ethylene glycol) (400) diglycidyl ether (PEGDGE, 68%, v/v) to which 5 μL of enzyme was added. An enzyme to Os-polymer mass ratio of 4:1 was used throughout all experiments. Cross-linking was allowed to proceed overnight at 4 °C in a humid atmosphere. Finally, the rods were rinsed with the running buffer and mounted into the FIA system.

Electrochemical Measurements. A wall-jet type flow-through electrochemical cell[30,31] was used for the characterization of the three different PDHs (gPDG, dgPDH, and fdgPDH). Graphite rods modified with the covalently bound enzyme−polymer complex were used as working electrodes, with Ag|AgCl (0.1 M KCl, 288 mV vs NHE) as the reference electrode and the platinum wire as the counter electrode. All three electrodes were connected to a three-electrode potentiostat (Zäta Electronics, Höör, Sweden) controlling the applied potential of the working electrode (468 mV vs NHE).

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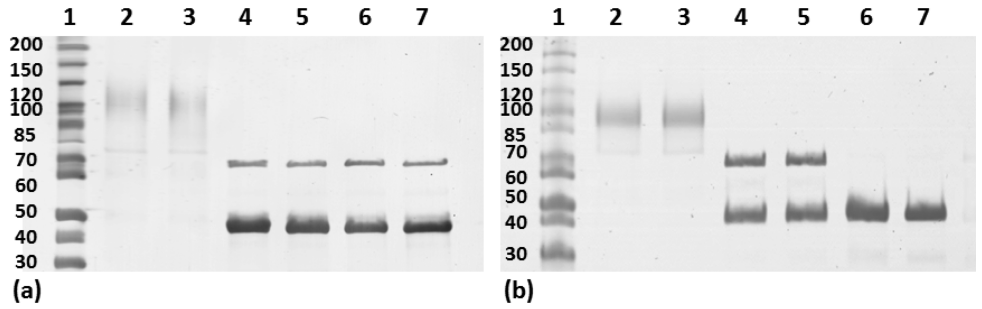

Figure 1. Characterization of enzyme fragmentation with time by 10% acrylamide SDS−PAGE with silver staining: lane 1, molecular weight standard; lanes 2 and 3, gPDH; lanes 4 and 5, dgPDH; lanes 6 and 7, fdgPDH. The difference between lanes 2 and 3, 4 and 5, and 6 and 7 can be observed after storing the enzymes for two weeks (a) at 4 °C: gPDH and fdgDPH and at −20 °C: dgPDH and for two months (b) at 4 °C: gPDH and fdgDPH and at −20 °C: dgPDH.

The current output was registered with a recorder (BD 112, Kipp & Zonen, Utrecht, The Netherlands). The electrochemical cell was connected to a FIA system[32] equipped with a peristaltic pump (Minipuls 3, Gilson, Villier-le Bel, France) and a six-port injection valve (Rheodyne, type 7125 LabPR, Cotati, CA) for introduction of 50 μL samples. All concentrations in the injected samples were corrected for the dispersion factor of the FIA system, which was determined to be 1.088. Sodium phosphate (50 mM, pH 7.4) containing 137 mM NaCl served as the running buffer, which was degassed prior to the measurements in order to avoid bubble formation in the system. The running buffer was pumped through the system at a constant flow rate of 0.5 mL min^{-1}. The kinetic parameters were calculated by fitting the data using the Michaelis−Menten equation in the OriginPro 8 program.

Cyclic voltammetry (CV) was performed using a BAS CV-50W potentiostat (Bioanalytical Systems, West Lafayette, IN) with the enzyme−polymer complex modified graphite rods used as the working electrode, a saturated calomel reference electrode (244 mV vs NHE), and a platinum foil counter electrode. Nitrogen was purged through the solutions prior to measurements for 15 min and over the solutions during the measurements in order to maintain an inert atmosphere. Cyclic voltammograms were recorded in a 50 mM sodium phosphate buffer containing 137 mM NaCl (pH 7.4) at a scan rate of 50 mV s^{-1}.

Gel Electrophoresis. Fragmentation of the deglycosylated enzyme was followed in time with one-dimensional SDS−PAGE, using a Bio-Rad system (Sundbyberg, Sweden) following the method of Laemmli.[33] Gels containing 10% acrylamide were used to resolve the proteins at a constant applied potential of 200 V. Subsequently, proteins were visualized on the gel with silver staining as described in refs 34 and 35. More detailed information can be found in the Supporting Information.

Trypsin Digestion. Prior to trypsin digestion, the disulfide bonds in the protein sample were reduced with DTT and blocked with IAA, according to a modified procedure described in ref 36 (Supporting Information).

MALDI-MS Measurements. The enzymatic digests (0.5 μL) were spotted onto the MALDI target in triplicate and an equal volume of a MALDI matrix solution consisting of 5 mg mL^{-1} α-cyano-4-cinnamic acid (α-CHCA) in 50% acetonitrile and 0.1% (v/v) phosphoric acid was added.[37] The matrix solution contained two peptide standards: des-Arg-bradykinin (m/z 904.468) and adrenocorticotropic hormone (ACTH) peptide fragment 18-39 (m/z 2465.199) that was used for internal mass calibration in every analyte/matrix position. MALDI-MS and MS/MS analyses of each sample were performed on a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer in the positive reflection mode (Applied Biosystems, Framingham, MA).

Dilutions of the digests were made at 1:5 and 1:10 fold with a mixture of 2% acetonitrile and 1% formic acid in deionized water and were spotted on the plate in the same manner. Uniprot database using Mascot (Matrix Sciences Ltd., Cheshire, U.K.) was used with the following parameters: carbamidomethylation of all cysteins, possible oxidation of methionine residues, three allowed trypsin misscleavages, and a mass tolerance of 30 ppm and 0.02 Da for MS and MS/MS search, respectively. Using the features of the GPMAW 8.1 software, sequence coverage for digested dgPDH and gPDH were compared with a S/N threshold of 15.

RESULTS AND DISCUSSION

Gel Electrophoresis Study of dgPDH Fragmentation. The present study focuses on the discovered fdgPDH: a fragmented form of the enzyme, which is formed from dgPDH when stored under certain conditions. In order to follow the fragmentation process, a portion of freshly purified and enzymatically deglycosylated dgPDH was stored at 4 °C instead of at −30 °C, at which the enzyme was shown to be stable.[22] All three forms of PDH (gPDH, dgPDH, and fdgPDH) were investigated using SDS−PAGE stained with silver.[35] The molecular weight of gPDH recombinantly expressed in *P. pastoris* was determined to be roughly ∼95−100 kDa (Figure 1, panels a and b), similar to previous reports by Sygmund et al.[22] It appeared in a broad band due to possible structural variations in the glycan moieties[38] but remained unchanged over a period of 3 months when stored at 4 °C (Figure 1, panels a and b).

Deglycosylation of gPDH was accomplished by incubation with Endo Hf as described in the Supporting Information. Purified dgPDH appeared in two bands on SDS−PAGE (Figure 1, panels a and b). A major band at ∼65 Da corresponds to a release of ∼30−35 kDa of the glycan moiety from the peptide chain. The other minor band at ∼46 kDa presumably is partially fragmented dgPDH, which is formed when the enzyme is stored at 4 °C. It appears due to the loss of a peptide of ∼20 kDa from the C-terminus of the protein, even in the absence of any proteolytic activity. The first observation of this effect was discovered by Sygmund et al. and was briefly described in ref 1. The smaller fragment was sequenced by Edman degradation and found to start with the sequence

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YRLLLQL, so cleavage must occur between amino acids 441 and 442 (number one being the Met from the start codon). This sequence does not conform to any known peptidase consensus site.

A gradual fragmentation of dgPDH can be seen in Figure 1 (panels a and b). dgPDH was initially represented by two bands at ~65 Da (dgPDH) and ~46 Da (fdgPDH). Changes in band intensity and thickness indicate that fragmentation progressed with time. After about two months, the upper band faded completely, and the lower band became the only one that could be clearly observed on the gel (Figure 1b). It corresponds to the fully fragmented product (fdgPDH). In terms of potential technological applications of PDH in biosensor development and EBFCs fabrication it was particularly important to study the effect of fragmentation on the activity of fdgPDH, as well as the current output in amperometric measurements when entrapped within an Os polymer matrix.

Spectrophotometric Activity Assay. The volumetric activity of fdgPDH was measured regularly for three months with ferrocenium ion as an electron acceptor and glucose as a substrate. The activity of the enzyme was gradually increasing, reaching a plateau after about two months (Figure 2). It can

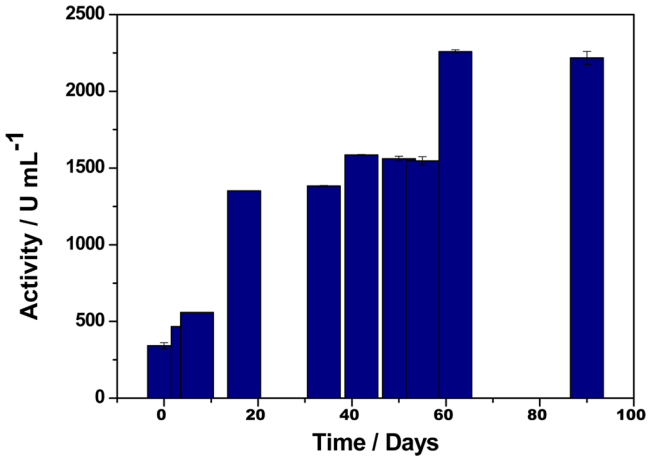

Figure 2. Change in activity of the solution originally containing freshly made dgPDH with time as a result of fragmentation of the enzyme. The volumetric activity was measured using glucose as a substrate and ferrocenium (Fc^+) as an electron acceptor (see the Experimental Section).

therefore be concluded that the loss of the C-terminal fragment does not play an important role in the activity of the whole enzyme. The remaining large part of dgPDH (fdgPDH) has an even higher specific activity due to the smaller molecular weight of the fragmented product.

Electrocatalytic Properties of Glycosylated, Deglycosylated, and Fragmented Deglycosylated PDH. In accordance with the Marcus theory, the rate of electron transfer between two species participating in a redox reaction decreases when the distance between them increases.[39] In the case of redox enzymes, it is thus possible to improve the electron transfer rate with an electrode by bringing the redox active site closer to the electrode surface. This can be practically accomplished by complete or partial removal of the glycan shell surrounding the protein peptide chain of glycosylated redox enzymes. Previous successful applications of this approach were demonstrated either by deglycosylation of redox enzymes[40−42] or by recombinant expression of enzymes in a prokaryotic host.[19−21,23−25,43] The electron transfer mechanism between fdgPDH and the Os-polymer matrix should also be improved compared to that of dgPDH and gPDH. The fragmented enzyme loses a part of its C-terminal sequence, which should make the distance between the active site and the mediator shorter. An additional increase in current output can be expected if the C-terminal fragment in dgPDH possibly interferes with the diffusion of the substrate into the active center of the enzyme. To investigate these matters, the electrochemical behavior of fdgPDH was measured using CV and amperometry in a FIA system, where the enzyme was coimmobilized with an Os-polymer using a diepoxy cross-linker (PEGDGE) and coprecipitated on the surface of a graphite working electrode in the same manner as described in refs 23−25. The catalytic properties of fdgPDH were compared to those obtained for gPDH and dgPDH in terms of current output.

Figure 3 depicts the current density response to glucose concentrations in the range of 0.1−20 mM of electrodes

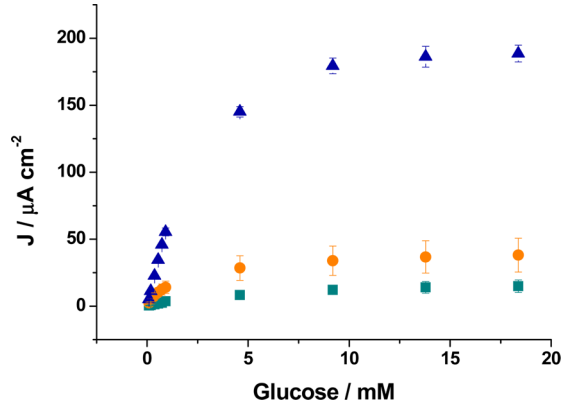

Figure 3. Catalytic response of (green ■) gPDH/Os(dmbpy)PVI; (orange ●) dgPDH/Os(dmbpy)PVI, and (blue ▲) fdgPDH/Os(dmbpy)PVI-modified graphite electrodes to different concentrations of glucose (0.1−20 mM) measured with the FIA system in 50 mM PBS, pH 7.4, at a flow rate of 0.5 mL min^{-1} and an applied potential of 468 mV vs NHE.

modified separately with the three forms of the enzyme (gPDH, dgPDH, fdgPDH) cross-linked with Os(dmbpy)PVI. The applied potential of 468 mV versus NHE was selected from the polarization curves, where the current density reaches a plateau for all three enzyme-Os-polymer-modified electrodes (Figure S-1 of the Supporting Information). The same working potential was selected for electrochemical characterization of the fdgPDH/Os(dmobpy)PVI-modified electrodes (Figure S-2 of the Supporting Information). A pronounced increase in current density is observed in the case of electrodes modified with fdgPDH/Os(dmbpy)PVI. When fitted to the Michaelis−Menten equation, J_{max} for fdgPDH is 13- and 6-fold higher than that for gPDH and dgPDH, respectively. The estimated apparent K_M^{app} is smaller for both dgPDH (1.7 mM) and fdgPDH (2.8 mM) compared to gPDH (5.1 mM), indicating that the substrate diffusion into the active site of the deglycosylated and fragmented forms of PDH is easier. Similar results were achieved when studying the catalytic properties of gPDH, dgPDH, and fdgPDH, with CV under conditions described in the Experimental Section and with 10 mM glucose as substrate (Figure S-3 in the Supporting Information). It is clear from the graphic output that the maximum of the anodic

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wave increases with a decrease in the molecular weight of the enzyme. Both the increase in current output for fdgPDH and the decrease in K_M^{app} support the theory that the spontaneous cleavage of the C-terminal fragment plays an important role in the catalytic performance of the remaining part of the fragmented enzyme. However, it is not clear if the improvement arises due to sterical reasons or to a faster electron communication between the redox active site and the Os-matrix. A contribution from both factors is possible.

When comparing the catalytic response of electrodes modified with fdgPDH coimmobilized with various PVI-bound Os complexes to different concentrations of glucose in the FIA system, higher current densities were obtained when using the Os(dmbpy)PVI polymer as a mediator (Figure 4).

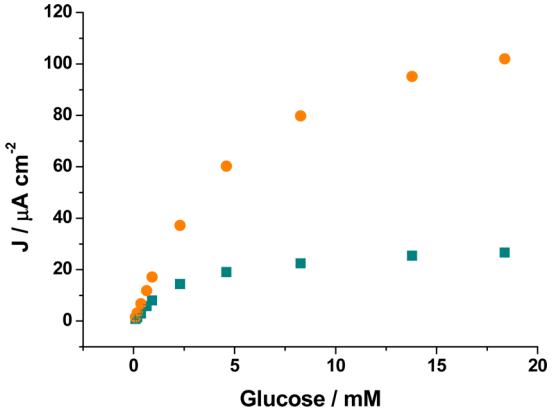

Figure 4. Catalytic response of (orange ●) fdgPDH/Os(dmbpy)PVI and (green ■) fdgPDH/Os(dmobpy)PVI-modified graphite electrodes to different concentrations of glucose (0.1−20 mM) measured with the FIA system in 50 mM PBS, pH 7.4, at a flow rate of 0.5 mL min^{-1} and an applied potential of 468 mV vs NHE.

The potential difference of the fdgPDH/Os(dmbpy)PVI pair is larger than that of the fdgPDH/Os(dmobpy)PVI pair. Therefore, the driving force for electron transfer is higher, and greater current densities could be obtained when the same applied potential of 468 mV versus NHE is used. Both of the Os-bound imidazole complexes should be considered for fabrication of bioanodes in combination with fdgPDH as a larger open circuit potential is obtained with Os(dmobpy)PVI having a lower formal potential, but higher current densities are obtained with the Os(dmbpy)PVI-based polymer.

The stability of fdgPDH "wired" with Os(dmbpy)PVI and Os(dmobpy)PVI was examined using the FIA system by reiterative injections of 5 mM glucose under those conditions specified in Figure 4 (Figure S-4 of the Supporting Information). A signal decrease of only 9% and 11% was observed for the fdgPDH/Os(dmbpy)PVI- and the fdgPDH/Os(dmobpy)PVI-modified electrodes, respectively, after 8 h of continuous operation. After 54 h, the electrodes retained 60% and 33% of their respective initial catalytic responses.

An attempt to produce a smaller PDH by expression without the C-terminal fragment has been made since the smaller size of the protein would allow more molecules to be incorporated into the polymer matrix without having to wait for spontaneously occurring fragmentation. Unfortunately, these attempts were unsuccessful and resulted only in an inactive enzyme (data not shown).

Analysis of Glycosites Using MALDI-MS. The improved electrocatalytic performance of fdgPDH makes that enzyme attractive for future biotechnological application. However, at present, the production of fragmented enzyme is time-consuming and requires enzymatic deglycosylation. Recombinant expression of the enzyme lacking (part of the) glycosylation in *P. pastoris* might be beneficial for such applications if the enzyme will retain its stability. This can be performed with site-directed mutagenesis, provided that the locations of the glycosites in the polypeptide chain are known.

Glycosylation site mapping using MALDI-MS in combination with site-specific endoglycosidase treatment was performed in order to unequivocally identify the glycosylation sites of *Am*PDH. In the first step, four possible glycosylation sites in the primary structure of *Am*PDH were located: N^{75}, N^{175}, N^{252}, and N^{319} based on the fact that carbohydrate moieties may only be attached to Asn in a tripeptide consensus sequon Asn-X-Ser/Thr/Cys (where X can be any amino acid except proline[44]). The carbohydrate chains were then removed from gPDH with Endo H, an endoglycosidase, which cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. Endo H hydrolyses the *O*-glycosidic bond between two *N*-acetylglucosamine residues (GlcNAc) attached to Asn in the polypeptide chain and the retained GlcNAc with a mass increase of 203.079 Da can therefore serve as a clear indication for glycosylation in the MS spectra (Figure 5).[45−47] In the following step, both gPDH and dgPDH were subjected to treatment with a protease (trypsin) and samples were analyzed with MALDI-MS. Finally, mass spectra of digested gPDH and dgPDH were compared using a peptide mass fingerprint (PMF) strategy.[48−50]

The PMF analysis gave a sequence coverage of 50% for the investigated glycosylated protein with 31 peptide hits and 50% for the deglycosylated protein with 28 peptides detected when using GPMAW 8.1. None of the peptides potentially carrying glycan moieties were covered when analyzing the mass list from both gPDH and dgPDH using GPMAW 8.1, which served as a preliminary indication that the Asn residues N^{75}, N^{175}, N^{252}, and N^{319} can be glycosylated.

Conformation of the glycosylation was performed by comparing the MALDI MS spectra for the control sample with the MALDI spectra of the deglycosylated-treated sample (Figure 5, panels a and b). The presence of a m/z peak with a mass of a suspected glycosylated peptide +203.079 Da clearly suggested that the peptide was glycosylated before treatment with Endo H (Figure 5b). The final confirmation was made when the suspected glycosylated peptide was submitted to MS/MS experiment with the following manual assignment of the fragmented ions (Figure 5c). Figure 5 illustrates the stepwise assignment of the N^{252} residue.

Three of four peptides carrying the N^{75}, N^{175}, and N^{252} residues with an increased mass of 203.079 Da were found in the mass spectra of dgPDH. Subsequent MS/MS analysis and sequencing of these precursor fragment ions indicated that the positions N^{75}, N^{175}, and N^{252} in *Am*PDH are indeed glycosylated. For the remaining residue N^{319}, no experimental data could be obtained either in the glycosylated peptide or in the nonmodified version, possibly due to the fact that the signal arising in MS for the corresponding peptide mass ion is suppressed by other, more abundant mass ions. Another mass spectrometric instrumentation was tried out in order to find the peptide sequence containing residue N^{319}. An Orbitrap-Velos Pro System was used and a data-dependent scan with an inclusion list of the peptide carrying N^{319} was performed. However, no MS/MS spectra could confirm the presence or

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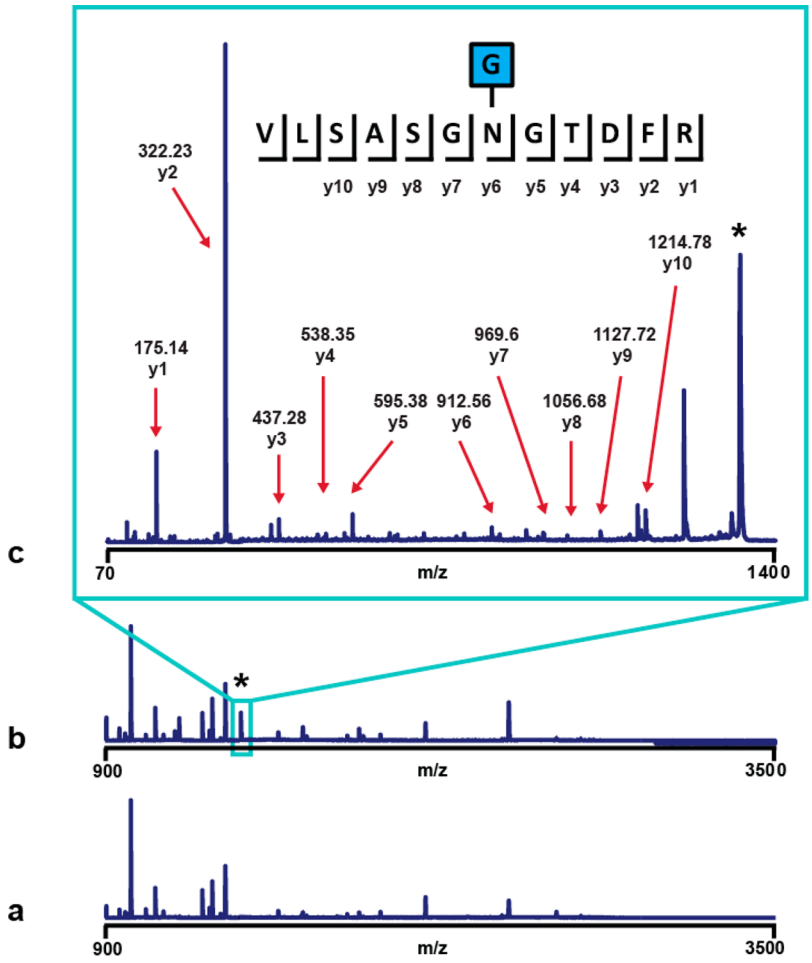

Figure 5. Stepwise glycosylation site mapping of N^{252} using MALDI-MS: (a) peptide mass fingerprint of the untreated glycosylated protein. Thirty-one peptides match the protein sequence, resulting in a sequence coverage of 50%. (b) MS spectrum for the Endo H digested protein; the *m/z* at 1246.69 (denoted with the star) corresponds to the *m/z* value of the VLSASGNGTDFR peptide carrying one GlcNAc residue (a mass increase of 203.079 Da). Twenty-eight peptides match the protein sequence resulting in a sequence coverage of 50%. (c) MS/MS spectrum of the peptide VLSASGNGTDFR from *Am*PDH deglycosylated with Endo H (the precursor fragment ion with *m/z* 1246.69 is denoted with a star). Y-ion series denoted in the figure, where the asparagine residue N^{252} carries the carbohydrate moiety (denoted with "G").

absence of glycosylation on this Asn residue. This indicates that the Endo H protocol did not work 100% efficiently for the peptide carrying the N^{319} residue or that this peptide had some post-translational modification that made it impossible to identify it. As expected, none of the precursor fragment ions were found in the gPDH sample (Figure 5a).

CONCLUSIONS

The discovered fdgPDH was characterized using SDS−PAGE with silver staining and a standard spectrophotometric assay. fdgPDH is formed from dgPDH, which loses a C-terminal fragment when stored for two months at 4 °C. The fragmented enzyme has a MW of ∼46 kDa and possesses a higher specific activity compared to the glycosylated and the deglycosylated forms of the same enzyme. The electrocatalytical properties of fdgPDH were compared to those of dgPDH and gPDH when coimmobilized with Os(dmbpy)PVI on the surface of the graphite electrode. Noticeably higher current densities were achieved for the fragmented enzyme in both FIA amperometry and cyclic voltammetry. A higher catalytic response was observed when "wiring" fdgPDH with the Os(dmbpy)PVI-based polymer compared to fdgPDH/Os(dmobpy)PVI-modified electrodes. Films prepared by coimmobilization of fdgPDH with the Os-polymer having a higher formal potential also exhibited a better operational and long-term stability. Glycosylation site mapping was performed using MALDI-MS in combination with endoglycosidase treatment and subsequent tryptic digestion. Three asparagine residues carrying carbohydrate moieties, N^{75}, N^{175}, N^{252}, were assigned in PMF analysis. The obtained information can serve as a starting point in recombinant production of mutant *Am*PDH lacking glycosylation.

ASSOCIATED CONTENT

Supporting Information

Detailed description of the methods used in the present study and additional experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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