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Effect of Deglycosylation of Cellobiose Dehydrogenases on the Enhancement of Direct Electron Transfer with Electrodes

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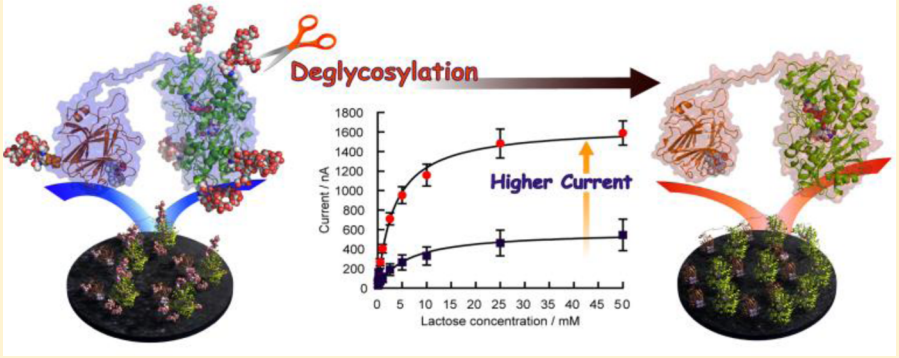
Supporting Information



ABSTRACT: Cellobiose dehydrogenase (CDH) is a monomeric extracellular flavocytochrome composed of a catalytic dehydrogenase domain (DH_{CDH}) containing flavin adenine dinucleotide (FAD), a cytochrome domain (CYT_{CDH}) containing heme *b*, and a linker region connecting the two domains. In this work, the effect of deglycosylation on the electrochemical properties of CDH from *Phanerochaete chrysosporium* (*Pc*CDH) and *Ceriporiopsis subvermispora* (*Cs*CDH) is presented. All the glycosylated and deglycosylated enzymes show direct electron transfer (DET) between the CYT_{CDH} and the electrode. Graphite electrodes modified with deglycosylated *Pc*CDH (d*Pc*CDH) and *Cs*CDH (d*Cs*CDH) have a 40−65% higher I_{max} value in the presence of substrate than electrodes modified with their glycosylated counterparts. *Cs*CDH trapped under a permselective membrane showed similar changes on gold electrodes protected by a thiol-based self-assembled monolayer (SAM), in contrast to *Pc*CDH for which deglycosylation did not exhibit any different electrocatalytical response on SAM-modified gold electrodes. Glycosylated *Pc*CDH was found to have a 30% bigger hydrodynamic radius than d*Pc*CDH using dynamic light scattering. The basic bioelectrochemistry as well as the bioelectrocatalytic properties are presented.

One of the major challenges in electrochemical research focusing on the communication between redox enzymes and electrodes is to obtain efficient electron transfer. In many instances this can only be reached by the use of redox mediators facilitating the electron transfer between the redox-active sites in the biological material and the electrode. Not so frequently, however, is efficient direct electron transfer (DET) between the biocomponent and the electrode reported, even if almost 40 years have passed since the first examples were reported.[1] DET is believed to occur at considerable rates only over distances shorter than 20 Å between prosthetic groups and electrode surfaces and is reported for only about 50 redox enzymes out of 3500.[2] For the application of redox enzymes in biosensors and biofuel cells, an efficient DET is certainly a great feature to achieve leading to a simple electrode setup and avoiding expensive or harmful mediators.

Despite increased research activities in the field, the original problem of insufficient communication between a redox enzyme and the supportive electrode material remains,[3] and thus, it is difficult to obtain a high current density based on DET, which is needed both for high sensitivity of a biosensor and high power output of a biofuel cell (BFC).[4] In order to improve the characteristics of such biodevices four different strategies are commonly used: (i) coimmobilization of redox-active compounds, using a mediated electron-transfer (MET) approach where the mediator acts as a potential electron-transferring bridge between the electrode and the enzyme,[5] (ii) increase in the electrode surface area using nano- or

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mesoporous structures to increase the amount of biomaterial in contact with the electrode possibly also increasing the percentage of enzyme molecules oriented for DET,[6] (iii) tailoring the electrode surface through modifications to achieve a better orientation or attachment of the enzyme facilitating DET,[7] or (iv) protein engineering of the enzyme to improve its catalytic properties, its orientation on the electrode, or to minimize the distance to the redox-active cofactor(s) of the enzyme and the electrode surface.[8]

In previous research it was shown that deglycosylation of horseradish peroxidase (HRP) and tobacco peroxidase shortens the distance between the prosthetic group (heme *b*) and the electrode and enhances the DET rate.[8d,9] More recently, different laboratories have used this strategy on glucose oxidase (GOx). GOx from *Aspergillus niger* is a glycosylated dimeric flavoenzyme containing two FAD (flavin adenine dinucleotide) cofactor units buried ∼15 Å below the protein surface.[10] The removal of the sugar residues from the enzyme surface allows a closer contact of the bound FAD in the active site and the electrode, which resulted in clear DET and glucose electrooxidation. When using a redox polymer the electrode modified with deglycosylated GOx also exhibited a higher electrooxidation current compared to electrodes modified with the fully glycosylated GOx, due to the downsizing of the enzyme's dimensions on the electrode surface.[11] Some other authors also used this approach on GOx but did not show clear direct electrocatalysis of glucose.[8b,12]

Cellobiose dehydrogenase [CDH, (cellobiose:acceptor) 1-oxidoreductase, EC 1.1.99.18] is a monomeric enzyme secreted by a variety of fungi from the phyla of Basidiomycota and Ascomycota.[13] CDH belongs, like GOx, to the glucose−methanol−choline (GMC) oxidoreductase superfamily[14] and is the only extracellular flavocytochrome known today. It is composed of a larger catalytic flavodehydrogenase domain (DH_{CDH}) containing one noncovalently bound FAD molecule as cofactor and a smaller cytochrome domain (CYT_{CDH}) containing heme *b* as cofactor, which are connected through a flexible linker.[15] CDHs from a variety of origins have shown efficient DET; however, the fact that CDH is glycosylated (about 9−16%, similar to GOx) implies that an improvement of its bioelectrocatalytic properties can be expected after deglycosylation. Because of their different catalytic properties, various CDHs have been used for the development of amperometric biosensors and biofuel cells based on both DET[16] and mediated electron transfer (MET).[5c,16a,17] Reviews on this work can be found in literature.[14,18] The reaction catalyzed by CDHs starts at the DH_{CDH} domain, which oxidizes cellobiose, cello-oligosaccharides, and some other carbohydrates (e.g., lactose) to their corresponding δ-lactones. The two electrons stored in the reduced FAD can be transferred onto small one- or two-electron acceptors, e.g., ferricyanide or quinones. Alternatively, the electrons can be transferred sequentially from the reduced FAD in the DH_{CDH} to the heme *b* in the CYT_{CDH} by an intramolecular electron-transfer (IET) step and further on to a terminal electron acceptor like cytochrome *c*. If CDH is immobilized in a favorable position, an electrode can also function as terminal electron acceptor. DET between CYT_{CDH} and the electrode has been shown to occur at a slower rate than the rate of the internal electron transfer (IET), identified to be the rate-limiting step of CDH in solution.[8e,19]

In this contribution, the DET of the glycosylated forms of *Phanerochaete chrysosporium* CDH (g*Pc*CDH) and *Ceriporiopsis subvermispora* CDH (g*Cs*CDH) and their deglycosylated counterparts (d*Pc*CDH and d*Cs*CDH) were compared. *Pc*CDH represents the most well-studied variant of CDH,[18b,20] with the crystal structures of its individual domains previously published,[21] and it is approximately 9% glycosylated for the enzyme produced by the fungi. *Cs*CDH is for the first time electrochemically characterized and with 16% of the total mass glycosylated when expressed from the fungus, one of the most heavily glycosylated CDHs.[22]

EXPERIMENTAL SECTION

Chemicals. 11-Mercaptoundecanol (MUOH) was obtained from Sigma-Aldrich GmbH (Sigma-Aldrich Chemicals, Steinheim, Germany). 11-Amino-1-undecanothiol ($MUNH_2$) was obtained from Dojindo Laboratories (Kumamoto, Japan). Absolute ethanol (>99% vol) used to prepare the thiol solutions was obtained from Solveco Chemicals AB (Stockholm, Sweden). Glutaraldehyde (GA) solution in water (50% weight), β-lactose (min 99%), cytochrome *c* (cyt *c*) from bovine heart, and hydrogen peroxide (30% w/w) were obtained from Sigma-Aldrich GmbH (Sigma-Aldrich Chemicals, Steinheim, Germany). Sulfuric acid (analytical grade) was obtained from VWR International (Poole, England). Buffer salts were of analytical grade and obtained from Fluka (Buchs, Germany). Water purified with a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.) to a specific resistivity (18.2 MΩ cm) was used to prepare all aqueous solutions.

Enzyme Preparation. Recombinant wild-type g*Pc*CDH was heterologously expressed in the methylotropic yeast *Pichia pastoris* and purified as described previously.[23] The purified enzyme was stored in 50 mM sodium citrate buffer (pH 5.5) at −20 °C. The specific cyt *c* activity for g*Pc*CDH and d*Pc*CDH was 17.1 and 12.5 U mg^{-1}, respectively. The g*Cs*CDH was produced and purified as described previously.[22] The purified enzyme was stored in 50 mM sodium citrate buffer (pH 5.0) at −20 °C. g*Cs*CDH and d*Cs*CDH exhibited a specific cyt *c* activity of 19.5 and 10.6 U mg^{-1}, respectively. The purified glycosylated CDHs were treated with α-1,2-mannosidase and endoglycosidase H (New England Biolabs, Ipswich, MA, U.S.A.) according to the manufacturer's instructions to obtain the deglycosylated CDHs a procedure previously described by Hallberg et al.[21a]

Kinetic Measurements. The cyt *c* assay was performed to determine the homogeneous activity of the enzymes used by following the reduction of 20 μM cyt *c* (ε_{550} = 19.6 mM^{-1} cm^{-1}) in 80 mM sodium acetate buffer, pH 4.50, containing 30 mM lactose at 30 °C. One unit of enzymatic activity is defined as the amount of enzyme that oxidizes 1 μmol of lactose per min under the assay conditions. The protein concentration was determined by the method of Bradford[24] using a prefabricated assay from Bio-Rad Laboratories (Hercules, CA, U.S.A.) and bovine serum albumin as the standard. An Applied Photophysics (Leatherhead, U.K.) SX-20 stopped-flow apparatus in the single-wavelength mode was used to measure the pre-steady-state reduction rates at 30 °C of FAD and heme *b* at 447 and 563 nm, respectively. Both substrate and enzyme solution were buffered in 80 mM sodium acetate buffer, pH 4.5. The final concentrations in the reaction cell were 50 mM lactose and 6 μM g*Pc*CDH or 4 μM d*Pc*CDH. The observed rates (k_{obs}) for both reactions are averages of three shots.

Preparation of Graphite Electrodes. Rods of spectrographic graphite (SPGE, Ringsdorff Werke GmbH, Bonn, Germany, type RW001, Ø 3.05 mm) were used as working

electrodes. The surface of the SPGE was prepared by polishing with fine emery paper (Tufback Durite, P1500), then thoroughly rinsed with Milli-Q water, and finally allowed to dry. The gCDH- and dCDH-modified electrodes were prepared by placing an aliquot of 5 μL of enzyme solution (all enzyme solutions were diluted to an equal volumetric activity of 18.2 U mL^{-1}) on the entire disk surface of the electrode. The electrodes were let to dry at room temperature and then stored overnight at 4 °C. Before each measurement, the electrodes were thoroughly rinsed with acetate buffer to remove weakly adsorbed enzyme molecules.

Preparation of Permselective Membrane Gold Electrodes. Gold disk electrodes (BASi, West Lafayette, IN, U.S.A., Ø 1.6 mm) were exposed for 3 min into freshly prepared Piranha solution (3/1 v/v H_2SO_4/H_2O_2; **CAUTION**: *Piranha solution is especially dangerous, corrosive, and may explode if contained in a closed vessel; it should be handled with special care*) followed by polishing in aqueous alumina slurry (0.1 μm, Struers, Copenhagen, Denmark), ultrasonicated in Milli-Q water for 5 min, and finally activated electrochemically by cyclic voltammetry (CV) in 0.5 M H_2SO_4 (300 mV s^{-1}, −0.1 to 1.7 V vs AgIAgCl, KCl(s), 20 cycles). In order to confirm the cleanliness of the electrodes the last scan of the CV was compared to that previously reported as clean gold.[25] The electrode was rinsed abundantly with water between every step. A self-assembled monolayer (SAM) of thiols was prepared by immersion into a 1 mM ethanolic thiol solution for 1 h at room temperature followed by rinsing with ethanol and drying with Ar. Modification of the electrode with CDH was made by trapping 11 μL of CDH solution in a Teflon holder between the electrode and a soaked permselective dialysis membrane (PME) (MWCO <12000−14000, Spectrum Laboratories, Inc., CA, U.S.A.).[26]

Preparation of CDH-Modified Gold Electrodes. Gold electrodes were cleaned and modified with SAM as already described above. A mixed SAM was assembled by immersing the cleaned gold electrode into a 1 mM thiol solution containing a mixture 1/50 (v/v) of $MUNH_2$ and MUOH for 1 h at room temperature.[6c] The electrodes were rinsed with ethanol and dried under Ar. Then 1 μL of a 0.25% GA solution and 2 μL of CDH solution were added and incubated for 2 h in a moist atmosphere at room temperature. Finally, the electrodes were rinsed with acetate buffer to remove unbound enzymes.

Carbon Nanoparticle Modified Graphite Electrodes. Carbon nanoparticles (CNPs) PRINTEX,[30] 27 nm diameter, were purchased from Degussa (Evonik Degussa Japan Co. Ltd., Tokyo, Japan) and used to modify GE working electrodes. CNP powder was mixed with dimethylformamide (DMF) (80:20, w/w) and suspended by ultrasonication for 30 min to prepare a CNP slurry. The slurry was applied (3 × 5 μL, allowed to dry between the steps) to a GE (electrode area, 0.073 cm^{-2}) and dried after the last addition in a drying oven at 60 °C for 12 h. The gCDH- and dCDH-modified electrodes were prepared by placing an aliquot of 5 μL of enzyme solution on the entire disk surface of the electrode. The electrodes were let to dry at room temperature and then stored overnight at 4 °C. Before measurements, the electrodes were thoroughly rinsed with buffer to remove any weakly adsorbed enzyme molecules.

Instrumental Procedures. All electrochemical measurements were performed at 25 °C. CV was performed using an AUTOLAB PGSTAT-30 (AUTOLAB Metrohm, Utrecht, The Netherlands). A three-electrode cell was used with the gCDH- or dCDH-modified electrode as the working electrode, a AgI AgCl, $KCl_{(S)}$ (+197 mV vs NHE) as the reference electrode, and a Pt foil as the auxiliary electrode. All CV measurements were performed in 100 mM acetate buffer (pH 4.50). Anaerobicity was achieved by purging with pure argon gas for at least 20 min before making measurements.

For detailed information about flow injection analysis (FIA) and dynamic light scattering (DLS) see the Supporting Information.

The prediction of the potential N-glycosylation sites was done by the NetNGlyc 1.0 server (www.cbs.dtu.dk/services/NetNGlyc)[27] for the two CDHs tested.

RESULTS AND DISCUSSION

To study the effect of glycosylation on the efficiency for DET, two basidiomycete class-I CDHs were chosen due to their generally good DET properties on graphite and gold electrodes.[16a,18a,28] *Pc*CDH is one of the best studied CDHs and has been electrochemically characterized previously.[29] When extracted from cultures from *P. chrysosporium* the enzyme contains 9.4% of natural carbohydrate content,[30] but when overexpressed in *P. pastoris* the glycosylation percentage increases. For the studied *Pc*CDH preparation the increase was low; according to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) 10% of the total mass was due to glycosyl residues (Supporting Information, Figure S-1).[14,30,31] *Cs*CDH was recently purified and kinetically characterized[22] and is here for the first time electrochemically characterized. *Cs*CDH is found to be present in two glycoforms of 98 and 87 kDa with carbohydrate contents of 16% and 4% in mass, respectively.[22] Both isoforms are visible on SDS−PAGE, and they converge into one single band when deglycosylated (Supporting Information, Figure S-2).

The homogeneous substrate kinetics is investigated for both the glycosylated and the deglycosylated forms. The steric effect of the glycosylation is expected to play an important role in the efficiency of DET as already shown by our group with HRP and pyranose dehydrogenase (PDH)[8d,9,32] and additionally by other groups for GOx.[8b,11a,b,12b]

Stopped-flow experiments showed that deglycosylation does not affect the intramolecular electron transfer (IET) of *Pc*CDH. The rates obtained for the reduction of the *b*-type heme depend on the IET and are similar for g*Pc*CDH (k_{obs} = 18.5 ± 0.4 s^{-1}) and d*Pc*CDH (k_{obs} = 17.3 ± 0.6 s^{-1}). Thus, it can be said that the presence or absence of the high-mannose-type glycosylation does not affect the IET in *Pc*CDH. g*Pc*CDH and d*Pc*CDH seem to be fully active by comparison of the reduced spectra obtained with lactose (reduction of catalytically active CDH) and dithionite (reduction of cofactors also in inactive CDH). Both spectra have the same shape and peak heights. The FAD reduction rates for g*Pc*CDH (k_{obs} = 102 ± 4 s^{-1}) and d*Pc*CDH (k_{obs} = 87 ± 5 s^{-1}) are also quite similar.

In order to investigate how deglycosylation affects the electrocatalytical currents of CDH-modified electrodes in detail, the dependence of bioelectrocatalysis of lactose on the amperometric responses of gCDH- and dCDH-modified electrodes was compared by FIA. The electrodes were modified with solutions containing g*Pc*CDH, d*Pc*CDH, g*Cs*CDH, or d*Cs*CDH adjusted to have the same volumetric activity. The amperometric responses for different lactose concentrations are plotted in Figure 1. The data were fitted to the Michaelis−Menten equation. All experiments were carried out using five

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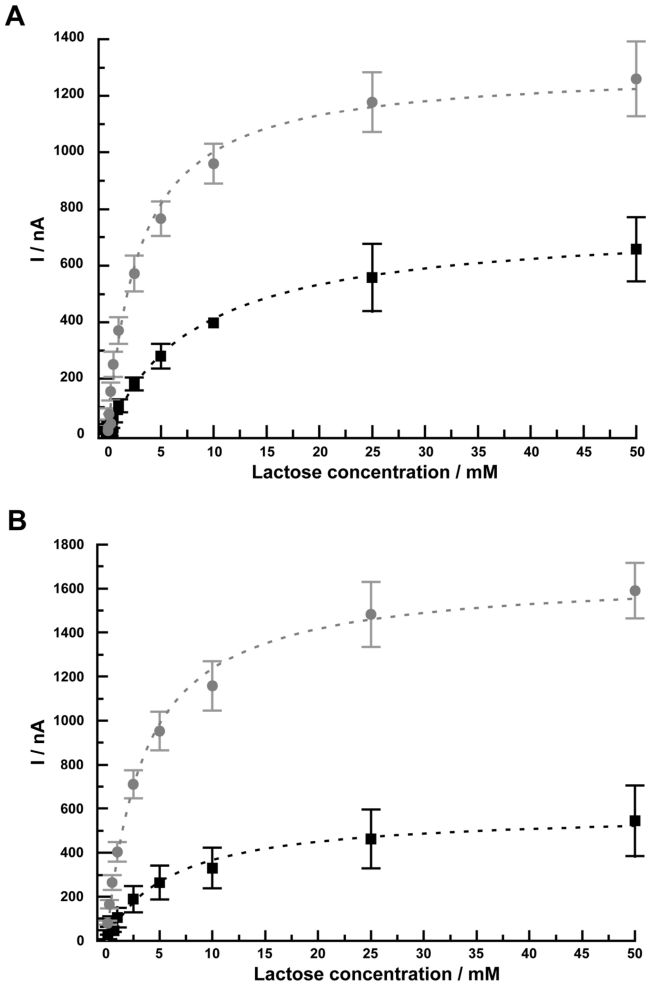

Figure 1. (A) Dependence of the amperometric response on the lactose concentration of g*Pc*CDH (■) and d*Pc*CDH (●). (B) Dependence of lactose concentration on the amperometric response of g*Cs*CDH (■) and d*Cs*CDH (●). E_{APPL} vs AglAgCl (0.1 M KCl) = +150 mV; flow rate 1 mL min^{-1}; acetate buffer (pH 4.5).

equivalent electrodes. The K_M values for lactose were estimated from the calibration curves (Table 1), and it was found that d*Pc*CDH (2.9 mM) has a 3.4 times lower value than g*Pc*CDH (8.2 mM). Also the I_{max} value for d*Pc*CDH (1300 nA) was twice higher than that for g*Pc*CDH (750 nA). A similar, but even more pronounced, effect of deglycosylation was observed for *Cs*CDH (Figure 1B and Table 1). The K_M value for d*Cs*CDH (3.4 mM) is around twice smaller than that for g*Cs*CDH (5.9 mM), and the I_{max} is 3 times higher for d*Cs*CDH (1660 nA) than for g*Cs*CDH (580 nA).

Table 1. Molecular and Kinetic Properties of Glycosylated and Deglycosylated *Pc*CDH and *Cs*CDH

	g*Pc*CDH	d*Pc*CDH	g*Cs*CDH	d*Cs*CDH
exptl MW [kDa]	89 ± 14[a,b]	82 ± 8	98[c]	84
amino acids	755	755	775	775
calcd mass [kDa]		80.1		80.7
glycosylation [%]	9.4[a]		16.0[c]	
isoelectric point	4.2[d]		3.0[c]	
lactose K_M [mM]/k_{cat} [s^{-1}]	1.1/13.4[b]		4.3/27[c]	
cellobiose K_M [mM]/k_{cat} [s^{-1}]	0.11/15.7[b]		0.082/26[c]	
lactose K_M electrochem [mM]	8.2	2.9	5.9	3.4
I_{max} [nA]	750	1300	580	1660

[a]Ref 30. [b]Ref 31. [c]Ref 22. [d]Ref 14.

The decrease in apparent K_M for electrodes based on the deglycosylated variants generated as expected a more restricted linear range of the calibration curves. The lower apparent K_M values at simultaneously higher I_{max} values indicate that the substrate flux to the electrode surface is not mass-transfer limited. However, the changes in the K_M are not that great meaning that the linear ranges found were rather similar to those obtained when using the glycosylated forms and also to what was shown previously for CDH adsorbed on graphite.[13c,33] The linear ranges for electrodes based on g*Pc*CDH and d*Pc*CDH were found to be from approximately 10 and 5 μM, respectively, up to about 500 μM, and for electrodes based on g*Cs*CDH and d*Cs*CDH the linear ranges were 10−500 to 0.5−500 μM, respectively. Thus, for electrodes modified with d*Cs*CDH a dramatic lowering of the lower limit of detection down to 0.5 μM was obtained, and that is the lowest limit of detection exhibited so far for any biosensors based on DET and CDH-modified graphite electrodes.[33a] If the linearity of the calibration curves was not so affected by the deglycosylation, the sensitivity for lactose increased drastically for both CDH variants. For the *Pc*CDH-modified electrodes the sensitivity increased around 5 times for the deglycosylated enzyme (from 98.5 to 478.5 nA mM^{-1}) and for *Cs*CDH around 4 times for the deglycosylated one (65.1 nA mM^{-1} for g*Cs*CDH and 250.1 nA mM^{-1} for d*Cs*CDH). CDH has already been shown to be a suitable enzyme in the biosensor field, and many reports can be found in the literature for analysis of lactose, glucose, hydroquinone, noradrenaline, and others, even in real samples.[13c,16d,33,34] This improvement in both linear range and sensitivity makes the deglycosylation technique suitable for future improvement of lactose biosensors based on CDH for the dairy industry.

Comparison of gCDH and dCDH by Cyclic Voltammetry on Graphite Electrodes. Parts A and B of Figure 2 show the CVs of g*Pc*CDH-, d*Pc*CDH-, g*Cs*CDH-, and d*Cs*CDH-modified GE electrodes in the absence and presence of 50 mM lactose. The volumetric activity of all enzyme solutions was made equal before modifying the electrodes (18.2 U mL^{-1}). The electrooxidation of lactose started for all variants at about +100 mV vs NHE, which is a potential similar as reported earlier for graphite electrodes modified with native *Pc*CDH.[29] Small but noticeable waves were observed in the CVs (Figure 2) on the variants tested in plain acetate buffer showing a direct communication of the CYT_{CDH} with the electrode. Calculation of the $E^{\circ\prime}$ values from these CVs shows an ~6 mV shift between the values for g*Pc*CDH and d*Pc*CDH, 161 and 167 mV, respectively. The values obtained are in agreement with the $E^{\circ\prime}$ value of 167.5 mV vs NHE for CYT_{PcCDH} calculated by redox titration at pH 4.50, considering a variation of the $E^{\circ\prime}$ value of the CYT_{CDH} with −15 mV per pH unit.[29b,35] The $E^{\circ\prime}$ of d*Cs*CDH was 135 mV and is for first time reported. This value differs with being about 30 mV more negative than the $E^{\circ\prime}$ of other class-I CDHs previously reported.[16a,c,28,29] Small waves in the CVs were also observed for g*Cs*CDH but not with enough good quality for calculating any $E^{\circ\prime}$ values.

Clear electrocatalytic currents for lactose oxidation in the CVs using a sweep rate of 2 mV s^{-1} were exhibited reflecting

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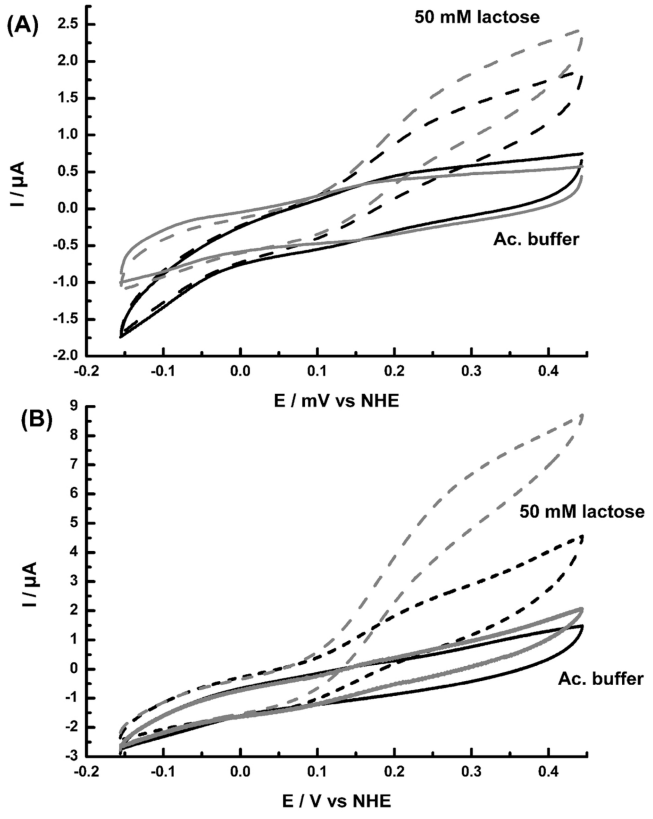

Figure 2. (A) CVs of a g*Pc*CDH-modified (black lines) and d*Pc*CDH-modified (gray lines) graphite electrodes at pH 4.50 in the presence (dashed line) and in the absence (solid line) of 50 mM lactose. (B) CVs of a g*Cs*CDH-modified (black lines) and d*Cs*CDH-modified (gray lines) graphite electrodes in the presence (dashed line) and in the absence (solid line) of lactose. Scan rate: 2 mV s^{-1}.

the electroactivity of the DH_{CYT} domain for all of the enzyme variants. A 2-fold increase in the catalytic current was observed for the d*Cs*CDH electrode compared with the electrode modified with its glycosylated counterpart, whereas a smaller increase was recorded for the d*Pc*CDH-modified electrode (Figure 2). Glycosylation has been shown to be around 9% for *Pc*CDH and 16% for *Cs*CDH, see Table 1. The decrease in size is relatively smaller for d*Pc*CDH compared with g*Pc*CDH than that for d*Cs*CDH compared with g*Cs*CDH leading to a higher number of enzyme molecules that are in DET contact with the electrode surface. Our suggestion is that the decrease in molecular mass of d*Pc*CDH is not big enough to cause such a dramatic change in the catalytic response of lactose as in the case of d*Cs*CDH and g*Cs*CDH and explains the smaller overall increase in the electrocatalytic activity.

No direct heterogeneous ET or catalysis was observed between the FAD-containing DH_{CDH} and the electrode even when running CVs between −300 and 0 mV versus NHE (data not shown). This is in disagreement with the previous study on deglycosylated GOx[8b] and PDH,[32] where deglycosylation enhanced the electrooxidation of glucose at a potential close to the $E^{\circ\prime}$ value of the bound FAD center in GOx or PDH and the electrode. The results suggest that (i) deglycosylation of either CDH did not shorten the distance sufficiently to enable any DET communication between the FAD in the active site of the DH_{CDH} domain and the electrode and/or (ii) that CDH was not oriented favorably on the graphite electrode for DET communication with the DH_{CDH}.

Effect of Deglycosylation on CNP-Modified Graphite Electrodes. Addition of CNPs onto the surface of graphite electrodes to nanostructure the electrode surface and to increase the surface area and possibly also to increase the possibility for correct contact between the electrode material and CDH to promote DET was investigated. Figure 3A shows

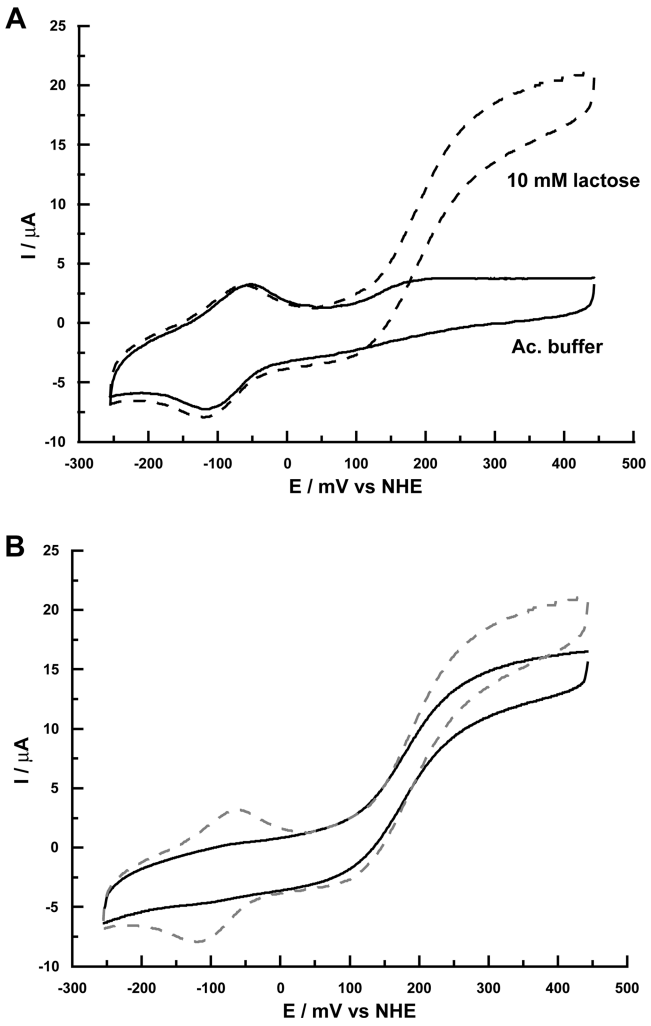

Figure 3. (A) CVs of a d*Cs*CDH-modified graphite electrode with CNPs at pH 4.50 in the presence (dashed line) and in the absence (bold line) of 50 mM lactose. (B) CVs of graphite electrodes with CNPs at pH 4.5 modified with g*Cs*CDH (bold line) and d*Cs*CDH (dashed line) in the presence of 50 mM lactose. Scan rate: 2 mV s^{-1}.

CVs for d*Cs*CDH absorbed onto such an electrode in the presence and absence of 50 mM lactose. In the absence of substrate the first redox wave at −90 mV can be referred to FAD from DH_{CDH}, whereas the second wave at a more positive potential (100 mV) can be assigned to the heme *b* in CYT_{CDH}. Clear electrocatalysis at approximately 100 mV versus NHE in the presence of 50 mM lactose was observed. No electrocatalytic current was observed that could be stated to emanate from the DH_{CDH} domain. Therefore, the wave seen at −90 mV in the CV is most likely caused by FAD released from the protein or a denaturized domain, as already reported.[5c,36] The $E^{\circ\prime}$ of adsorbed free FAD (−86 mV vs NHE at pH 4.50) coincides well with the values obtained here.[37] Electrocatalysis was observed also for g*Cs*CDH starting at the same potential as for d*Cs*CDH (Figure 3B). In this case no waves were found

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corresponding to the free FAD for the g*Cs*CDH-modified electrodes.

As observed on GE electrodes the electrodes modified with deglycosylated enzyme showed higher current density at the plateau region (25%) on the presence of substrate than when modified with g*Cs*CDH.

Effect of Deglycosylation of CDH on Gold Electrodes. Gold electrodes modified with functionalized thiols forming a SAM were chosen to study the heterogeneous electron transfer in an environment where the enzyme has a partially diffusive behavior. This approach has been shown to reveal clear and defined CV waves and catalysis in the absence and in the presence of substrate, respectively, for a number of CDHs and other redox proteins and enzymes.[26,28,29,38]

In these experiments CDH was kept between a MUOH-modified gold electrode and a permselective dialysis membrane, which allowed the diffusion of buffer and substrate through the membrane but kept the enzyme at a high concentration at the electrode surface.[26] The choice of MUOH was done according to previous reports on CDH using CV that showed high electrocatalytic current and clear redox waves for the CYT_{CDH} of CDHs in the absence of substrate.[6c,28,38e] The choice of the thiol for activation of the gold electrode was crucial, since the functionalization facilitates ET between the heme *b* contained in CYT_{CDH} and the electrode.

All tested CDH variants exhibited clear redox waves in their CVs, which indicates DET between CDH_{CYT} and the SAM-protected electrode. The $E^{\circ\prime}$ for the CYT_{CDH} of g*Pc*CDH was 152 mV at pH 4.50, which is in accordance with the value (154 mV at pH 4.50) previously found.[29a] The $E^{\circ\prime}$ for the CYT_{CDH} of g*Cs*CDH was 163 mV at pH 4.50, which is close to the values found for other class-I CDHs. As already shown for *Pc*CDH-modified graphite electrodes the deglycosylated variant showed a +8 mV shift compared to g*Pc*CDH. The $E^{\circ\prime}$ of d*Pc*CDH was 160 mV and that of d*Cs*CDH was 171 mV at pH 4.50. The CDH_{CYT}-containing domains of *Pc*CDH and *Cs*CDH have a number of both anionic and cationic amino acid residues distributed on their surfaces,[21a,22] and the shift can be attributed to a closer interaction of them with the hydroxyl-functionalized SAM. A shift of the potential of the heme group has already been observed with thiolic SAMs with different functional groups.[28]

The dependence of the peak current on scan rate showed a linear relationship at low scan rates for the anodic peak (data not show), which is a typical thin-layer behavior of membrane-entrapped proteins.[26,29a] A value of ΔE_p less than 59 mV indicates that there are relatively strong interactions between the enzyme and the SAM.[39]

When using all the different variants of the CDHs on the SAM−Au electrode a high catalytic current for all variants was found in the presence of substrate (10 mM lactose). Figure 4 shows that the current at the plateau region of the CV was 30% higher for d*Cs*CDH (372 nA) than that for g*Cs*CDH (225 nA). This behavior is in accordance with the difference observed using graphite electrodes. On the other hand g*Pc*CDH and d*Pc*CDH showed virtually the same catalytic current at the plateau of the respective CVs for both variants (approximately 275 nA). The reason is believed to be due to as previously indicated, that *Pc*CDH is less glycosylated (~9%)[14,30,31] than *Cs*CDH (~16%)[22] and therefore the deglycosylation of d*Pc*CDH results in a less noticeable increase in catalytic current at the SAM-protected Au electrode.

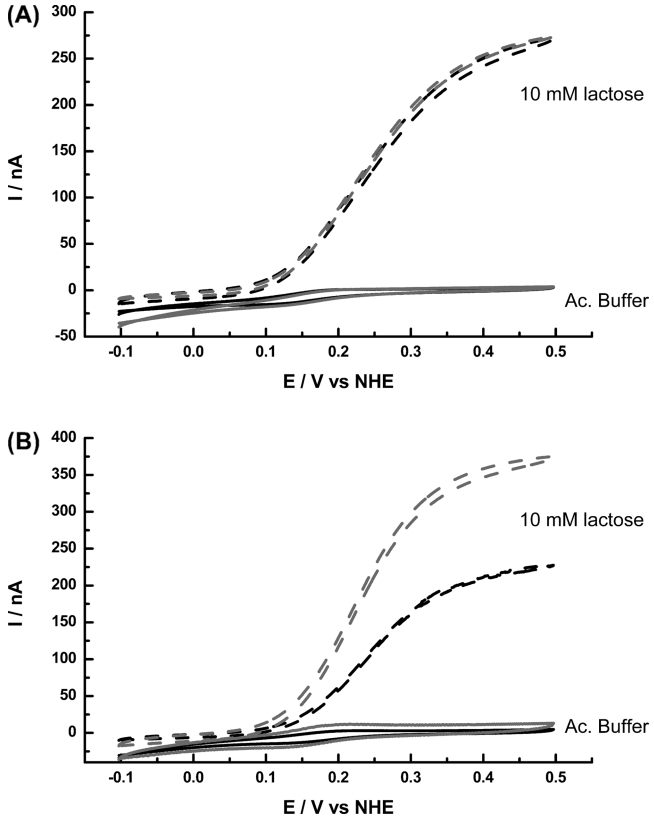

Figure 4. (A) CVs of g*Pc*CDH (black lines) and d*Pc*CDH (gray lines) trapped under a permselective membrane at a MUOH-modified Au electrode at pH 4.50 in the presence (dashed line) and in the absence (solid line) of 10 mM lactose. (B) CVs of a g*Cs*CDH (black lines) and d*Cs*CDH (gray lines) trapped under a permselective membrane at pH 4.50 in the presence (dashed line) and in the absence (solid line) of 10 mM lactose. Scan rate: 2 mV s^{-1}.

No direct heterogeneous ET or catalysis was observed either between the electrode and the separate and free DH_{CDH} domain even when running CV between −300 and 0 mV versus NHE (data not shown). On the basis of the $E^{\circ\prime}$ values calculated by redox titration the potential for DH_{CDH} should be around 17.5 mV versus NHE considering the variation on the $E^{\circ\prime}$ of the flavin with −59 mV per pH unit.[35]

Additional experiments with covalently bound *Pc*CDH were performed in order to study how the electrocatalytic currents measured using CV were affected by the deglycosylation of *Pc*CDH. All attempts to absorb CDH directly on unmodified gold electrodes failed due to the low stability of adsorbed CDH on the surface of the electrode as already shown previously.[6c,28,29b] The immobilization technique used has been shown previously and consisted of a mixed SAM of $MUNH_2$/MUOH, onto which CDH was covalently bound using GA.[6c] As GA contains two reactive aldehyde groups, it will very rapidly react under mild conditions with the primary amino groups of the SAM and on the lysine residues on the enzyme surface. The −OH terminated headgroups act both to orient the enzyme and to facilitate DET. Different ratios of the mixture were used, from 1/10 to 1/99 $MUNH_2$/MUOH and enzyme solutions of g*Pc*DH and d*Pc*DH. The modified electrodes were studied using CV, and in the presence of 10 mM lactose catalysis was observed for all the tested mixtures. Small redox waves in the CVs were observed in the absence of substrate but not with enough good quality for $E^{\circ\prime}$ calculations or kinetic studies.

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Figure 5 shows baseline-subtracted currents at the catalytic current plateau region (+250 mV vs NHE) of the CVs obtained

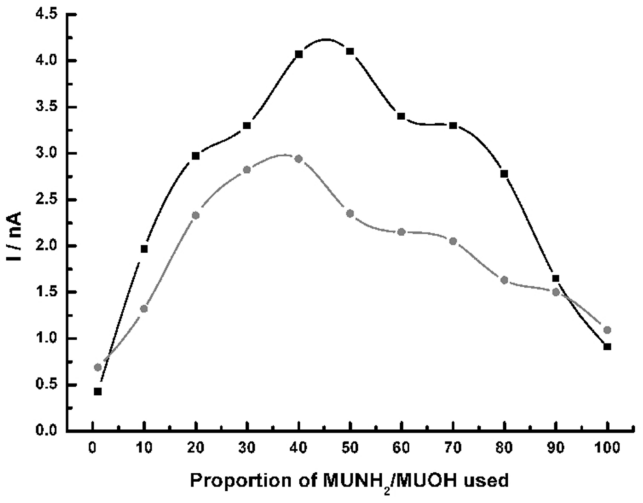

Figure 5. Dependence of the catalytic current at pH 4.50 on the molar ratio of mixed $MUNH_2$/MUOH: g*Pc*CDH (■) and d*Pc*CDH (●). Scan rate: 2 mV s^{-1}.

in the presence and the absence of 10 mM lactose in acetate buffer for the different ratios of the used SAMs. The optimal SAM mixture ratio for g*Pc*CDH was found to be between 1/50 and 1/60. For d*Pc*CDH the optimal SAM mixture ratio was found to be higher for the amino-functionalized thiol (1/40−1/50). The ∼10% difference in the optimal ratio observed is similar to the amount of glycosylation in weight of the enzyme compared with that of the deglycosylated one.

Using DLS, the hydrodynamic radius can be determined and a qualitative analysis of the volume occupied by the glycosylated sites can be checked. The hydrodynamic radius was calculated in terms of volume and intensity (Table 2).

Table 2. Calculated Hydrodynamic Radii from DLS Measurements

	g*Pc*CDH	d*Pc*CDH
hydrodynamic radius determined by intensity (nm)	7.3	5.0
hydrodynamic radius determined by volume (nm)	6.1	4.3

d*Pc*CDH showed a smaller hydrodynamic radius than g*Pc*CDH. The difference observed in the hydrodynamic radius was bigger than the difference in mass between the glycosylated and the deglycosylated enzyme (∼10%). As already mentioned CDH is composed of a *b*-type CYT_{CDH} connected through a linker to a much bigger DH_{CDH}.[18a] Early investigations on CDH showed that it is mainly N-glycosylated with none or little O-linked sugars.[40] A prediction of the N-glycosylation sites from the NetNGlyc 1.0 server (www.cbs.dtu.dk/services/NetNGlyc),[27] shows that *Pc*CDH contains nine possible putative N-glycosylation sites and six of them are over the threshold. NetNGlyc 1.0 server finds 10 possible putative sites for *Cs*CDH and seven of them are over the threshold, respectively (Supporting Information, Tables S-1 and S-2). Using these estimated glycosylation sites a representation based on the crystal structures published up to now of the separate CYT_{CDH} and DH_{CDH} of *Pc*CDH are shown[21] (Supporting Information, Figure S-3). The length of the carbohydrate chains was considered as a high-mannose-type glycosylation with an approximate MW of 1800 Da.[40] The hydrodynamic radius measured with DLS is influenced by the shape of the enzyme globule, and in this case the absence of symmetry creates a bigger hydrodynamic radius than the one really occupied by the folded CDH.[41] The values obtained from the DLS measurements are in our opinion then just qualitative values to be compared between the glycosylated and deglycosylated variants. g*Pc*CDH appears to be approximately 30% bigger in its hydrodynamic radius than d*Pc*CDH, 7.3 and 5 nm, respectively (Table 2). This is approximately in accordance with the ∼40% increase in I_{max} observed for graphite electrodes, and this it is attributed to an increase on the amount of CDH molecules packed on the surface.

■ CONCLUSIONS

The effect of deglycosylation of *Pc*CDH and *Cs*CDH on their electrocatalytic properties was studied. It was found that deglycosylation improves the catalytic current density, I_{max}, and the sensitivity for lactose. This increase can be attributed to a higher number of electroactive CDH molecules on the electrode surface due to the downsizing of the enzyme's dimensions and a facilitated DET due to deglycosylation. Despite the increase in DET between CYT_{CDH} and the electrodes no DET between DH_{CDH} and the electrodes was observed. The increased current density of dCDH-modified electrodes is certainly an effect of the reduced hydrodynamic radius of the deglycosylated CDHs. Interestingly; deglycosylation decreased the apparent K_M values for the two CDHs tested. The $E^{\circ\prime}$ of the CYT_{CDH} showed a small positive shift of ∼7−8 mV due to the removal of the glycosylation on the enzymes.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. 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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REFERENCES

(1) (a) Eddowes, M. J.; Hill, H. A. O. *J. Chem. Soc., Chem. Commun.* **1977**, 771−772. (b) Yeh, P.; Kuwana, T. *Chem. Lett.* **1977**, 1145−1148.

(2) Leger, C.; Bertrand, P. *Chem. Rev.* **2008**, *108*, 2379−2438.

(3) Heller, A. *J. Phys. Chem.* **1992**, *96*, 3579−3587.

(4) (a) Barton, S. C.; Gallaway, J.; Atanassov, P. *Chem. Rev.* **2004**, *104*, 4867−4886. (b) Willner, I.; Yan, Y. M.; Willner, B.; Tel-Vered, R. *Fuel Cells* **2009**, *9*, 7−24. (c) Leech, D.; Kavanagh, P.; Schuhmann, W. *Electrochim. Acta* **2012**, *84*, 223−234.

(5) (a) Heller, A. *Acc. Chem. Res.* **1990**, *23*, 128−134. (b) Zafar, M. N.; Tasca, F.; Boland, S.; Kujawa, M.; Patel, I.; Peterbauer, C. K.; Leech, D.; Gorton, L. *Bioelectrochemistry* **2010**, *80*, 38−42. (c) Tasca, F.; Gorton, L.; Harreither, W.; Haltrich, D.; Ludwig, R.; Nöll, G. *Anal. Chem.* **2009**, *81*, 2791−2798.

(6) (a) Katz, E.; Willner, I. *Angew. Chem., Int. Ed.* **2004**, *43*, 6042−6108. (b) Pingarron, J. M.; Yanez-Sedeno, P.; Gonzalez-Cortes, A. *Electrochim. Acta* **2008**, *53*, 5848−5866. (c) Matsumura, H.; Ortiz, R.; Ludwig, R.; Igarashi, K.; Samejima, M.; Gorton, L. *Langmuir* **2012**, *28*, 10925−10933.

(7) (a) Gooding, J. J.; Ciampi, S. *Chem. Soc. Rev.* **2011**, *40*, 2704−2718. (b) Zimmermann, H.; Lindgren, A.; Schuhmann, W.; Gorton, L. *Chem. Eur. J.* **2000**, *6*, 592−599.

(8) (a) Holland, J. T.; Lau, C.; Brozik, S.; Atanassov, P.; Banta, S. *J. Am. Chem. Soc.* **2011**, *133*, 19262−19265. (b) Courjean, O.; Gao, F.; Mano, N. *Angew. Chem., Int. Ed.* **2009**, *48*, 5897−5899. (c) Xiao, Y.; Patolsky, F.; Katz, E.; Hainfeld, J. F.; Willner, I. *Science* **2003**, *299*, 1877−1881. (d) Castillo, J.; Ferapontova, E.; Hushpulian, D.; Tasca, F.; Tishkov, V.; Chubar, T.; Gazaryan, I.; Gorton, L. *J. Electroanal. Chem.* **2006**, *588*, 112−121. (e) Harreither, W.; Nicholls, P.; Sygmund, C.; Gorton, L.; Ludwig, R. *Langmuir* **2012**, *28*, 6714−6723.

(9) (a) Ferapontova, E. E.; Grigorenkoa, V. G.; Egorova, A. M.; Börchers, T.; Ruzgas, T.; Gorton, L. *Biosens. Bioelectron.* **2001**, *16*, 147−157. (b) Presnova, G.; Grigorenko, V.; Egorov, A.; Ruzgas, T.; Lindgren, A.; Gorton, L.; Börchers, T. *Faraday Discuss.* **2000**, *116*, 281−289. (c) Lindgren, A.; Tanaka, M.; Ruzgas, T.; Gorton, L.; Gazaryan, I.; Ishimori, K.; Morishima, I. *Electrochem. Commun.* **1999**, *1*, 171−175. (d) Ferapontova, E. E.; Castillo, J.; Hushpulian, D.; Tishkov, V.; Chubar, T.; Gazaryan, I.; Gorton, L. *Electrochem. Commun.* **2005**, *7*, 1291−1297.

(10) Wohlfahrt, G.; Witt, S.; Hendle, J.; Schomburg, D.; Kalisz, H. M.; Hecht, H.-J. *Acta Crystallogr., Sect. D* **1999**, *55*, 969−977.

(11) (a) Prevoteau, A.; Courjean, O.; Mano, N. *Electrochem. Commun.* **2010**, *12*, 213−215. (b) Courjean, O.; Flexer, V.; Prevoteau, A.; Suraniti, E.; Mano, N. *ChemPhysChem* **2010**, *11*, 2795−2797. (c) Fraser, D. M.; Zakeeruddin, S. M.; Grätzel, M. *Biochim. Biophys. Acta* **1992**, *1099*, 91−101.

(12) (a) Liu, G.; Paddon-Row, M. N.; Gooding, J. *Electrochem. Commun.* **2007**, *9*, 2218−2223. (b) Demin, S.; Hall, E. A. H. *Bioelectrochemistry* **2009**, *76*, 19−27.

(13) (a) Christenson, A.; Dimcheva, N.; Ferapontova, E. E.; Gorton, L.; Ruzgas, T.; Stoica, L.; Shleev, S.; Yaropolov, A. I.; Haltrich, D.; Thorneley, R. N. F.; Aust, S. D. *Electroanalysis* **2004**, *16*, 1074−1092. (b) Zamocky, M.; Hallberg, M.; Ludwig, R.; Divne, C.; Haltrich, D. *Gene* **2004**, *338*, 1−14. (c) Harreither, W.; Coman, V.; Ludwig, R.; Haltrich, D.; Gorton, L. *Electroanalysis* **2007**, *19*, 172−180.

(14) Zamocky, M.; Ludwig, R.; Peterbauer, C.; Hallberg, B. M.; Divne, C.; Nicholls, P.; Haltrich, D. *Curr. Protein Pept. Sci.* **2006**, *7*, 255−280.

(15) (a) Ander, P. *FEMS Microbiol. Rev.* **1994**, *13*, 297−311. (b) Morpeth, F. F. *Biochem. J.* **1985**, *228*, 557−564.

(16) (a) Tasca, F.; Gorton, L.; Harreither, W.; Haltrich, D.; Ludwig, R.; Nöll, G. *J. Phys. Chem. C* **2008**, *112*, 13668−13673. (b) Stoica, L.; Lindgren-Sjölander, A.; Ruzgas, T.; Gorton, L. *Anal. Chem.* **2004**, *76*, 4690−4696. (c) Lindgren, A.; Stoica, L.; Ruzgas, T.; Ciucu, A.; Gorton, L. *Analyst* **1999**, *124*, 527−532. (d) Zafar, M. N.; Safina, G.; Ludwig, R.; Gorton, L. *Anal. Biochem.* **2012**, *425*, 36−42.

(17) Shao, M.; Zafar, M. N.; Sygmund, C.; Guschin, D. A.; Ludwig, R.; Peterbauer, C. K.; Schuhmann, W.; Gorton, L. *Biosens. Bioelectron.* **2013**, *40*, 308−314.

(18) (a) Ludwig, R.; Harreither, W.; Tasca, F.; Gorton, L. *ChemPhysChem* **2010**, *11*, 2674−2697. (b) Henriksson, G.; Johansson, G.; Pettersson, G. *J. Biotechnol.* **2000**, *78*, 93−113. (c) Cameron, M. D.; Aust, S. D. *Enzyme Microb. Technol.* **2001**, *28*, 129−138.

(19) Ludwig, R.; Ortiz, R.; Schulz, C.; Harreither, W.; Sygmund, C.; Gorton, L. *Anal. Bioanal. Chem.* **2012**, submitted for publication.

(20) Cameron, M. D.; Timofeevski, S.; Aust, S. D. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 751−758.

(21) (a) Hallberg, B. M.; Bergfors, T.; Bäckbro, K.; Pettersson, G.; Henriksson, G.; Divne, C. *Structure* **2000**, *8*, 79−88. (b) Hallberg, M. B.; Henriksson, G.; Pettersson, G.; Divne, C. *J. Mol. Biol.* **2002**, *315*, 421−434.

(22) Harreither, W.; Sygmund, C.; Duenhofen, E.; Vicuna, R.; Haltrich, D.; Ludwig, R. *Appl. Environ. Microbiol.* **2009**, *75*, 2750−2757.

(23) Yoshida, M.; Ohira, T.; Igarashi, K.; Nagasawa, H.; Aida, K.; Hallberg, B. M.; Divne, C.; Nishino, T.; Samejima, M. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2050−2057.

(24) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.

(25) Hoare, J. P. *J. Electrochem. Soc.* **1984**, *131*, 1808−1815.

(26) Haladjian, J.; Bianco, P.; Nunzi, F.; Bruschi, M. *Anal. Chim. Acta* **1994**, *289*, 15−20.

(27) Blom, N.; Sicheritz-Pontén, T.; Gupta, R.; Gammeltoft, S.; Brunak, S. *Proteomics* **2004**, 1633−1649.

(28) Stoica, L.; Dimcheva, N.; Haltrich, D.; Ruzgas, T.; Gorton, L. *Biosens. Bioelectron.* **2005**, *20*, 2010−2018.

(29) (a) Lindgren, A.; Larsson, T.; Ruzgas, T.; Gorton, L. *J. Electroanal. Chem.* **2000**, *494*, 105−113. (b) Larsson, T.; Lindgren, A.; Ruzgas, T.; Lindquist, S. E.; Gorton, L. *J. Electroanal. Chem.* **2000**, *482*, 1−10.

(30) Bao, W.; Usha, S. N.; Renganathan, V. *Arch. Biochem. Biophys.* **1993**, *300*, 705−713.

(31) Henriksson, G.; Sild, V.; Szabo, I. J.; Pettersson, G.; Johansson, G. *Biochim. Biophys. Acta* **1998**, *1383*, 48−54.

(32) Yakovleva, M.; Killyeni, A.; Ortiz, R.; Schulz, C.; MacAodha, D.; Conghaile, P. Ó; Leech, D.; Popescu, I. O.; Gonaus, C.; Peterbauer, C. K.; Gorton, L. *Electrochem. Commun.* **2012**, *24*, 120−122.

(33) (a) Stoica, L.; Ludwig, R.; Haltrich, D.; Gorton, L. *Anal. Chem.* **2006**, *78*, 393−398. (b) Yakovleva, M.; Buzas, O.; Matsumura, H.; Samejima, M.; Igarashi, K.; Larsson, P.-O.; Gorton, L.; Danielsson, B. *Biosens. Bioelectron.* **2012**, *31*, 251−256.

(34) (a) Safina, G.; Ludwig, R.; Gorton, L. *Electrochim. Acta* **2010**, *55*, 7690−7695. (b) Tasca, F.; Zafar, M. N.; Harreither, W.; Nöll, G.; Ludwig, R.; Gorton, L. *Analyst* **2011**, *136*, 2033−2036. (c) Glithero, N.; Clark, C.; Gorton, L.; Schuhmann, W.; Pasco, N. *Anal. Bioanal. Chem.* **2012**, submitted for publication.

(35) (a) Clark, W. M. *Oxidation−Reduction Potentials of Organic Systems*; Willams and Wilkins: Baltimor, Md., 1960. (b) Igarashi, K.; Momohara, I.; Nishino, T.; Samejima, M. *Biochem. J.* **2002**, *365*, 521−526.

(36) Tasca, F.; Gorton, L.; Harreither, W.; Haltrich, D.; Ludwig, R.; Nöll, G. *J. Phys. Chem. C* **2008**, *112*, 9956−9961.

(37) Gorton, L.; Johansson, G. *J. Electroanal. Chem.* **1980**, *113*, 151−158.

(38) (a) Guo, L.-H.; Hill, H. A. O. *Adv. Inorg. Chem.* **1991**, *36*, 341−375. (b) Gooding, J. J.; Hibbert, D. B. *TrAC, Trends Anal.Chem.* **1999**, *18*, 525−533. (c) Finklea, H. O. In *Electroanalytical Chemistry*; Bard, A. J., Rubinstein, I., Eds.; Marcel Dekker: New York, 1996; Vol. *19*, pp 110−337. (d) Ostuni, E.; Yan, L.; Whitesides, G. M. *Colloids Surf., B* **1999**, *15*, 3−30. (e) Coman, V.; Harreither, W.; Ludwig, R.; Haltrich, D.; Gorton, L. *Chem. Anal. (Warsaw)* **2007**, *52*, 945−960.

(39) Laviron, E. In *Electroanalytical Chemistry*; Marcel Dekker: New York, 1982; Vol. *12*, pp 53−157.
(40) Raices, M.; Paifer, E.; Cremata, J.; Montesino, R.; Ståhlberg, J.; Divne, C.; Szabo, I. J.; Henriksson, G.; Johansson, G.; Pettersson, G. *FEBS Lett.* **1995**, *369*, 233−238.
(41) (a) Lehner, D.; Zipper, P.; Henriksson, G.; Pettersson, G. *Biochim. Biophys. Acta, Protein Struct.* **1996**, *1293*, 161−169. (b) Zipper, P.; Durchschlag, H. *Phys. Scr.* **2005**, *2005*, 228.